The Journal of Experimental Biology 210, 4399-4410
Published by The Company of Biologists 2007
doi:10.1242/jeb.008722

Unphosphorylated twitchin forms a complex with actin and myosin that may contribute to tension maintenance in catch

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Accepted 18 September 2007

Summary

Molluscan smooth muscle can maintain tension over extended periods with little energy expenditure, a process termed catch. Catch is thought to be regulated by phosphorylation of a thick filament protein, twitchin, and involves two phosphorylation sites, D1 and D2, close to the N and C termini, respectively. This study was initiated to investigate the role of the D2 site and its phosphorylation in the catch mechanism. A peptide was constructed containing the D2 site and flanking immunoglobulin (Ig) motifs. It was shown that the dephosphorylated peptide, but not the phosphorylated form, bound to both actin and myosin. The binding site on actin was within the sequence L10 to P29. This region also binds to loop 2 of the myosin head. The dephosphorylated peptide linked myosin and F-actin and formed a trimeric complex. Electron microscopy revealed that twitchin is distributed on the surface of the thick filament with an axial periodicity of 36.25 nm and it is suggested that the D2 site aligns with the myosin heads. It is proposed that the complex formed with the dephosphorylated D2 site of twitchin, F-actin and myosin represents a component of the mechanical linkage in catch.

Key words: catch contraction, twitchin, ABRM, myosin, actin.

Introduction

Molluscan smooth muscles, such as the bivalve adductor muscle and mussel *Mytilus* anterior byssus retractor muscle (ABRM) are capable of maintaining tension for extended periods with little energy consumption. This phenomenon is called catch. Catch muscles contract by stimulation with acetylcholine and the resulting increase in intracellular [Ca^{2+}]. Subsequent removal of acetylcholine results in the maintenance of tension even at low intracellular [Ca^{2+}] (Ishii et al., 1989; Twarog, 1976). Secretion of serotonin abolishes the catch state by increasing intracellular [cAMP] and activation of cAMP-dependent protein kinase (PKA). These various phases (Watabe and Hartshorne, 1990) can be reproduced in skinned catch muscle fibers, i.e. an increase in [Ca^{2+}] initiates contraction, a decrease in [Ca^{2+}] promotes catch and addition of cAMP terminates the catch state.

Since the discovery of catch there has been considerable interest in defining the molecular basis responsible for force maintenance with very low energy expenditure. Early ideas suggested that two types of linkages were formed, the catch linkage consisting of paramyosin–paramyosin interactions and the contractile linkages of actin and myosin (Ruegg, 2001; Watabe and Hartshorne, 1990). In this scheme, phosphorylation of paramyosin by PKA would reduce or eliminate the paramyosin association and in the presence of Ca^{2+} allow cross-bridge cycling. Subsequently, the paramyosin hypothesis became less popular and catch was suggested to reflect attached non-cycling cross-bridge–actin interactions, the so-called 'linkage' hypothesis (Lowy et al., 1964). It was suggested that phosphorylation of the myosin rod was the critical regulatory step (Castellani and Cohen, 1987). It was found subsequently that the only protein phosphorylated by PKA in ABRM (catch) fibers was the high molecular mass protein, twitchin (Siegman et al., 1997). These and subsequent studies (Siegman et al., 1998; Funabara et al., 2001a) proposed that twitchin is an integral component of the catch mechanism and is a phosphorylation-dependent regulator of the catch state. Dephosphorylated twitchin promotes catch and phosphorylation of twitchin by PKA releases catch. From *in vitro* studies, involving isolated thick and thin filaments under relaxing conditions, it was further suggested that the only components necessary for catch were actin, myosin and twitchin (Yamada et al., 2001). As a working hypothesis it was assumed that catch reflected attached non-cycling cross-bridges that bear the mechanical load and whose formation is regulated by phosphorylation of twitchin (Butler et al., 2001). But, other observations made it necessary to reconsider a basic tenet of the 'linkage' hypothesis. Galler and coworkers found that catch depends on cAMP- and pH-sensitive linkages but not

on the cross-bridge–actin interactions (Galler et al., 2005). It has also been suggested that the mechanical link in catch could be the interaction between dephosphorylated twitchin and actin, where phosphorylation of twitchin abolishes interaction (Shelud'ko et al., 2004). Binding of synthetic myosin filaments to F-actin filaments and dephosphorylated twitchin has been shown, but not direct binding of twitchin to actin (Yamada et al., 2001). The idea that alternative links may be involved was further developed and although regulation of catch by twitchin phosphorylation is accepted there is considerable evidence to support the idea that the mechanical links in catch are not attached cross-bridges (Andruchova et al., 2005; Hopflinger et al., 2006; Butler et al., 2006).

The *Mytilus* ABRM twitchin is composed of a single polypeptide of 530 kDa containing multiple repeats of immunoglobulin (Ig) and fibronectin type III motifs and a single kinase domain (Funabara et al., 2003) and is very similar to twitchin and twitchin-related proteins from other species (Funabara et al., 2005). *In vitro* phosphorylation assays with PKA indicate that ABRM twitchin incorporates 3 mol phosphates mol^{-1} twitchin (Funabara et al., 2001a). Two of the three phosphorylation sites are referred to as D1 and D2 and are located at S1075 and S4316 near the N and C termini, respectively. It was suggested that both sites are involved in the regulation of catch contraction *in vivo* (Funabara et al., 2003).

In light of the suggestion that the mechanical links in catch involve interactions other than the force-generating actin–myosin complex, this study was initiated to examine interactions with twitchin, with an emphasis on effects mediated by phosphorylation at the D2 site. Because of the high mass of twitchin (and complexity in binding assays) a D2 peptide (composed of the D2 site plus two Ig motifs) was used. Important observations were that the dephosphorylated D2 peptide binds to myosin and F-actin and promoted complex formation between myosin and F-actin. In addition the localization of twitchin on myofilaments of ABRM was examined. These cumulative data are interpreted to indicate that the D2 site of twitchin mediates interaction between myosin and F-actin.

Materials and methods

Protein preparation

The twitchin D2 region was expressed in bacteria as TWD2-S and consists of the 21st to 22nd Ig motifs from the N terminus including the D2 region (Fig. 1A). The cDNA fragment coding for TWD2-S was amplified by PCR using a primer set of 5′-CCCTCGAGCGCAAAGAAGCAGCTCCCAG-3′ and 5′-GGCTCGAGTCAGTCAAAGTCATGAGCTCGGTC-3′ and inserted into an expression vector pET-15b (Novagen, Darmstadt, Germany) after digestion with *Xho*I. The mutants where the D2 phosphorylatable serine (S4316 in the full sequence) was replaced by alanine (TWD2-A) or aspartic acid (TWD2-D) were constructed by PCR (Ito et al., 1991). ABRM actomyosin was prepared as previously reported from *Mytilus galloprovincialis* Lamarck (Funabara et al., 2001b). Chicken skeletal actomyosin was prepared by the same method as that for ABRM actomyosin. Myosin was prepared from chicken fast skeletal and scallop *Patinopecten yessoensis* Jay striated muscles (Stafford et al., 1979). Paramyosin was isolated from ABRM (Watabe et al., 1989). Actin was prepared from acetone powder of chicken fast skeletal muscle (Mommaerts, 1951). Twitchin was prepared as described (Funabara et al., 2001a).

Mg^{2+}-ATPase assay

ATPase assays were carried out in 20 mmol l^{-1} 3-(*N*-morpholino)propanesulfonic acid (MOPS)-NaOH (pH 7.4) containing 30 mmol l^{-1} KCl, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} ATP, 80 μg ml^{-1} actomyosin, 16 μg ml^{-1} D2 peptide mutant and 0.1 mmol l^{-1} $CaCl_2$ or 1 mmol l^{-1} ethyleneglycol bis(2-aminoethylether)tetraacetic acid (EGTA). ATPase of chicken fast skeletal actomyosin was also assayed in the presence of 80 μg ml^{-1} ABRM paramyosin. Liberated inorganic phosphate was determined by the Malachite Green method (Kodama et al., 1986).

