Projectin-Thin Filament Interactions and Modulation of the Sensitivity of the Actomyosin ATPase to Calcium by Projectin Kinase*

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The insect muscle protein projectin (900 kDa) belongs to a novel family of cytoskeleton-associated protein kinases (titin, twitchin, and projectin) that are members of the immunoglobulin superfamily. The functions of these kinases are still unknown although recent data suggest a role in modulating muscle activity and generating passive elasticity. An important question is what are the in vivo substrates for these enzymes. We found a thin filament-associated 30 kDa protein that acts as an in vitro substrate for projectin kinase from Locusta migratoria. However, we did not find activators for projectin kinase. Neither calcium, calcium with calmodulin, nor cAMP activated the in vitro activity of projectin kinase. Binding studies revealed a strong interaction between projectin and thin filaments comparable with that of the projectin-myosin interaction. That an interaction might be possible in vivo is suggested by immunological studies showing that projectin is attached to the surface of myosin filaments. Since the molecular weights indicate that the 30 kDa protein might be tropinin I, which is known to play a central role in modulating cardiac contractile activity, we studied whether phosphorylation of this protein by projectin changes the calcium sensitivity of the actomyosin ATPase. We found a significant increase in the calcium sensitivity. Thus, our results indicate the existence of a novel mechanism of regulation of muscle activity by a cytoskeleton-associated kinase.

Accessory proteins closely associated with actin filaments, tropinin, and tropomyosin, mediate the Ca$^{2+}$ regulation of skeletal muscle contraction (reviewed in Ref. 1). Moreover, there is evidence for the involvement of a myosin-linked regulatory system in vertebrate skeletal and cardiac muscle during modulation of muscle contraction (2–4). Most invertebrate muscles are characterized by both myosin and actin-linked regulation (5). The molecular mechanism of myosin filament mediated regulation of skeletal muscle is still little understood. Phosphorylation of the myosin light chains (MLC)\(^1\) is supposed to play a central role in the regulation of skeletal muscle activity as in smooth muscle contraction (3, 6).

The discovery of a novel family of myosin filament-associated protein kinases, such as titin, twitchin, and projectin, homologous to MLC kinases of smooth and skeletal muscles (7–9) raises the question of whether these participate in the regulation of muscle contraction. These extraordinarily large protein kinases are classified as intracellular members of the immunoglobulin protein superfamily on the basis of their molecular structure (7, 8, 10). Members of this family are the giant mammalian titin (3 MDa, reviewed in Ref. 11) and the invertebrate mini-titins (0.6–1.2 MDa, reviewed in Ref. 12).

Mini-titins are distributed throughout the A band of invertebrate muscles, but in insect flight muscles, they are located in the I band (15–21). First indications of the possible functions of these proteins have been derived from genetic studies on the nematode Caenorhabditis elegans. Mutants of the unc-22 gene encoding twitchin are characterized by a repetetive twitching of the body wall muscle and by an abnormal sarcomere structure (7), suggesting that twitchin-like molecules are directly involved in modulating muscle activity and are also essential for ordered sarcomere assembly. Findings that projectin is able to determine the length of myosin filaments in vitro confirm the idea that these proteins are molecular directives for myosin filament assembly (22). Nevertheless, the function of the kinase domain is still unknown. The recent report on the x-ray crystal structure of the kinase domain of twitchin has given insight into the regulatory power of this kinase and provides evidence for an intrasteric mechanism of protein kinase regulation employing an autoregulatory sequence (23). In vitro autophosphorylation has been described for the bacterially expressed kinase of twitchin from C. elegans (24) and for other members of this family including insect projectin kinase from Drosophila leg and flight muscle (8), crustacean projectin kinase from crayfish tail muscle (25) and mollusc twitchin kinase from Aplysia (26). An endogenous in vitro substrate for mollusc twitchin is the regulatory MLC (27). In contrast, in vitro studies on the kinase catalytic core of twitchin from C. elegans expressed in Escherichia coli, indicate that this twitchin differs in substrate specificity (24). Although synthetic MLC peptides homologous to a part of chicken smooth muscle MLC proved a useful model substrate, MLC peptides derived from C. elegans itself were weakly phosphorylated by twitchin kinase (24). In addition, intact chicken smooth muscle MLC failed as a substrate (24). So far, in vivo substrates for twitchin and other members of the twitchin and titin-like kinases are still unknown. Projectin kinase of insects has been less well studied with respect to potential substrates, even though identification of substrates for mini-titin of different invertebrate species would provide an essential clue for understanding their functions.

