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Enzymatic activity and filament assembly of *Acanthamoeba* myosin II are regulated by adjacent domains at the end of the tail

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Polyclonal antibodies raised against a synthetic peptide consisting of the last 19 amino acids at the end of the coiled-coil region of the heavy chains inhibited the actin-activated Mg^{2+}-ATPase activity of myosin II and its ability to form filaments. Antibodies against a synthetic peptide corresponding to the 21 adjacent amino acids at the beginning of the non-helical tailpiece, which include the three regulatory phosphorylatable serines, had no effect on either activity.

Myosin; Actin activation; ATPase; Filament formation

1. INTRODUCTION

Each of the two heavy chains of *Acanthamoeba* myosin II [1–3] consists of a globular head region of molecular mass about 90 kDa, which contains the actin- and ATP-binding sites [4], and an extended α-helical tail of molecular mass about 81 kDa, which joins with the tail of a second heavy chain to form a coiled-coil rod approx. 90 nm long [5]. An endogenous kinase phosphorylates three serine residues [6] that are located at positions 11, 16 and 21 from the COOH-terminus [5,7] of each heavy chain within a 29-amino-acid residue non-helical tailpiece. Phosphorylation reversibly inhibits actin-activated Mg^{2+}-ATPase activity [6,8–10].

Partial digestion of myosin II with α-chymotrypsin [7] releases a 66-amino-acid peptide (comprising the last 37 amino acids of the helical region and the non-helical tailpiece) from the COOH-terminus of each heavy chain. Chymotrypsin-cleaved myosin II is unable to form bipolar filaments and has no actin-activated Mg^{2+}-ATPase activity, although it retains full Ca^{2+}-ATPase activity and can associate into parallel dimers [11,12]. These, and other [5,11–15], data suggest that a small region at the COOH-terminus of the heavy chains is essential for the formation of bipolar filaments, that bipolar filaments are necessary for actin-activated Mg^{2+}-ATPase activity, and that phosphorylation regulates this enzymatic activity by affecting filament conformation. Kiehart and Pollard's observations [16–18] that monoclonal antibodies, that were shown by electron microscopy to bind near the end of the tail, inhibit the actin-activated Mg^{2+}-ATPase activity of myosin II and cause the filaments to depolymerize are consistent with these ideas.

Now, we have raised polyclonal antibodies to two synthetic peptides with sequences corresponding to the very end of the helical region and to the adjacent beginning of the non-helical tailpiece of the myosin II heavy chain. These antibodies have provided sensitive site-specific probes with which to investigate the roles of these two regions of the molecule in regulating filament assembly and actin-activated enzymatic activity of myosin II.

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2. EXPERIMENTAL

Acanthamoeba myosin II [19], dephosphorylated myosin II [8] and actin [20] were prepared as described. Peptides were synthesized on a Vega coupler 250 solid-phase peptide synthesizer using the standard Merrifield procedure [21,22]. Peptide purity was confirmed by reverse-phase HPLC, amino acid analysis and amino acid sequencing.

Antibodies were raised in rabbits by injecting the peptides cross-linked to bovine serum albumin [23,24]. Antibodies were purified by ammonium sulfate precipitation, chromatography on DE52 (Whatman) to remove non-IgG components, and removal of anti-albumin antibodies by passage through an albumin-Sepharose CL-4B column, and concentrated by ultrafiltration. Rabbit polyclonal antibodies were also raised against native myosin II. Actin-activated Mg^{2+}-ATPase and Ca^{2+}-ATPase assays [8,9], protein determinations [25], polyacrylamide gel electrophoresis [26], and electroblotting [27] were performed by published procedures.

3. RESULTS

3.1. *Peptides and antibodies*

The sequence of the COOH-terminal 79 amino acids of the heavy chains of *Acanthamoeba* myosin II and the locations of the sequences corresponding to the two synthesized peptides are shown in fig.1: peptide P1 corresponds to the first 21 amino acids of the predicted non-helical tailpiece (including the 3 phosphorylatable serines), and peptide P2 corresponds to the immediately preceding 19 amino acids at the end of the long segment that defines the α-helical coiled coil region. The antibodies raised against P1 (αP1) and P2 (αP2) had the expected specificities. Both reacted in immunoblots with intact myosin II heavy chain but not with the chymotrypsin-cleaved heavy chain, which lacks the COOH-terminal 66 amino acids (fig.1), whereas antibodies raised against native myosin II (αMII) bound to both the intact and modified heavy chains (not shown).