Solid-phase binding assay

Solid-phase binding assay was carried out according to published methods (Weitkamp et al., 1998) with some modifications. In this assay, washing, blocking reaction, dilution of samples and detection were carried out using reagents included in Protein Detector ELISA kit (KPL, Gaithersburg, MD, USA). Wells of an enzyme-linked immunosorbent assay (ELISA) microplate were coated with 100 μl of 10 μg ml^{-1} scallop striated adductor myosin, ABRM paramyosin or chicken fast skeletal F-actin in 1× Coating Solution included in the kit for 1 h at room temperature. After blocking, 100 μl of 10 μg ml^{-1} TWD2-S or thiophosphorylated

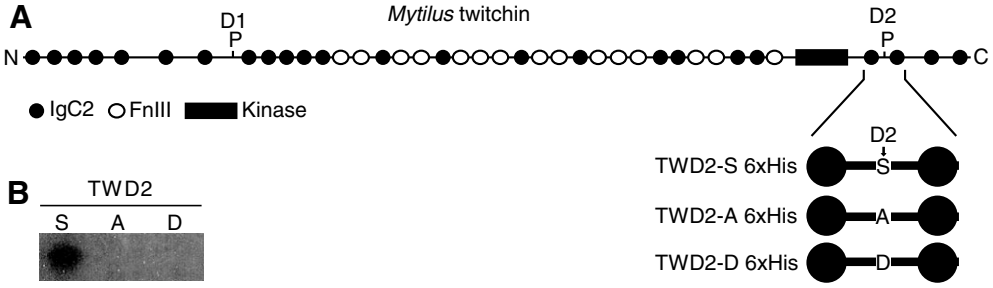

Fig. 1. A schematic representation of the ABRM twitchin molecule and mutants of the D2 peptide. (A) The motif structure of ABRM twitchin is shown together with the region expressed as various 6xHis-fusion proteins that are used in the present study. The D1 and D2 sites are S1075 and S4316, respectively. (B) Phosphorylation of twitchin D2 peptide mutants by PKA. TWD2-S was phosphorylated, whereas TWD2-A and TWD2-D were not phosphorylated. Phosphorylation of TWD2-S was detected by SDS-gel electrophoresis as described previously (Funabara et al., 2001a).

TWD2-S were hybridized in 1× Coating Solution for 1 h at room temperature with proteins coated on the plate. Anti-6xHis and anti-mouse IgG antibodies conjugated with alkaline phosphatase were used, respectively, as the primary and secondary antibodies. The reaction was traced by measuring absorbance at 630 nm after the substrate solution included in the kit was added to wells.

Cosedimentation binding assay

30 μg ml^{-1} scallop striated adductor myosin, 300 μg ml^{-1} chicken fast skeletal F-actin and 10 μg ml^{-1} TWD2-S were mixed in 20 mmol l^{-1} MOPS-NaOH (pH 7.4), containing 30 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} ATP and 1 mmol l^{-1} EGTA, on ice for 30 min and centrifuged at 3000 ***g***. The supernatants and precipitates obtained were subjected to SDS-PAGE.

Identification of twitchin binding region of actin

Chicken fast skeletal actin was extensively digested by trypsin in 20 mmol l^{-1} Tris-maleate (pH 7.0) containing 50 mmol l^{-1} KCl and 10 mmol l^{-1} $CaCl_2$ for 24 h at 37°C. The digests were subjected to reverse-phase high-performance liquid chromatography with a TSKgel ODS-80T column (4.6 mm×15 cm) (Tosoh, Tokyo, Japan) and adsorbed peptides were eluted with 0%–40% acetonitrile linear gradient. The isolated peptides were subjected to the solid-phase binding assay using TWD2-S as a probe. The peptide bound to TWD2-S was sequenced using the Applied Biosystems Procise 492 HT protein sequencer (Applied Biosystems, Foster City, CA, USA).

Competitive binding assay

A peptide AGFAGDDAP synthesized based on the sequence A21–P29 of chicken fast skeletal actin was subjected to competition binding assay for TWD2-S according to the method of the solid-phase binding assay using the same ELISA kit. Wells of a microplate were coated with 100 μl of 50 μg ml^{-1} chicken fast skeletal F-actin in 1× Coating Solution. After washing and blocking, 100 μl of 5 μg ml^{-1} TWD2-S was hybridized with the adsorbed F-actin for 1 h at room temperature. After washing, 100 μl of 0, 50, 100 and 150 μg ml^{-1} synthetic peptide were added to the wells and hybridized in 1× BSA Diluent/Blocking Solution included in the kit for 1 h at room temperature. Detection was performed as described in the solid-phase binding assay. Assays were done in the absence of added Ca^{2+}.

Production of anti-TWD2-S antibody and its specificity

Anti-twitchin D2 antibody used for electron microscopy was raised against TWD2-S in rabbit. The specificity of the antibody to twitchin was confirmed by immunoblotting. To confirm whether anti-TWD2-S antibody used in the present study reacts only to twitchin in ultrathin sections and isolated thick filaments, immunoblotting analysis was carried out. Myofibrillar proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane after separation on SDS-PAGE. The membrane was hybridized with anti-TWD2-S antibody for 1 h at room temperature and reacted with anti-rabbit IgG antibody conjugated with horseradish peroxidase as the secondary antibody after washing. Detection was carried out using 0.2 mg ml^{-1} 3,3′-diaminobenzidine tetrahydrochloride and 0.005% H_2O_2.

To examine the reactivity of the antibody to twitchin and its phosphorylated form, immunoblotting analysis and ELISA using TWD2-S and its phosphorylated form were performed. TWD2-S was phosphorylated as described previously (Funabara et al., 2001a). Immunoblotting was performed as described above except for twitchin peptides. ELISA was carried out using the same kit used in the solid-phase binding assay in the present study. Wells of a microplate were coated with 10, 20 and 30 μg ml^{-1} twitchin peptide, TWD2-S or its phosphorylated form, and anti-TWD2-S antibody was added to the wells after blocking. Anti-rabbit IgG antibody conjugated with alkaline phosphatase was used as the secondary antibody. All solutions for ELISA were included in the kit.

Electron microscopy

Ultrathin sections for electron microscopy were prepared from ABRM fibers fixed with 4% paraformaldehyde (pH 7.2) in active contraction raised by 1 mmol l^{-1} acetylcholine, catch state provoked by subsequent washing with artificial seawater, and relaxation after treatment with 1 μmol l^{-1} serotonin. These contraction–relaxation stages were monitored by tension measurement. Fibers showed maximum tension at 50 s after treatment with 1 mmol l^{-1} acetylcholine (at 25°C), and at this point were fixed with paraformaldehyde and represented fibers in the active state of contraction. Paraformaldehyde-fixed fibers were dehydrated with ethanol and treated with Lowicryl K4M resin. Ultrathin sections of about 80 nm were placed on Ni-150 meshes covered with collodion membranes, treated with the anti-twitchin D2 primary antibody at 0.1 mg ml^{-1} for 1 h, and then with AuroProbe EM goat anti-rabbit IgG conjugated with colloid gold as the secondary antibody for 30 min each on ice. The ultrathin sections thus prepared were stained with 1% uranyl acetate and 1% lead citrate each for 10 min at room temperature, and observed using a JEOL JEM 2000EX transmission electron microscope.

ABRM thick filaments for electron microscopy were prepared according to Nonomura's method (Nonomura, 1974). Briefly, ABRM relaxed by 1 μmol l^{-1} serotonin was treated with 0.05% saponin and the resulting skinned fibers were homogenized on ice in an appropriate volume of 10 mmol l^{-1} phosphate buffer (pH 6.8) containing 0.1 mol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} ATP. The homogenate containing thick and thin filaments was mixed with the anti-twitchin D2 antibody at 0.1 mg ml^{-1} for 5 min on ice, followed by reaction with the above secondary antibody conjugated with colloid gold for 5 min on ice. The filaments were negatively stained with 1% uranium acetate on Ni-400 meshes. A part of the negatively stained preparation was rotary shadowed with platinum, using a BAF060 freeze-etch machine (BAL-TEC AG, Liechtenstein).