In this paper, we demonstrate that projectin from insect muscles is able to autophosphorylate in vitro. Moreover, we
identify thin filament-associated 30-kDa protein, not a MLC, as a substrate for projectin. We show that phosphorylation affects the sensitivity of actomyosin ATPase to Ca^{2+}. Immunoelectron microscopy studies provide evidence that projectin attaches to the surface of myosin filaments. Finally, in vitro binding studies reveal a strong interaction between projectin and thin filaments.

**EXPERIMENTAL PROCEDURES**

**Purification of Projectin and Myosin—**Myofibrils were prepared from front and hind leg muscles from the grasshopper Locusta migratoria as described by Ettlinger et al. (28). Projectin and myosin were isolated from leg and flight muscle myofibrils as described by Kölsch et al. (22). Washed myofibrils were extracted in 20 mM Na2PO4, 1 mM CaCl2, and 10 mM Tris-HPO4, pH 8.3, for 1 h by stirring on ice. The insoluble material was removed by centrifugation for 10 min at 20,000 × g. For projectin purification, the supernatant was subjected to chromatography on Mono-Q-FPLC (Amersham Pharmacia Biotech). Subsequently, the pellet was used for myosin extraction in 40 mM Na2PO4, 10 mM ATP, 10 mM MgCl2, 5 mM EGTA, and 10 mM Tris-HCl, pH 7.5. Unsoluble proteins were removed by centrifugation for 2 h at 20,000 × g, and the supernatant was applied to Mono-Q-FPLC. Equilibration buffer for projectin chromatofocusing contained 10 mM Na2PO4, 10 mM Tris-HPO4, pH 7.5. For myosin chromatography, it contained 40 mM Na2PO4, 10 mM NaCl, 10 mM Tris-HCl, pH 7.5. Proteins were eluted from the column with a linear salt gradient (0–0.5 M NaCl in equilibration buffer). All preparation steps were done at 4°C. Protein concentration was measured according to Lowry et al. (29). The purity of protein preparations was judged by SDS-PAGE using silver and Coomassie Blue staining for protein detection. Proteins were electrophoresed in SDS-poly-acrylamide slab gels with the Laemmli buffer system (30) and 2–14% acrylamide gradient.

**Generation of Antibodies against Projectin—**Rabbits were immunized subcutaneously with 0.5 mg of purified projectin in complete Freund’s adjuvant. They were boosted twice at 3-week intervals with 0.25 mg of projectin in incomplete Freund’s adjuvant. Serum was collected 2 weeks after the last boost. Immunoglobulins were precipitated with 50% (w/v) ammonium sulfate, resuspended in PBS (1/8 of starting volume) and dialyzed against PBS. Antibodies specific for projectin were isolated by passing the dialyzed probe over a projectin affinity column and eluting the bound antibodies with 0.2 M NaCl in equilibration buffer. All preparation steps were done at 4°C. Protein concentration was measured according to Lowry et al. (29). The purity of protein preparations was judged by SDS-PAGE using silver and Coomassie Blue staining for protein detection. Proteins were electrophoresed in SDS-poly-acrylamide slab gels with the Laemmli buffer system (30) and 2–14% acrylamide gradient.

**Rabbit Anti-Projectin Antibodies—**Rabbits were immunized subcutaneously with 0.5 mg of purified projectin in complete Freund’s adjuvant. They were boosted twice at 3-week intervals with 0.25 mg of projectin in incomplete Freund’s adjuvant. Serum was collected 2 weeks after the last boost. Immunoglobulins were precipitated with 50% (w/v) ammonium sulfate, resuspended in PBS (1/8 of starting volume) and dialyzed against PBS. Antibodies specific for projectin were isolated by passing the dialyzed probe over a projectin affinity column and eluting the bound antibodies with 0.2 M NaCl in equilibration buffer. All preparation steps were done at 4°C. Protein concentration was measured according to Lowry et al. (29). The purity of protein preparations was judged by SDS-PAGE using silver and Coomassie Blue staining for protein detection. Proteins were electrophoresed in SDS-poly-acrylamide slab gels with the Laemmli buffer system (30) and 2–14% acrylamide gradient.