The antibody reactions were quantified by ELISA (fig.2). Each antibody had higher titer with the peptide against which it was raised than with myosin II (fig.2A,B), and neither reacted with the other peptide (not shown). Whereas αMII had identical titer against phosphorylated and dephosphorylated myosin (fig.2A), αP1 had a 2-fold higher titer against dephosphorylated than phosphorylated myosin (fig.2B), and αP2 consistently showed a slightly higher titer (1.5-fold) against phosphorylated than dephosphorylated myosin (fig.2C). The higher titer of αP1 with

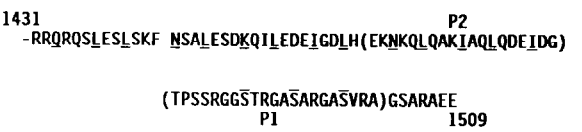

Fig.1. Amino acid sequence of the COOH-terminal 79 amino acids of the heavy chains of *Acanthamoeba* myosin II, residues 1431–1509 [5,7]. The lines beneath the sequence identify the predominantly hydrophobic amino acids that occur at alternating intervals of 3 and 4 beginning at residue 847 and ending abruptly at residue 1479 [5]. The lines above the sequence identify the three serine residues whose phosphorylation inactivates the actin-activated Mg^{2+}-ATPase activity of native myosin II. The space following Phe 1443 indicates the site of cleavage by α-chymotrypsin that removes the COOH-terminal 66 residues. The sequences corresponding to the synthesized peptides P1 and P2 are shown in parentheses.

dephosphorylated myosin II is not surprising as this antibody was raised against an unphosphorylated peptide. The same differential affinity of αP1 was obtained with myosins denatured by heat or guanidine (not shown). This indicates that the slightly lower affinity of αP1 for phosphorylated myosin is due directly to the substitution of phosphoserines for serines rather than to different conformational states of this epitope in the phosphorylated and dephosphorylated proteins. On the other hand, the differential binding of αP2 to the phosphorylated and dephosphorylated myosins was eliminated when the myosins were denatured. This suggests that phosphorylation of the non-helical tailpiece may cause a slight unfolding or increase in mobility of the adjacent helical, coiled-coil region which is the epitope for αP2. Results consistent with these conclusions were obtained in competition experiments using soluble monomeric and filamentous dephosphorylated and phosphorylated myosin II (not shown).

3.2. *Effects of αP1 and αP2 on the enzymatic activity of myosin II*

In the experiments described in fig.3, monomers of dephosphorylated myosin II were incubated with serial dilutions of αP1 and αP2 for 30 min and then assayed for actin-activated Mg^{2+}-ATPase activity after dilution into the assay buffer. Enzymatic activity was strongly inhibited by αP2 whereas αP1 had no effect. The inhibition was not the result of non-specific cross-linking of myosin molecules since monovalent Fab fragments of αP2 also inhibited enzymatic activity (not shown). The