Results

Inhibition of actomyosin Mg^{2+}-ATPase activity by TWD2-S

To examine the function of the D2 phosphorylation site in twitchin, a recombinant twitchin fragment (TWD2-S) was prepared that consists of the linker region including the D2 phosphorylation site (S4316) and neighboring Ig motifs

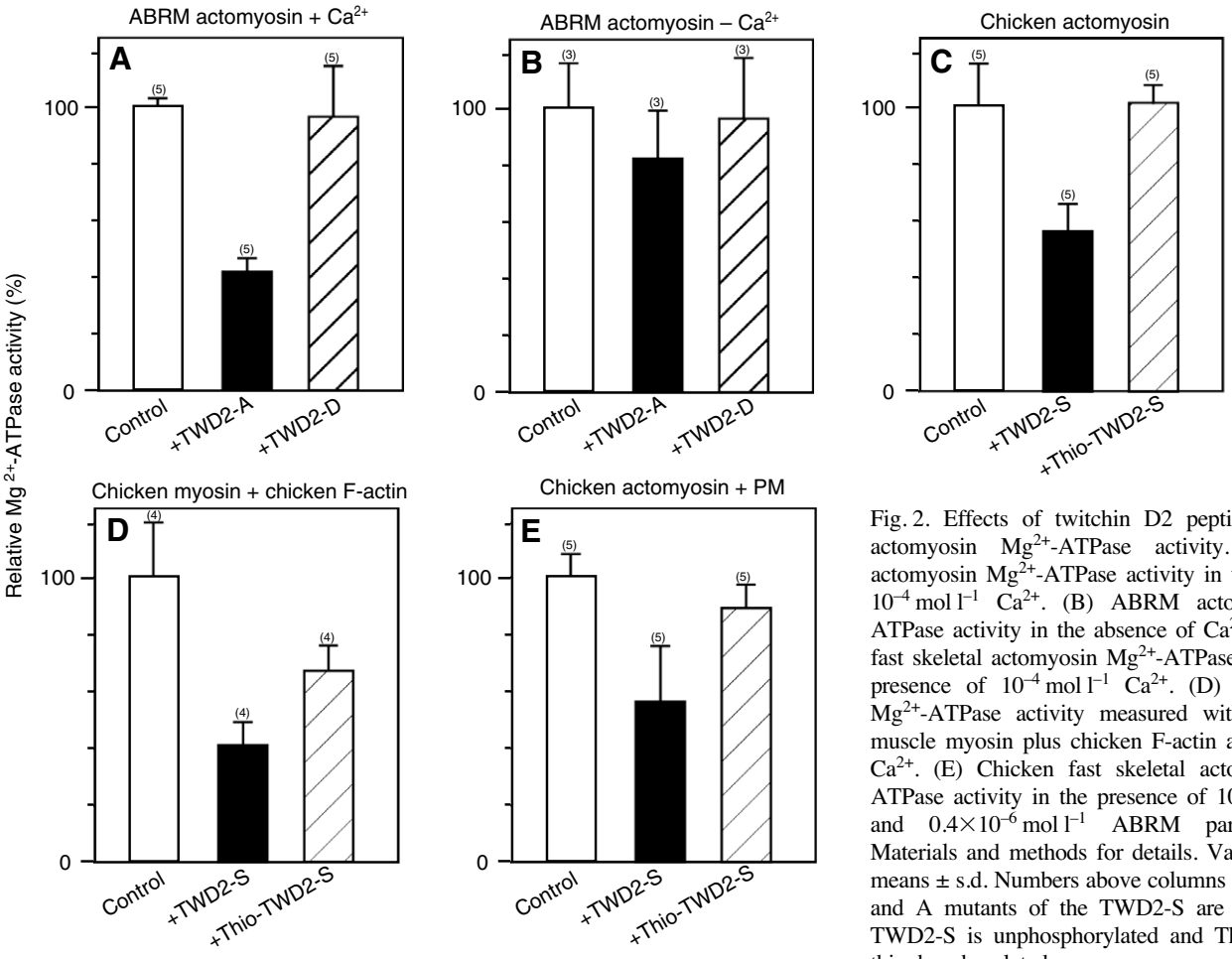

Fig. 2. Effects of twitchin D2 peptide mutants on actomyosin Mg^{2+}-ATPase activity. (A) ABRM actomyosin Mg^{2+}-ATPase activity in the presence of 10^{-4} mol l^{-1} Ca^{2+}. (B) ABRM actomyosin Mg^{2+}-ATPase activity in the absence of Ca^{2+}. (C) Chicken fast skeletal actomyosin Mg^{2+}-ATPase activity in the presence of 10^{-4} mol l^{-1} Ca^{2+}. (D) Actin-activated Mg^{2+}-ATPase activity measured with chicken fast muscle myosin plus chicken F-actin and 10^{-4} mol l^{-1} Ca^{2+}. (E) Chicken fast skeletal actomyosin Mg^{2+}-ATPase activity in the presence of 10^{-4} mol l^{-1} Ca^{2+} and 0.4×10^{-6} mol l^{-1} ABRM paramyosin. See Materials and methods for details. Values shown are means ± s.d. Numbers above columns are *N* values. D and A mutants of the TWD2-S are indicated; here TWD2-S is unphosphorylated and Thio-TWD2-S is thiophosphorylated.

(Fig. 1A). Variants of this construct were prepared in which the D2 serine was replaced by alanine (TWD2-A) or aspartic acid (TWD2-D) to represent unphosphorylated and phosphorylated D2 peptides, respectively. The constructs were subjected to phosphorylation assays with PKA to confirm these substitutions. The mutants TWD2-A and TWD2-D were not phosphorylated, as predicted. In addition these data show that no other phosphorylation sites are present in the D2 peptide (Fig. 1B).

ABRM actomyosin Mg^{2+}-ATPase activity in the presence of TWD2-A and Ca^{2+} was determined to be 40% of the original activity (i.e. in the absence of twitchin peptides) whereas TWD2-D had no effect on activity (Fig. 2A). The molar ratio of the TWD2 peptide to myosin heads in this and subsequent assays was 12:1. In the absence of Ca^{2+}, where molluscan myosin is inactivated, TWD2-A also tended to inhibit Mg^{2+}-ATPase activity, but this effect was not significantly different from that observed with TWD2-D (Fig. 2B). However, these effects were difficult to establish unequivocally since the Mg^{2+}-ATPase activity was low (Table 1). The Mg^{2+}-ATPase activity of chicken skeletal muscle actomyosin (isolated as the complex) in the presence of Ca^{2+} was decreased to 55% of the original activity, whereas thiophosphorylated TWD2-S had no effect (Fig. 2C). Similar results were obtained with chicken skeletal muscle myosin plus actin (isolated individually) as shown in Fig. 2D. The inhibitory effect of the unphosphorylated TWD2-S on chicken actomyosin Mg^{2+}-ATPase activity was the same in the presence of paramyosin (Fig. 2E) and show that paramyosin has no effect on inhibition by the peptide. Data from these assays are summarized in Table 1.

Binding to myosin, actin and paramyosin of twitchin D2 peptides

It has been claimed that intact twitchin binds to both myosin (Yamada et al., 2001) and actin (Shelud'ko et al., 2004). Thus, the binding of actin, myosin and paramyosin to the TWD2 constructs was investigated using a solid-phase binding assay (see Materials and methods). Thiophosphorylated TWD2-S was used as a probe instead of TWD2-D, because the thiophosphorylated TWD2-S mimicked the phosphorylation form more effectively than TWD2-D. For convenience (i.e. higher yield) we prepared chicken fast skeletal actin and scallop striated adductor myosin. Fig. 3A,C,E show typical results in the solid-phase binding assay for unphosphorylated TWD2-S and thiphosphorylated TWD2-S with actin, myosin and paramyosin, respectively. Unphosphorylated TWD2-S bound to each of the proteins, but not to the thiophosphorylated construct. Relative binding abilities of 10 μg ml^{-1} twitchin D2 peptides are shown in Fig. 3B,D,F. These results clearly indicate that the twitchin D2 site interacts with actin, myosin and paramyosin in a phosphorylation dependent-manner.

Table 1. *The inhibitory effects of twitchin D2 peptide on actomyosin Mg^{2+}-ATPase activity*

Actomyosin Mg^{2+}-ATPase activity (nmol Pi min^{-1} mg^{-1} actomyosin)	Control	TWD2-A	TWD2-D
ABRM actomyosin + Ca^{2+}	1.96±0.0560	0.760±0.101	1.88±0.330
ABRM actomyosin – Ca^{2+}	0.240±0.0394	0.180±0.0363	0.230±0.0532

	Control	TWD2-S	Thiophosphorylated TWD2-S
Chicken actomyosin	74.9±12.5	42.5±4.11	76.5±5.98
Chicken actomyosin + paramyosin	17.3±1.59	9.5±2.08	15.8±1.38
Chicken F-actin + chicken myosin*	35.4±8.52	15.7±1.36	23.6±2.36

*Actomyosin activity measured as nmol Pi min^{-1} mg^{-1} myosin.