**Renaturing Blotting Assay—**Autoanphosphorylating endogenous kinases of a myofibril extract, which was also used for chromatographic purification of projectin, were detected using a modified renaturing blotting assay as described by Kato et al. (31). The extract was mixed with an equal volume of sample buffer which contained 5.5% (w/v) SDS, 12.5% (v/v) 2-mercaptoethanol, 12.5 mM EDTA, 156 mM Tris, pH 6.8, 4 mM urea, and 0.5% (w/v) DTT. Protein preparations were transferred to polyvinylidene difluoride (Immobilon P; Millipore, 0.45 μm pore size) membranes in 192 mM glycine, 25 mM Tris at 150 mM for 30 min (semidyel blot). Blots were incubated for 1 h at room temperature with gentle rocking in 7 ml guanidine hydrochloride, 50 mM Tris, 50 mM dithiothreitol, 2 mM EDTA, 0.1% (v/v) Triton X-100, pH 8.3. Enzymes were renatured in 50 mM Tris, 2 mM dithiothreitol, 2 mM EDTA, 0.1% (v/v) Tween 20 for 12 h at 4°C. We did not use casein in the renaturing solution and did not block the membrane after renaturation of enzymes as originally described. To determine unspecific binding of ATP to proteins on the membrane, blots were incubated with [γ-32P]ATP and various Ca^{2+} concentrations (5 mM CaCl2 or 5 mM CaCl2 and 5 mM EGTA or 5 mM EGTA). Projectin was added to a final concentration of 1.0 μg/ml and myosin or filaments or crude extracts to a final concentration of 0.5 mg/ml. Proteins were preincubated for 5 min, and the reactions were stopped after 15 min at 30°C by the addition of an equal volume of a 2-fold concentrated sample buffer (4% (w/v) SDS, 12.5% (v/v) 2-mercaptoethanol, 12.5 mM EDTA, 120 mM Tris, pH 6.8, 0.001% (w/v) bromophenol blue and 4 μM urea). Proteins were separated by SDS-PAGE. Gels were fixed, stained with Coomassie Brilliant Blue, dried, and used for autoradiography (exposure overnight with intensifying screens).

**Solid Phase Binding Assay—**Purified projectin from locust flight muscles was biotinylated with N-biotinyl-e-aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim) for 20 min at 4°C. After centrifugation, the supernatant was separated from unbound biotin by gel filtration on a SephAdex G-25. Unlabeled proteins were immobilized on polyacrylamide ELISA wells at an amount of 300 ng well for saturation. After blocking with 1% (w/v) BSA, biotinylated projectin was added to each well at different concentrations in high ionic strength buffer containing 0.5 mM NaCl in PBS, pH 7.2, and incubated for 1 h at 37°C. The wells were washed five times with PBS, pH 7.2. After reaction with avidin-peroxidase in PBS with 0.1% (w/v) BSA and with 0.05% (v/v) Tween 20, detection was started with a substrate solution using H2O2 and O-phenylenediamine (Sigma) as the chromogen. One molar H2SO4 was used to stop the reaction. The absorbance was measured with a micro-ELISA reader (Dynatech) at 492 nm.

**ATPase Assays—**Thin filaments (250 μg/ml), isolated from locust flight leg muscles as described previously (33), were preincubated with active or with heat-inactivated (1 min at 65°C) projectin kinase (25 μg/ml) in 150 mM NaCl, 10 mM histidine, pH 7.0, 50 mM MgCl2, 10 mM ATP, and variable calcium concentrations for 30 min at 30°C. The free Ca^{2+} concentration was adjusted according to Portzehl et al. (34). ATPase reactions were started by the addition of myosin (250 μg/ml) and stopped after 10 min with an equal volume of 10% (v/v) trichloroacetic acid. ATPase activity of myosin was determined by measuring the amount of inorganic phosphate as described previously (35).