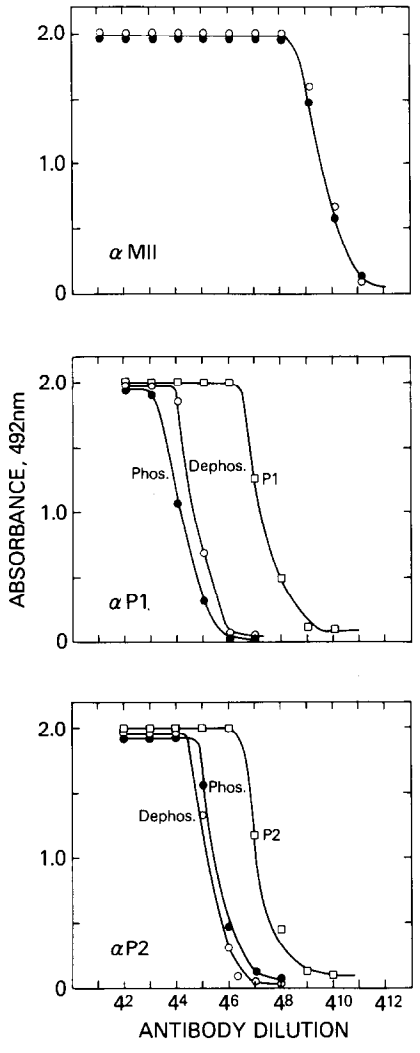

Fig.2. ELISA of antibodies against dephosphorylated and phosphorylated myosin II and synthetic peptides P1 and P2. 100 μl/well of 20 μg/ml dephosphorylated (○) or phosphorylated (●) myosin II or synthetic peptides P1 or P2 (□) in 20 mM phosphate (pH 7.3) and 120 mM NaCl were incubated at 37°C for 12 h in Immulon II microtiter plates (Dynatech, Alexandria, VA). Unbound antigen was removed by washing with 20 mM phosphate (pH 7.3) and 120 mM NaCl containing 0.05% Tween 20 (Sigma). 100 μl of 4-fold serial dilutions of αMII, purified αP1, or purified αP2 in the washing buffer were added to the wells and the plates were incubated for 1 h at 37°C. The unbound antibody was washed off, and 100 μl of a standard dilution of horseradish peroxidase conjugated to goat anti-rabbit IgG antibody (Boehringer) added to each well. After incubation at 37°C for 1 h and washing, the bound antibody was visualized using *o*-phenylenediamine (Sigma) as a substrate. The color was quantified by absorbance at 492 nm using a Chromoscan EIA reader.

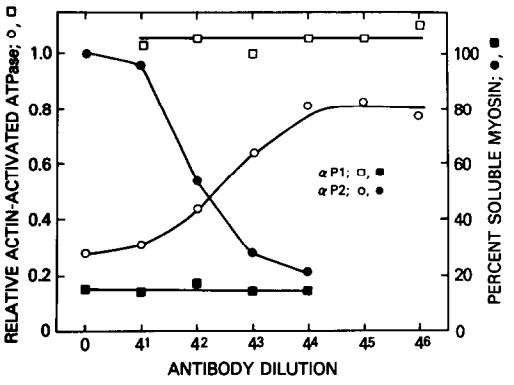

Fig.3. Effect of αP1 and αP2 on actin-activated Mg^{2+}-ATPase activity and filament formation of dephosphorylated myosin II. For Mg^{2+}-ATPase assays, 10 μg dephosphorylated myosin II in a final volume of 25 μl was incubated with 4-fold serial dilutions of αP1 or αP2 in 10 mM phosphate (pH 7.3) for 30 min at room temperature. The myosin was diluted to a concentration of 40 μg/ml in the enzyme assay buffer in which the F-actin concentration was 50 μg/ml. For filament formation assays, 30 μg dephosphorylated myosin II in a final volume of 75 μl was incubated with 4-fold serial dilutions of αP1 or αP2 in 10 mM phosphate (pH 7.3) for 30 min at room temperature. Samples were then diluted into Mg^{2+}-ATPase assay conditions and duplicate samples were then either centrifuged at 30 lb/inch^2 for 10 min in a Beckman airfuge or allowed to stand at room temperature. Aliquots (75 μl) of the supernatant of each sample were carefully aspirated and analyzed by polyacrylamide gel electrophoresis together with equal aliquots of the samples that were not centrifuged. The gels were stained and destained, the protein-bound Coomassie blue was eluted with 1 ml of 25% pyridine in H_2O, and its absorbance measured at 595 nm to estimate the percentage of myosin that remained soluble.

non-physiological, unregulated Ca^{2+}-ATPase activity of myosin II was not inhibited by either αP1 or αP2 at the highest concentrations that could be tested (not shown). Neither αP1 nor αP2 inhibited the actin-activated Mg^{2+}-ATPase activity when they were added to filaments of myosin II.

Since only filamentous myosin II expresses actin-activated Mg^{2+}-ATPase activity, we tested the abilities of αP1 and αP2 to block the formation of filaments which normally occurs when monomeric myosin II is diluted into the enzyme assay buffer (fig.3); αP1 had no detectable effect but αP2 inhibited filament formation. However, the concentration of αP2 necessary to prevent 50% of the myosin from forming filaments was about 3-fold higher than that required to inhibit the actin-activated Mg^{2+}-ATPase activity. In a control

experiment, the Fab fragment of αP2 also blocked formation of myosin II filaments (not shown).

4. DISCUSSION

Our data are consistent with the previous evidence that only filaments of myosin II express actin-activated Mg^{2+}-ATPase activity [11,14], and that antibodies that bind close to the carboxyl end of the heavy chain can inhibit both filament formation and actin-activated Mg^{2+}-ATPase activity [16–18]. The present data extend those earlier results by showing that antibodies to the 19 amino acids at the end of the α-helical coiled-coil region are inhibitory while antibodies to the immediately following 21 amino acids at the beginning of the non-helical tailpiece, which include the regulatory phosphorylatable serines, are without effect. The importance of the very end of the helical region for both filament formation and enzymatic activity suggests that phosphorylation of the regulatory serines in the non-helical tailpiece may inactivate myosin II by affecting the conformation of this neighboring helical region. Some support for this idea comes from the fact that phosphorylation of myosin II increases its affinity for αP2 whose epitope lies within that helical region.

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