Twitchin binding site on actin

The location of the D2-binding site on actin was determined. Chicken fast skeletal muscle was used as source material to obtain a higher yield. Enzymatic digests of chicken actin were separated by reverse-phase high performance liquid chromatography and each fraction was subjected to solid-phase binding assay with unphosphorylated TWD2-S (Fig. 4A,B). Only fraction 16 reacted with unphosphorylated TWD2-S (Fig. 4C) and this was subjected to a second reverse-phase chromatography. Fraction 16 was separated into three peaks and only fraction 16-2 reacted with unphosphorylated TWD2-S. Its sequence was LVCDNGS, located at the N-terminal end of actin, L10–S16. An adjacent sequence in actin, D26–D27, also is required for the binding of actin to the loop 2 region of myosin and is important for ATP-driven motility (Johara et al., 1993) (Fig. 4D). To investigate whether this region of actin (D26–D27) might be involved in binding to the D2 peptide a synthetic peptide, A21–P29, was prepared and shown to bind to the unphosphorylated TWD2-S but not the thiophosphorylated form (data not shown). This synthetic peptide competed with actin for binding to unphosphorylated TWD2-S (Fig. 4E). The cumulative evidence indicates that L10–P29, exposed on the surface of actin, is involved in interaction with twitchin (Fig. 4F).

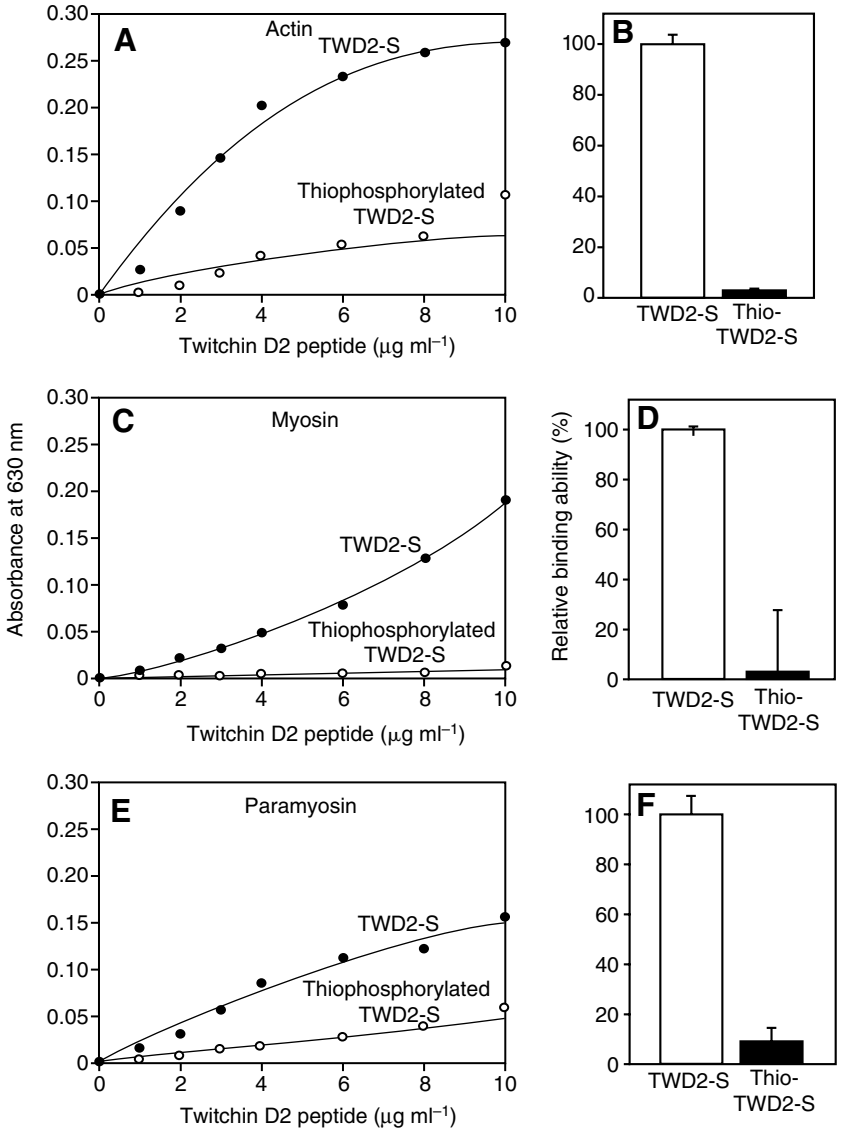

Fig. 3. Binding of twitchin D2 peptide to actin, myosin and paramyosin. (A,C,E) Typical results of solid-phase binding assays for unphosphorylated TWD2-S and thiophosphorylated TWD2-S (Thio-TWD2-S) peptides against chicken fast skeletal actin, scallop myosin and ABRM paramyosin, respectively. (B,D,F) Relative binding abilities of twitchin D2 peptide to chicken fast skeletal actin, scallop myosin and ABRM paramyosin, respectively. Values are means ± s.d. (*N*=6).

Formation of a complex of myosin, actin and TWD2-S

An essential feature of the catch mechanism is the maintenance of tension at low intracellular [Ca^{2+}] where myosin is not active. Do myosin and actin maintain a connection with twitchin during catch? To address this question, myosin and actin were mixed with unphosphorylated TWD2-S under low [Ca^{2+}] and high [ATP] to mimic catch conditions. Due to the requirements for relatively