**RESULTS**

To characterize the pattern of protein phosphorylation by endogenous kinases, extracts of purified myofibrils were incubated with [γ-32P]ATP and various Ca^{2+} concentrations. Fig. 1 shows the protein composition of myofibrils. Reaction mixtures...
were analyzed by SDS-PAGE and autoradiography (Fig. 4). Several phosphoproteins were detected in these experiments, and projectin was labeled strongly with $^{32}$P using leg and flight muscle extracts. The pattern of protein phosphorylation was not influenced by calcium, but phosphorylation of projectin decreased slightly in the presence of calcium (results not shown).

In an initial step, we tested whether locust projectin exhibits kinase activity. The kinase activity of projectin was determined with a renaturing blotting assay. Myofibrillar extracts were subjected to SDS-PAGE, and separated proteins were transferred to membranes. After renaturation of proteins, blot strips were incubated with $[^{32}P]ATP$. Autoradiography revealed three autophosphorylating kinases in myofibrillar extracts from leg and flight muscles (Fig. 2A). Control strips that had been incubated with $[^{32}P]ATP$ showed that the radioactive labeling was not caused by unspecific binding of ATP to proteins (Fig. 2A). The high molecular weight band on the autoradiogram (Fig. 2A) shows the SDS-PAGE analyses of the projectin preparation. The corresponding autoradiogram (Fig. 2A) shows the India ink-stained blots (from left to right: lanes 1 and 4) and autoradiograms (from left to right: lanes 2, 3, 5, and 6). Positions of protein molecular weight standards analyzed in parallel are indicated on the left, and numbers indicate the molecular mass in kDa. Note that projectin and myosin preparations were free of contaminating proteins. Considering the molecular weight, the thin filament preparation consists predominantly of actin (42 kDa), troponin T (55 kDa), tropomyosin (38 kDa), proteins in the range of 30 kDa (probably troponin I isoforms), troponin C (20 kDa), and some protein of higher molecular mass of unknown identity (42 kDa). The 200-kDa band was not a myosin contamination as determined by immunoblots with anti-myosin antibodies (results not shown).

[Fig. 1. SDS-PAGE analyses of myofibrils, thin filaments, myosin, and projectin preparations. Lanes 1–8 (from left to right) show Coomassie Blue-stained gels (2–14% gradient), and lanes 9 and 10 show silver-stained gels (2–14% gradient). Note that lanes 9 and 10 are derived from different gels than lanes 6–8, and therefore the mobility of projectin differs slightly. Samples are indicated on top. Position of protein molecular weight standards analyzed in parallel are indicated on the left, and numbers indicate the molecular mass in kDa. Note that projectin and myosin preparations were free of contaminating proteins. Considering the molecular weight, the thin filament preparation consists predominantly of actin (42 kDa), troponin T (55 kDa), tropomyosin (38 kDa), proteins in the range of 30 kDa (probably troponin I isoforms), troponin C (20 kDa), and some protein of higher molecular mass of unknown identity (42 kDa). The 200-kDa band was not a myosin contamination as determined by immunoblots with anti-myosin antibodies (results not shown).]

[Fig. 2. Autoradiography of renaturated projectin. Western blots of myofibrillar extracts from locust leg and flight muscle are indicated on top. Renaturation of kinases was performed as described under “Experimental Procedures” and incubated with $[^{32}P]ATP$ (α) or $[^{32}P]ATP$ (γ). Panel A shows the India ink-stained blots (from left to right: lanes 1 and 4) and autoradiograms (from left to right: lanes 2, 3, 5, and 6). Positions of protein molecular weight standards analyzed in parallel are indicated on the left, numbers indicate the molecular mass in kDa. In addition to projectin, autophosphorylation (radioactively labeled high molecular weight protein on the top of the blot) at least two other kinases were detected by this method. Panel B shows autophosphorylation of renaturated projectin from leg muscle in the presence of 5 mM EGTA, or 5 mM Ca$^{2+}$, or 5 mM Ca$^{2+}$ and 0.5 μg/ml calmodulin, or 10 μM AMP. Autophosphorylation of projectin did not change significantly under these conditions.