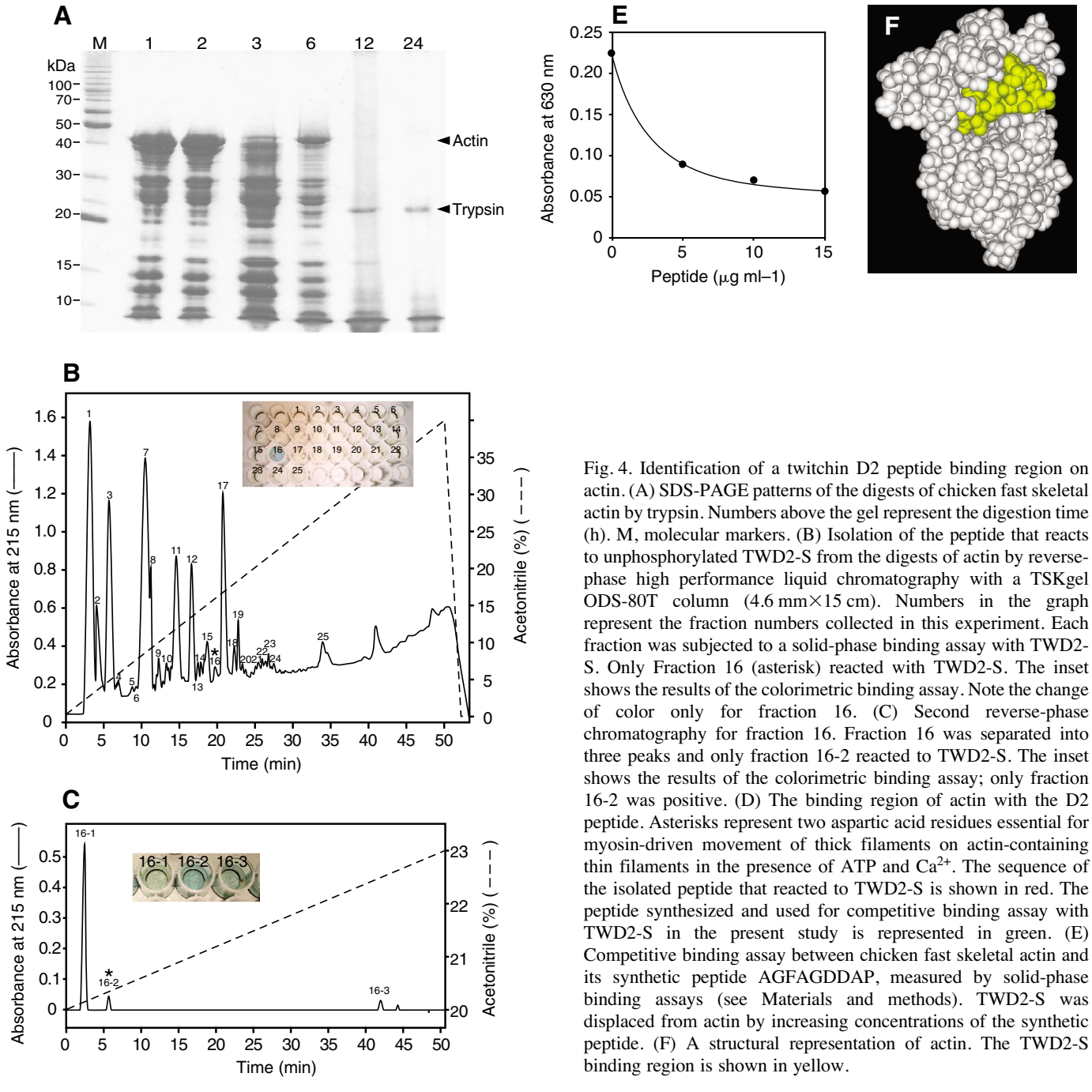

Fig. 4. Identification of a twitchin D2 peptide binding region on actin. (A) SDS-PAGE patterns of the digests of chicken fast skeletal actin by trypsin. Numbers above the gel represent the digestion time (h). M, molecular markers. (B) Isolation of the peptide that reacts to unphosphorylated TWD2-S from the digests of actin by reverse-phase high performance liquid chromatography with a TSKgel ODS-80T column (4.6 mm×15 cm). Numbers in the graph represent the fraction numbers collected in this experiment. Each fraction was subjected to a solid-phase binding assay with TWD2-S. Only Fraction 16 (asterisk) reacted with TWD2-S. The inset shows the results of the colorimetric binding assay. Note the change of color only for fraction 16. (C) Second reverse-phase chromatography for fraction 16. Fraction 16 was separated into three peaks and only fraction 16-2 reacted to TWD2-S. The inset shows the results of the colorimetric binding assay; only fraction 16-2 was positive. (D) The binding region of actin with the D2 peptide. Asterisks represent two aspartic acid residues essential for myosin-driven movement of thick filaments on actin-containing thin filaments in the presence of ATP and Ca^{2+}. The sequence of the isolated peptide that reacted to TWD2-S is shown in red. The peptide synthesized and used for competitive binding assay with TWD2-S in the present study is represented in green. (E) Competitive binding assay between chicken fast skeletal actin and its synthetic peptide AGFAGDDAP, measured by solid-phase binding assays (see Materials and methods). TWD2-S was displaced from actin by increasing concentrations of the synthetic peptide. (F) A structural representation of actin. The TWD2-S binding region is shown in yellow.

large amounts of myosin and F-actin, scallop striated adductor and chicken fast skeletal muscle were used as starting materials, respectively. The binding of myosin, actin and unphosphorylated TWD2-S was assayed under conditions mimicking catch using low-speed sedimentation at low ionic strength where myosin is insoluble and actin soluble (Fig. 5A). Initially, an excess of unphosphorylated TWD2-S or thiophosphorylated TWD2-S was used (molar ratio for twitchin construct:myosin of 50:9). Thiophosphorylated TWD2-S had no effect on the distribution of actin in the supernatant fraction (Fig. 5A). Cosedimentation of each of the three components was detected only when unphosphorylated TWD2-S was used. Subsequently, it was found that lower ratios of unphosphorylated TWD2-S:myosin also promoted cosedimentation. Even at a ratio of 1:150 (unphosphorylated TWD2-S:myosin) a trimeric complex formation was detected (Fig. 5B). It is proposed that the *in vivo*

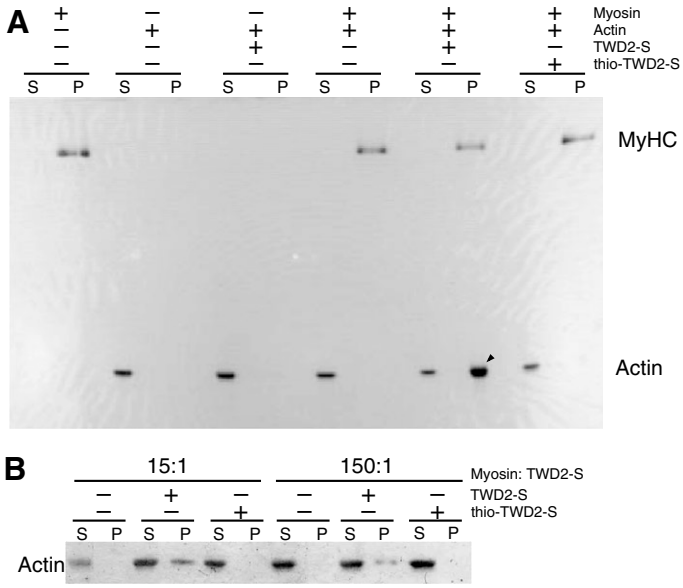

Fig. 5. The binding between scallop striated adductor myosin and chicken fast skeletal F-actin and the influence of the twitchin D2 peptide under catch conditions. SDS-PAGE results for the mixtures indicated are shown. S, supernatant; P, pellet (see Materials and methods). (A) Results using a molar ratio of peptide:myosin of 50:9. TWD2-S facilitated the binding between actin and myosin, whereas the thiophosphorylated form (thio-TWD2-S) did not. Arrowheads indicate F-actin, which co-sedimented with myosin. (B) The concentration-dependence of F-actin binding with myosin *via* TWD2-S. Samples for SDS-PAGE contained 10 μg actin (S) or myosin (P). For the actin–myosin coprecipitate, samples applied contained 10 μg myosin.

molar ratio of twitchin:myosin in catch muscle is about 1:15 (Siegman et al., 1997). These results suggest that under catch conditions the unphosphorylated D2 peptide mediates interaction between myosin and F-actin.

Twitchin localization on myofilaments

We raised an antibody against the twitchin D2 peptide using the recombinant protein (see Materials and methods). The antibody reacted to twitchin, but not to the other major components of the thick filaments, i.e. myosin heavy chain, myorod and paramyosin, as revealed by immunoblotting (Fig. 6A). The antibody reacted with both the dephosphorylated and phosphorylated D2 peptide, but the sensitivity with the dephosphorylated peptide was 10–20-fold higher (Fig. 6B,C).

Transmission electron microscopy was employed to localize twitchin in ultrathin sections of ABRM using the anti-D2 peptide antibody. Different experimental conditions with ABRM were adopted to simulate active contraction, catch state and relaxed state. The abundance of gold particles was highest for ABRM in the catch state (Fig. 7C,D) followed by a decreased distribution in the contracted state (Fig. 7A,B) and a further reduction for the relaxed state (Fig. 7E,F). These data, summarized in Table 2, suggest that twitchin in the catch state is unphosphorylated. Gold particles, which were observed only around thick filaments and not thin filaments, appeared marginally separated from the thick filament surface in active and catch states (Fig. 7B,D), while gold particles in the relaxed state were closer to the thick filaments (Fig. 7F). This slight shift of gold particles indicates that the location of the twitchin D2 site may move at different stages of muscle contraction. No gold particles were observed when ABRM was treated in the absence of the antibody (Fig. 7G,H).

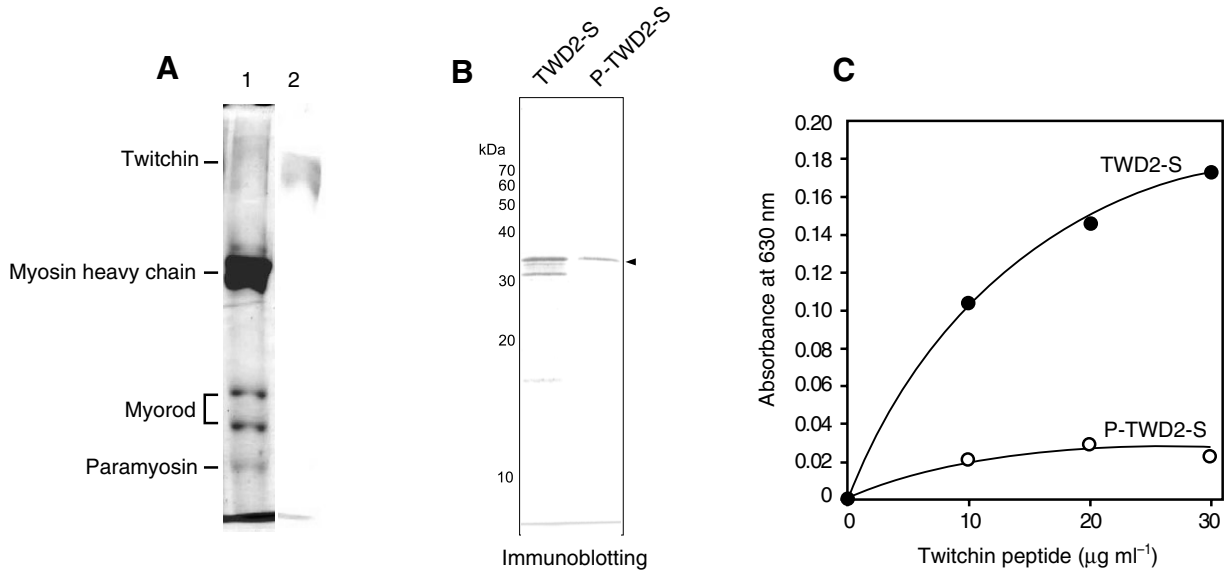

Fig. 6. The specificity of anti-twitchin D2 antibody. (A) Immunoblotting using the anti-twitchin D2 antibody against ABRM myofibrillar proteins. Lane 1, protein-loaded gel stained with Commassie Brilliant Blue; lane 2, antibody loaded onto the PVDF membrane reacted only with twitchin and not to the major components of thick filaments, myosin heavy chain, myorod and paramyosin. (B) Immunoblotting using the anti-twitchin D2 antibody against TWD2-S and its phosphorylated form (P-TWD2-S). Arrowheads indicate twitchin peptide and the positions of molecular mass markers (kDa) are shown. (C) Differences in the reactivity of the anti-twitchin D2 antibody against TWD2-S and P-TWD2-S. Closed and open circles represent TWD2-S and P-TWD2-S, respectively.

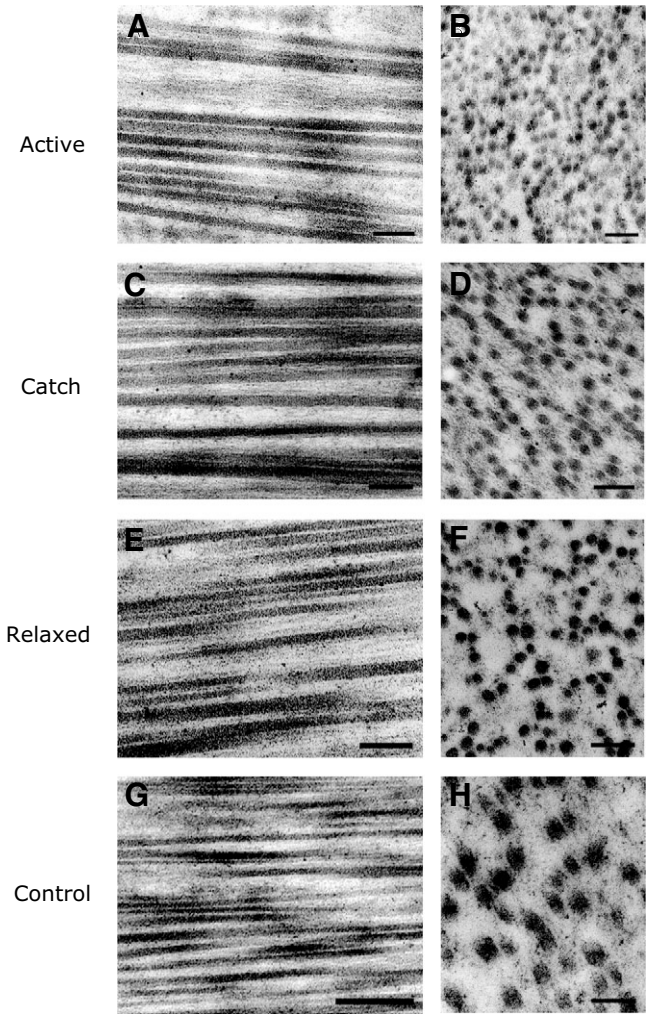

Fig. 7. Electron microscopic observation on ultrathin sections of ABRM reacted with anti-twitchin D2 peptide antibody in active contraction, catch and relaxation stages. Longitudinal (A,C,E,G) and cross-sectional (B,D,F,H) views for ABRM in active contraction (A,B), ABRM in catch (C,D), ABRM in relaxation (E,F) and ABRM without the antibody (control) (G,H). Bars, 200 nm (A–F), 500 nm (G), 100 nm (H).

Table 2. *Colloidal gold distribution on thick filaments in active, catch and relaxed fibers*

	Contractile condition			
	Active	Catch	Relaxed	Control
Gold particles (per 10 μm^2)	3.9±0.9[a]	8.5±4.5[b]	3.7±0.9[a]	0

Gold particles were measured on thick filaments per 10 μm^2 in electron micrographs. Different suffixes represent significant differences in the number of gold particles on thick filaments from different contractile conditions, as revealed by Student's *t*-test ($P<0.01$). Values are means ± s.d. for 11–12 different areas from three ultrathin sections each from active, catch and relaxed state samples except for the antibody-free control, the data of which were obtained from five different areas of three sections.

Photographs of ultrathin sections are shown in Fig. 7.

To investigate the arrangement of twitchin on the ABRM thick filaments (isolated from ABRM in the catch state) the distances between closest neighboring two gold particles (recognizing the twitchin D2 site) were measured. These were measured by modifying a measurement position perpendicular to the thick filament axis. A total of 223 measurements were made. Since larger distances are considered to be less reliable for statistical analysis, distances between two gold particles of not more than 400 nm (N=155) were subjected to frequency distribution analysis (Hasselblad, 1966) and separated into a mixture of multiple normal distributions (Fig. 8A). The distribution showed a periodicity of 36.25 nm and the estimated slope of the regression line (36.29) through the origin (Fig. 8B) was not significantly different from the observed period (F-test, P=0.91). This interval of 36.25 nm on the thick filaments is half of the 72.5 nm periodicity of paramyosin filaments (Squire, 1981).

Furthermore, electron microscopy of isolated ABRM thick filaments reacted with the anti-D2 peptide antibody demonstrated that gold particles conjugated with the secondary antibody were located periodically on the surface of the thick filaments (Fig. 9A), confirming the above observations with ultrathin sections. In fact the homogenate of ABRM prepared

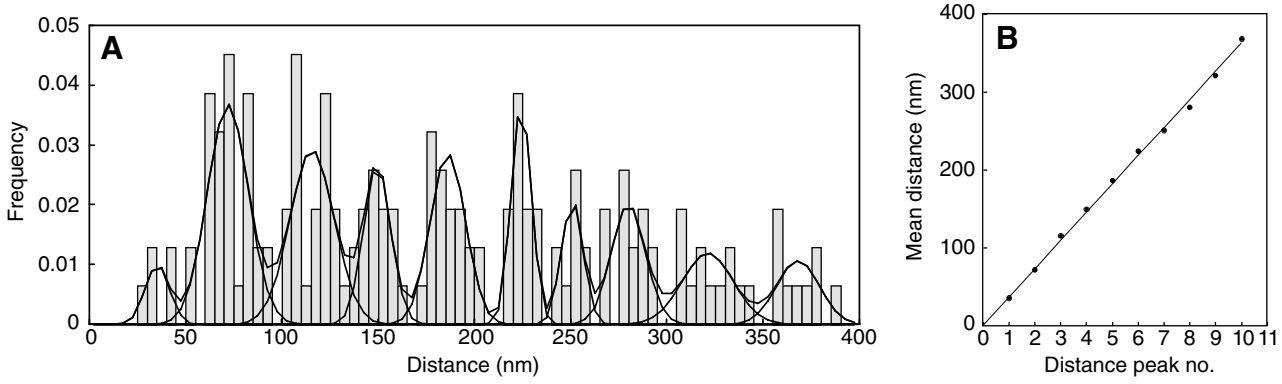

Fig. 8. Histogram showing frequency distribution of distances between any two gold particles (corresponding to the location of the D2 antibody) on the same thick filament, as seen by electron microscopy, and the statistical significance analysis. (A) Frequency was set up for a total of 155 measurements of not more than 400 nm and analyzed on the assumption of compound normal distributions with parameters indicated by the maximum likelihood method. (B) Estimated slope (36.29) of the regression line through the origin, compared to 36.25 (see text for details). The two values were not statistically different (F-test, P=0.91).