In a next step we isolated thin (native actin) filaments to determine whether thin filament-associated proteins are targets for projectin kinase. The protein composition of the thin filaments was analyzed by SDS-PAGE (see Fig. 1). The major proteins of the locust thin filaments were actin (42 kDa) and the regulatory proteins: troponin T (65 kDa), tropomyosin (38 kDa), troponin I (32 and 30 kDa), and troponin C (approximately 20 kDa) (41). The higher molecular mass proteins were not characterized. Immunoblots with anti-myosin antibodies as well as electron microscopy revealed that the preparation was not contaminated with myosin (results not shown). Projectin was incubated with thin filaments, $[^{32}P]ATP$ and varying calcium concentrations. Reaction mixtures were analyzed by SDS-PAGE (Fig. 5A) and autoradiography (Fig. 5B). Fig. 5B shows that a protein with a molecular weight of 30 kDa (arrow in Fig. 5B) was phosphorylated in the presence of projectin. Multiple bands including a 32-kDa band are phosphorylated in the control, where thin filaments were incubated without projectin, indicating that the thin filaments contain associated kinases. A 30-kDa phosphoprotein, however, is only visible if...]

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Projectin is added to a thin filament phosphorylation assay. The 30-kDa protein is not a contamination of the projectin preparation as shown in the autophosphorylation assay (Fig. 5). The 55-kDa protein that has been weakly phosphorylated in the autophosphorylation assay (Fig. 5) is possibly a degradation product of projectin. The intensity of this band is slightly enhanced in the presence of calcium, suggesting a calcium-dependent degradation of projectin by proteases (results not shown).

We have not been able to identify the 30-kDa protein up to now because there are few data about the protein composition of thin filaments from *Locusta migratoria*, and sequence data on locust troponin are lacking. However, for thin filaments of other invertebrates, proteins with a molecular mass of approximately 30 kDa have been identified as troponin I, the inhibitory component of the troponin complex (36–40). Since the molecular mass of the 30 kDa protein, phosphorylated by projectin, indicated that it might be troponin I, we reasoned that phosphorylation of this protein might affect the actomyosin ATPase activity. To determine whether this is the case, we performed ATPase assays with isolated myosin and the same thin filaments we used for phosphorylation studies at varying calcium concentrations. In Fig. 6 the ATPase activity of protein preparations of flight and leg muscles is plotted against the calcium concentration. To rule out that the Ca$^{2+}$-sensitivity might be influenced by steric parameters, the experiments were performed with heat-inactivated projectin kinase (80 °C for 1 min) before preincubation as control. The half-maximal ATPase activity ($pC_{50}$) was increased from 5.85 to 6.35 when thin filaments were preincubated with active projectin kinase before starting the reaction by the addition of myosin (Fig. 6A). For proteins of leg muscles, $pC_{50}$ was increased from 5.77 to 6.22
The binding of projectin was achieved when 30 nM projectin was added per well (Fig. 7). Higher projectin concentrations resulted in decreased binding of projectin to thin filaments or to myosin, represented as % 

**DISCUSSION**

The data presented here demonstrate the kinase activity of projectin, a twitchin-like muscle protein of insects. In addition to autophosphorylation, which has already been described for Drosophila projectin (8), we identified an in vitro substrate for projectin kinase activity. Using isolated thin filaments as a substrate in kinase assays, we showed that projectin phosphorylates thin filament-associated 30 kDa protein. On the basis of its molecular mass, we tentatively assume this protein to be troponin I. Furthermore, we have shown that phosphorylation of the 30-kDa protein increases the Ca^{2+} sensitivity of the actomyosin ATPase activity. We did not find a significant effect of Ca^{2+}-calmodulin, cAMP, and calcium on autophosphorylating activity.

Earlier studies on the molecular structure of the kinase domain have indicated that MLC may be an in vivo substrate for projectin because the kinase domains resemble strongly the catalytic domain of MLC kinase (7, 8). However, in our studies, we failed to phosphorylate MLC with projectin. In contrast, mollusc twitchin does phosphorylate MLC (27). So far it is unclear whether this different substrate specificity in vitro reflects physiological differences in the activation of muscle contraction in mollusc and insect muscles. Mollusc muscles contain only a myosin filament-associated regulatory system (41), whereas in most other invertebrates, regulatory mecha-
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C. elegans electron microscopy (indirect immunogold labeling).

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Control sections were exposed to nonimmune rabbit IgG. Note the deposition of gold particles at both sides of the Z disc. Aplysia asynchronous flight muscles of Phormia terrea novea. Immunogold labeling of an ultrathin section of LR White-embedded flight muscles (5). Immunogold labeling of isolated locust flight muscle thick (arrow) and thin (arrowheads) filaments with anti-projectin IgG results in deposition of gold particles along the thick filaments (G). Panel H shows control. No gold particles were found on thin filaments. The average diameter of the gold particles is 10 nm. Scale bars = 500 nm.

Fig. 8. Localization of projectin in situ revealed by immunoelectron microscopy (indirect immunogold labeling). A and B, asynchronous flight muscles of Phormia terrea novea. Panel B shows the labeling of an ultrathin section of LR White-embedded flight muscles with anti-projectin IgG. Panel A shows a serial section that was exposed to nonimmune rabbit IgG. Note the deposition of gold particles at both sides of the Z disc. C—H, synchronous flight muscle and leg muscle of L. migratoria. Ultrathin sections of LR White resin embedded leg (D) and flight (F) muscles were exposed to anti-projectin antibodies. Gold particles are clearly restricted to the A band in both muscle types. Control sections were exposed to nonimmune IgG (C and E). Immunogold labeling of isolated locust flight muscle thick (arrow) and thin (arrowheads) filaments with anti-projectin IgG results in deposition of gold particles along the thick filaments (G). Panel H shows control. No gold particles were found on thin filaments. The average diameter of the gold particles is 10 nm. Scale bars = 500 nm.

nisms exist that are mediated by both actin and myosin filaments (5).

For the understanding of the regulation of twitchin-like kinases and the signaling pathways involved, it is important to obtain insight into the functions of these enzymes. It has been suggested that calmodulin could activate twitchin and titin because the sequence of the autoinhibitory region of twitchin kinase is similar to that of the calmodulin binding domain and because binding of calmodulin to twitchin and titin has been shown (42). However, although calmodulin binds to Aplysia twitchin, it does not activate it (26). We likewise did not find an activation of projectin kinase by Ca\(^{2+}\)-calmodulin. Heierhorst et al. (43) have recently reported that Ca\(^{2+}\)-S100A1, a member of the large Ca\(^{2+}\)-binding protein family, greatly enhances the enzyme activity of the autoinhibited Aplysia twitchin fragment TWK-43 and C. elegans twitchin. These authors also demonstrated that the S100A1-binding site is a part of the autoregulated sequence positioned in the active site responsible for intrasteric autoinhibition of twitchin kinase. Twitchin is the first enzyme to be described that is activated by a member of the S100 family. Because twitchin and projectin are closely related proteins, it will be interesting to determine whether S100A1 protein also activates insect projectin.

cAMP and Ca\(^{2+}\) intracellular signaling pathways interact at several levels in the hierarchy of control (44). Regarding this aspect, it is noteworthy that mollusc twitchin has been identified as the major substrate for neuropeptide-activated cAMP-dependent protein kinase and that the phosphorylation state was shown to correlate with the extent of neuropeptidergic modulation of muscle relaxation in vivo (45). We have not yet determined whether locust projectin is a target for cAMP-dependent kinases. In vertebrate cardiac muscles, cAMP pathways are known to be involved in the regulation of muscle contraction. cAMP-dependent phosphorylation of troponin I decreases the Ca\(^{2+}\)-sensitivity of the ATPase of cardiac actomyosin (46). Despite the evolutionary divergence between vertebrates and invertebrates, a similar mechanism, with troponin as a target for protein kinases, might exist in invertebrate muscles. Our result that phosphorylation of a 30-kDa protein associated with thin filaments increases the sensitivity of actomyosin to Ca\(^{2+}\) points to this new aspect of invertebrate muscle regulation.