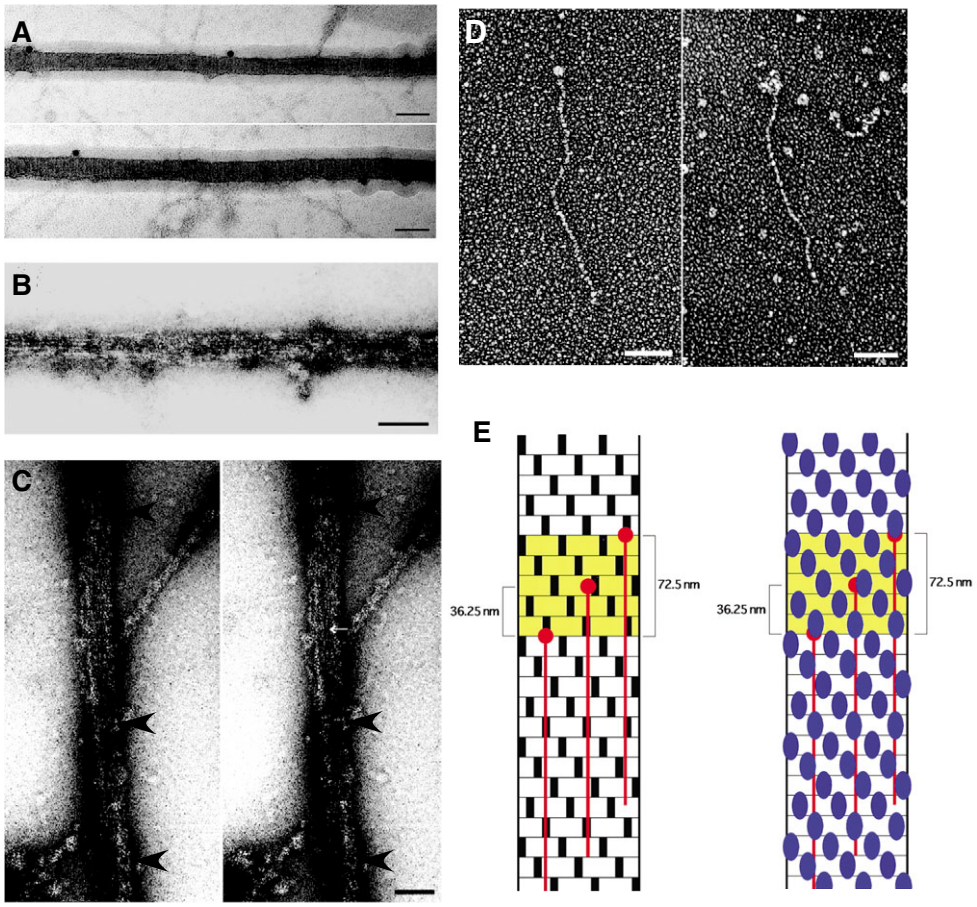

Fig. 9. Electron microscopic observation on thick filaments labeled with anti-twitchin D2 peptide antibody. (A) Electron micrographs of ABRM thick filaments labeled with the anti-twitchin D2 peptide antibody and negatively stained. Antibodies conjugated with gold particles, indicating localization of twitchin, are distributed on the surface of the filaments at intervals (upper panel) and at helical turns (lower panel). (B) Electron micrograph of a thick filament treated with low angle rotary shadowing after negative staining. The secondary antibody-conjugated gold particles are localized on globular structures. (C) Stereo views of negatively stained thick filaments. Ultrathin filaments, possibly representing twitchin molecules, expand longitudinally on the thick filament as indicated by the white arrow. Arrowheads indicate location of the antibody-conjugated gold particles. (D) Electron microscopic observation of twitchin molecules by rotary shadowing. Twitchin molecule (left) and after treatment with anti-twitchin kinase domain antibody (right). Twitchin (0.06 mg ml^{-1}) was reacted with the anti-twitchin kinase domain antibody (Funabara et al., 2001a) and mixed with 40% glycerol. This preparation, and a sample of twitchin without antibody, were sprayed onto mica and subjected to rotary shadowing using platinum and carbon as described above. (E) Models of the parallel array of twitchin molecules (red) superimposed on the Bear-Selby net pattern (Bear and Selby, 1956) and relative to myosin head distribution (blue). Bars, 100 nm (A–C), 50 nm (D). The Bear-Selby net reflects the arrangement of paramyosin molecules in the thick filament. The paramyosin molecules assemble into fibers with an axial repeat of 72.5 nm and staggering of these filaments generates the characteristic ‘checkerboard’ array of nodes. In negatively stained samples the nodes are the gaps between molecules where stain is trapped (Squire, 1981; Cohen, 1982).

in the relaxed state contained both thick and thin filaments, but only thick filaments were selected and subjected to analysis. Gold particles attached to the secondary antibody also were associated with granular structures having an average diameter of 20.2 nm, i.e. about the diameter of the myosin head (Fig. 9B), suggesting an interaction between the D2 peptide and the myosin head. Also noted were ultrathin structures of over 50 nm in length and 3.8 nm diameter that extended along the axis of the thick filaments (Fig. 9C). These dimensions suggest, tentatively, that the 50 nm structures may be part of twitchin filaments, extending along the surface of the thick filament. Resolution was not adequate to obtain more accurate estimates of the filament length. ABRM twitchin as observed by rotary shadowing is an elongated molecule approximately 225 nm in length with a spherical head (Fig. 9D), as reported elsewhere (Vibert et al., 1993). The antibody against the kinase domain (Funabara et al., 2001a) reacted to this head region (Fig. 9D). [For these experiments the antibody to the kinase domain was used since its titer for twitchin was higher than the D2 peptide antibody (data not shown).] Models of the parallel array of twitchin molecules (red) are superimposed on the Bear-Selby net pattern (Bear and Selby, 1956) and shown relative to the myosin head distribution (blue) (Fig. 9E). The D2 site is located adjacent to the kinase domain at its C-terminal edge (Fig. 1A) and thus may be positioned close to the myosin head.

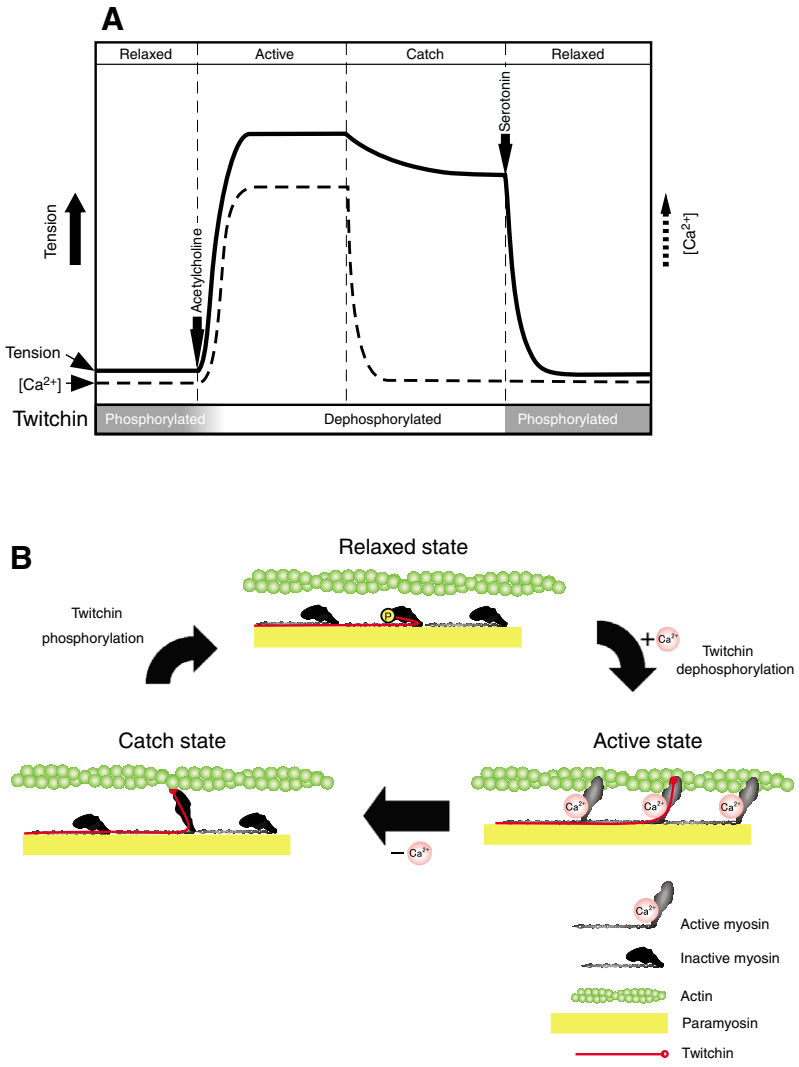

Fig. 10. A model of interactions of twitchin with myosin and actin for different stages in the contractile cycle. For explanation, see text. Tropomyosin in thin filaments is not shown.