Our finding that projectin does not phosphorylate MLC raises the question how does projectin interact with thin filaments in vivo. To determine whether such an interaction as we have observed in in vitro binding studies is possible with projectin and thin filaments in vivo, we studied the arrangement of projectin within myosin filaments in more detail. Previous information on the localization of projectin in synchronous insect flight muscles is based on immunofluorescence studies (12). However, interpretation of fluorescence staining may be complicated since immunoreactivity may reflect either abundance or accessibility of the labeled species. We have tried to overcome this problem using two different approaches, labeling of isolated filaments and postembedding immunogold labeling. The results of these studies indicate that projectin binds to the surface of the myosin filaments along their whole length. Isolated thin filaments were not labeled with antibodies against projectin. This may be because of the isolation conditions for myosin and thin filaments used in this study. Confirmatory results have recently arisen from experiments which show that titin inhibits thin filament motility in vitro in a calcium-dependent manner (47). The authors also demonstrated that titin interacts with native thin filaments and F-actin and that increasing calcium concentration results in enhanced binding.

Another aspect of the possible physiological function of the thin filament-projectin interaction is the proposed elasticity of this class of molecules. The molecular structure of these proteins makes them a plausible candidate for generating passive elasticity in muscles, and for titin, new data corroborating this idea exist (13, 14). The sticky proteins consist predominantly of fibronectin type III-like domains and IgG-like domains (7, 8, 10). The fibronectin type III-like domains of this protein class are thought to constitute the molecular basis for elasticity as one domain can reversibly unfold from 4 to 29 nm (48). Because projectin in many invertebrate muscles obviously does not anchor the myosin filaments in the Z disc as titin does, it is difficult to explain how projectin should generate passive tension. An elastic connection would be possible, however, if projectin binds to thin filaments.

If troponin I is an in vivo substrate for projectin, both enzyme and substrate would be fixed within the cell. Interaction could
be controlled by the dynamics of the cytoskeleton, which therefore would be involved in the regulation of the enzyme activity. We postulate that the projectin-thin filament interaction could represent an intracellular sensor that could provide dynamic feedback to the cytoskeleton by transforming mechanical into biochemical signals.

Our results on projectin and thin filament interactions may give new insight into the function of these cytoskeleton-associated protein kinases. Studies with specific inhibitors like antibodies which inhibit projectin kinase activity in muscles will be a powerful tool for investigating whether a correlation exists between the phosphorylation of actin filament proteins by projectin kinase and muscle activity.