Discussion

In the present study we have shown that the dephosphorylated D2 peptide (consisting of the D2 phosphorylation site, S4316, flanked by two Ig motifs) binds to both F-actin and myosin, but the phosphorylated form does not bind. The binding site on actin was located within the sequence L10–P29 and this region also binds to loop 2 of the myosin head. This interaction between myosin and actin is essential to initiate cross-bridge cycling (Johara et al., 1993). Thus it is expected that binding of either myosin or twitchin to this N-terminal region of actin would be competitive. However, this competition would occur only with unphosphorylated D2 peptide. The binding site for the D2 peptide on myosin remains to be determined, but the electron microscopy data suggests an interaction with the myosin head. As emphasized, the D2 peptide interaction with myosin is phosphorylation-dependent, and it is not known if other phosphorylation-independent links are formed between intact twitchin and the contractile apparatus. The finding that twitchin remains attached to thick filaments isolated under various conditions suggest that there are other twitchin–thick filament interactions (with myosin and/or paramyosin). We propose that a critical feature of the catch mechanism is the phosphorylation-dependent interaction of the twitchin D2 region with both F-actin and myosin. This complex does not involve cross-bridge–actin interactions. The locations of the actin-binding and the myosin-binding sites on the D2 peptide are not known. It is assumed that distinct sites are involved (possibly in the two flanking Ig motifs) and that both are regulated by phosphorylation of D2. It is not necessary that direct binding to the D2 site (S4316) is involved, but this could occur for one of the sites. In addition to twitchin there are several muscle proteins that contain Ig motifs [e.g. titin, myosin light chain kinase (MLCK), telokin and the myosin-binding proteins (MyBPs)] and the ability of certain Ig motifs to bind to myosin has been documented. For example, in cardiac MyBP-C the interaction with myosin was mapped to the C-terminal Ig motif (Alyonycheva et al., 1997). Subsequently, it was found that the unphosphorylated MyBP-C motif bound to the S2 region of myosin and influenced cardiac mechanics. Interestingly, phosphorylation of the MyBP-C motif by PKA abolished these effects by eliminating binding to myosin (Kunst et al., 2000). In cardiac muscle the stoichiometry of MyBP-C to myosin heads is about 1:8 (Kunst et al., 2000). The binding of Ig motifs to actin is not well documented, although the N-terminal two Ig motifs of long MLCK have been implicated in actin binding (Yang et al., 2006).

These results are consistent with those of other investigators (see Introduction) and, for example, may explain the myofilament interconnections proposed by Andruchova et al. (Andruchova et al., 2005). The competition between the unphosphorylated D2 peptide and loop 2 of myosin for the binding site on actin also may be pertinent to the results of Butler et al. regarding the low- and high-force states (Butler et al., 2006). A higher affinity of myosin for actin in the high-force state would effectively displace the twitchin–actin interaction. This is consistent with the observation (Butler et al., 2006) that activation of the catch muscle by Ca^{2+} increases the proportion of myosin in the high-force state and decreases interaction of twitchin with actin. Transition to the low-force state, by decreasing Ca^{2+} levels, would favor the twitchin–actin interaction. It should be emphasized that a direct role for Ca^{2+} in binding of the D2 peptide to either actin or myosin is not suggested and there is no evidence from our data to implicate a Ca^{2+} sensitive step, other than the regulation of actin-activated ATPase by Ca^{2+} binding to myosin. Dephosphorylation of twitchin by calcineurin (Castellani and Cohen, 1992; Yamada et al., 2004) would be coincident with initiation of contraction since both events are Ca^{2+} dependent, and thus for most of the contractile phase twitchin is dephosphorylated and theoretically

available to compete with myosin for actin. In practice, twitchin does not inhibit the *in vivo* contraction of ABRM fibers in the presence of Ca^{2+} (Siegman et al., 1998). A competition between twitchin and myosin might explain the *in vitro* inhibition of Mg^{2+} ATPase activity of actomyosin by relatively high concentrations of TWD2-S.

Results from the localization of twitchin on the thick filament indicate that the twitchin molecules are distributed on the surface of the filament at a periodicity of 36.25 nm, half of the 72.5 nm periodicity of paramyosin filaments (Squire, 1981). It is suggested that the D2 site at the C-terminal end of twitchin aligns with myosin heads, i.e. at half of the 72.5 nm axial periodicity (Cohen, 1982). In the model proposed in Fig. 9E the twitchin molecule could extend through about three of the 72.5 nm repeats or nodes, if arranged parallel to the filament axis. The myosin rod extends axially through two nodes. Thus there is a possibility of other interactions between twitchin and the myosin rod. The molar ratio of twitchin to myosin is about 1:15 in ABRM catch muscle (Siegman et al., 1997). Therefore, even if all of the twitchin molecules interact with myosin, the number of trimeric complexes is small compared with total myosin. It is not known if there are cooperative effects on neighboring myosin molecules induced by the binding of twitchin to myosin. The ultrathin filaments observed above on the surface of the thick filaments are thought to be twitchin and these reflect the underlying Bear-Selby net (Bear and Selby, 1956) (see Fig. 9C).

There are several aspects of the catch mechanism to be resolved. In an earlier report (Funabara et al., 2003) it was stated that phosphorylation of both the D1 and D2 sites is required for relaxation from the catch state. The D1 site is unusual in that relatively high levels of phosphorylation (40–50%) are found while catch is maintained. The D2 site is more sensitive to effects of phosphorylation; low levels of phosphorylation are found during catch and high levels of phosphorylation accompany the release from catch (Funabara et al., 2003). The more marked phosphorylation dependence of the D2 site prompted this present study, but the role of the D1 site is not understood and should be resolved by further studies. Another area for future study is to identify the phosphorylation-dependent binding site on myosin for the D2 peptide and also to determine if other interactions between native twitchin and myosin occur. If present, these could anchor the twitchin molecule to the surface of the thick filament. Based on the *in vitro* results of Yamada et al. (Yamada et al., 2001) it is assumed that paramyosin is not an essential component of the catch mechanism, but whether it has any influence on catch under *in situ* conditions remains to be determined. The phosphorylation-dependent binding of the D2 peptide to paramyosin is shown above. It is known that in fibers of molluscan smooth muscle the catch state is sensitive to pH (Hopflinger et al., 2006) and is reduced on moderate alkalinization to pH 7.2–7.7, i.e. at those pH values used for the binding experiments described above. It is important to determine if any of the interactions between isolated proteins show a marked pH dependence and this can be tested experimentally. On the other hand, the sensitivity to pH may be associated only with the intact contractile system. Recently, it was found that striated muscles from oyster and scallop contain twitchin and this regulates interaction between thin and thick filaments at low [Ca^{2+}] (Tsutsui et al., 2007), as it does in molluscan catch muscle. These results suggest a similar role for twitchin in striated and smooth molluscan muscle and the molecular scheme outlined above may be applicable to both muscle types.

In summary, our results provide novel data on the molecular interactions involved in catch. In Fig. 10A the different phases of the contractile cycle in catch muscle are shown, with associated changes in [Ca^{2+}], tension and twitchin phosphorylation. Starting with relaxed muscle (Fig. 10B), twitchin is phosphorylated (by PKA) and the D2 peptide does not interact with either actin or myosin. It is assumed that in the relaxed state there are no interfilament connections. Stimulation by acetylcholine increases [Ca^{2+}], which promotes contraction (by binding to and activating myosin) and activates calcineurin. Twitchin is dephosphorylated but does not bind to actin (although theoretically this interaction is still possible) because of competition with the increased population of high-force states, i.e. the cross-bridge–actin interaction predominates. The onset of catch follows a reduction in [Ca^{2+}] to close to resting levels and inactivation of myosin. The low-force state (the detached cross-bridge being part of the low-force state) allows the D2 twitchin region to bind to F-actin and myosin and form a mechanical force-bearing complex. In this model there is no direct effect of Ca^{2+} on the binding of the unphosphorylated D2 peptide to either myosin or actin and our hypothesis is that the obligatory role for a reduction in Ca^{2+} to promote the catch state reflects the inactivation of myosin and an increase in low-force state. This complex is proposed to represent at least part of the mechanical connection (catch bridge) between the myofilaments. Serotonin release causes an increase in [cAMP] and activation of PKA. One of the PKA sites on twitchin is the D2 site and its phosphorylation eliminates binding of the D2 peptide to actin and myosin. The catch connection is lost and the muscles enters the relaxed phase.

We thank Dr M. J. Siegman and Dr T. M. Butler for their advice and Dr Takashi Yamakawa for help in statistical analysis. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to D.F.) and Japan-US Cooperative Science Program from Japan Society for the Promotion of Science (to S.W.) and NIH grant HL-23615 (to D.J.H.).

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