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REFERENCES
1. Farah, C. S., and Reinach, F. C. (1995) FASEB J. 9, 755–767
2. Vandenoorn, R., Grange, R. W., and Houston, M. E. (1995) Am. J. Physiol. 37, 596–603
3. Grange, R. W., Vandenoorn, R., and Houston, M. E. (1993) Can. J. Appl. Physiol. 18, 229–242
4. Gautel, M., Zuffardi, O., Freiburg, A., and Labeit, S. (1995) EMBO J. 14, 1952–1960
5. Lehman, W., and Szent-Györgyi, A. (1975) J. Gen. Physiol. 66, 1–30
6. Tothong, R., Yamashita, H., Graham, M., Haeberle, J., Simcox, A., and Maughan, D. (1995) Nature 374, 650–653
7. Benian, G. M., Kiff, J. E., Neckelmann, N., Moereman, D. G., and Waterston, R. H. (1989) Nature 342, 45–50
8. Ayne-Southgate, A., Southgate, R., Saide, J., Benian, G. M., and Pardue, M. L. (1995) J. Cell Biol. 128, 393–403
9. Olson, N.-J., Pearson, R. B., Needleman, D., Hurwitz, M. Y., Kemp, B. E., and Means, A. R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2284–2288
10. Labeit, S., Gautel, M., Lakey, A., and Trinick, J. (1992) EMBO J. 11, 1711–1716
11. Keller, C. S. (1995) Curr. Opin. Cell Biol. 7, 32–38
12. Ziegler, C. (1994) Comp. Biochem. Physiol. A. 109, 823–833
13. Horowitz, R., Kemper, E. S., Bishop, M. E., and Podolsky, R. J. (1986) Nature 323, 160–164
14. Horowitz, R., Maruyama, K., and Podolsky, R. J. (1989) J. Cell Biol. 109, 2169–2176
15. Hu, D. H., Matsuura, A., Teraoka, K., Matsuura, T., Kimura, S., and Maruyama, K. (1990) J. Muscle Res. Cell Motil. 11, 497–511
16. Lakey, A., Ferguson, C., Labeit, S., Reedy, M., Larkins, A., Butler, G., Leonard, K., and Bullard, B. (1996) EMBO J. 9, 3459–3467
17. Vigoreaux, J. O., Saide, J. D., and Pardue, M. L. (1991) J. Muscle Res. Cell Motil. 12, 340–354
18. Manabe, T., Kawamura, Y., Higuchi, H., Kimura, S., and Maruyama, K. (1993) J. Muscle Res. Cell Motil. 14, 655–665
19. Vibert, P., Edelstein, S. M., Castellani, L., and Elliott, B. W., Jr. (1993) J. Muscle Res. Cell Motil. 14, 598–607
20. Kawamura, Y., Suzuki, K., Kimura, S., and Maruyama, K. (1994) J. Muscle Res. Cell Motil. 15, 623–632
21. Ohtani, Y., Maki, S., Kimura, S., and Maruyama, K. (1996) Tissue Cell. 28, 1–8
22. Kolesch, C., Ziegler, C., and Beinbrech, G. (1995) Naturwissenschaften 82, 239–241
23. Hu, S. H., Parker, M. W., Lei, J. Y., Wilce, M. C., Benian, G. M., and Kemp, B. E. (1994) Nature 369, 581–584
24. Lei, J., Tang, X., Chambers, T. C., Pohl, J., and Benian, G. M. (1994) J. Biol. Chem. 269, 21078–21085
25. Maroto, M., Vinos, J., Marco, R., and Cervera, M. (1992) J. Mol. Biol. 224, 287–291
26. Heierhorst, J., Probst, W. C., Vilim, F. S., Buku, A., and Weiss, K. R. (1994) J. Biol. Chem. 269, 21086–21093
27. Heierhorst, J., Probst, W. C., Kohnazki, R. A., Buku, A., and Weiss, K. R. (1995) Eur. J. Biochem. 233, 426–431
28. Etlinger, J. D., Zak, R., and Fishman, D. A. (1976) J. Cell Biol. 74, 123–143
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. Kato, M., Hagihara, M., and Hidaka, H. (1992) Biochem. J. 281, 339–342
32. Huxely, H. E. (1965) Sci. Am. 213, 18–27
33. Kendrick-Jones, J., Lehman, W., and Szent-Györgyi, A. G. (1970) J. Mol. Biol. 44, 313–326
34. Portzehl, H., Caldwell, P. C., and Ruegg, J. C. (1965) Biochim. Biophys. Acta 79, 581–591
35. Rockstein, M., and Herron, R. E. (1951) Anal. Chem. 23, 1500–1501
36. Ojima, T., Toyoguchi, T., and Nishita, K. (1995) Fisheries 61, 871–875
37. Barbas, J. A., Galceran, J., Krab-Jentgens, I., De La Pompa, J. L., Canal I., Pungo, O., and Ferrus, A. (1991) Genes Dev. 5, 132–140
38. Beall, C. J., and Fryberg, E. (1991) J. Biol. Chem. 266, 941–951
39. Nishita, K., and Ojima, T. (1990) J. Biochem. (Tokyo) 108, 677–683
40. Lehman, W. B., Bullard, B., and Hammond, K. (1974) J. Gen. Physiol. 63, 553–563
41. Szent-Györgyi, A. G., and Szentkirály, E. M. (1973) J. Mol. Biol. 74, 179–203
42. Gautel, M., Castiglione-Morelli, M. A., Pfühl, M., Motta, A., and Pastore, A. (1995) Eur. J. Biochem. 230, 752–759
43. Heierhorst, J., Heer, B., Feil, S. C., Parker, M. W., Benian, G. M., Weiss, K. R., and Kemp, B. E. (1996) Nature 380, 636–639
44. Cohen, P. (1988) Proc. R. Soc. Lond. B Biol. Sci. 234, 115–144
45. Probst, W. C., Cropper, E. C., Heierhorst, J., Hooper, S. L., Jaffe, H., Vilim, F., Beuhsenau, S., Kupfermann, I., and Weiss, K. R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8487–8491
46. Buss, J. E., and Stall, J. T. (1977) FEBS Lett. 73, 101–104
47. Herold, P. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10114–10118
48. Kellermayer, M. S. Z., and Granzier, H. L. (1996) FEBS Lett. 380, 281–286