Dictyostelium Myosin Heavy Chain Kinase A Subdomains

COILED-COIL AND WD REPEAT ROLES IN OLIGOMERIZATION AND SUBSTRATE TARGETING*

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Myosin heavy chain kinase A (MHCK A) participates in the regulation of cytoskeletal myosin assembly in Dictyostelium, driving filament disassembly via phosphorylation of sites in the myosin tail. MHCK A contains an amino-terminal coiled-coil domain, a novel central catalytic domain, and a carboxyl-terminal domain containing a 7-fold WD repeat motif. We have overexpressed MHCK A truncation constructs to clarify the roles of each of these domains. Recombinant full-length MHCK A, MHCK A lacking the predicted coiled-coil domain, and MHCK A lacking the WD repeat domain were expressed at high levels in Dictyostelium cells lacking endogenous MHCK A. Biochemical analysis of the purified proteins demonstrates that the putative coiled-coil domain is responsible for the oligomerization of the MHCK A holoenzyme. Removal of the WD repeat domain had no effect on catalytic activity toward a synthetic peptide, but did result in a 95% loss of protein kinase activity when native myosin filaments were used as the substrate. Cellular analysis confirms that the same severe loss of activity against myosin occurs in vivo when the WD repeat domain is eliminated. These results suggest that the WD repeat domain of MHCK A serves to target this enzyme to its physiological substrate.

Conventional myosin (myosin II) is involved in a wide range of contractile events in eukaryotic cells. In Dictyostelium, genetic and cellular analyses have demonstrated roles for myosin in the maintenance of cortical tension, cytokinesis, morphogenesis, capping of receptors, and cell locomotion (1–5). Although the roles of myosin II appear similar in many cell types, the in vivo mechanisms regulating myosin II assembly and activity in nonmuscle cells are not well understood.

Assembly of Dictyostelium myosin II bipolar filaments can be regulated by phosphorylation on the myosin II heavy chain (MHC). Two distinct MHC kinases (MHCKs) have been purified and cloned from Dictyostelium. A 130-kDa MHCK (MHCK A), discussed below, is expressed during both growth and development. An 84-kDa MHCK that is expressed only during development appears to contain two distinct catalytic domains, one related to protein kinase C (6–8) and one related to diacylglycerol kinases (9). Both MHCKs are capable of phosphorylating threonine residues on the myosin tail and can drive myosin bipolar filament disassembly in vitro. A third kinase, related to MHCK A, has recently been cloned from Dictyostelium (10), but it is not yet established whether this protein regulates myosin assembly in vivo. The in vitro target sites of MHCK A have been mapped to threonines 1823, 1833, and 2029 in the tail region of the MHC (11, 12). The physiological significance of these sites was demonstrated by mutating the sites either to alanine (3X ALA myosin) to create a non-phosphorylatable MHC or to aspartic acid (3X ASP myosin) to mimic phosphorylated MHC (13). In vivo, cells expressing 3X ALA myosin display severe myosin II overassembly in the cytoskeleton, whereas cells expressing 3X ASP myosin display severely reduced myosin II assembly in the cytoskeleton, resulting in a block in cytokinesis and development. The 3X ASP cells are also unable to complete development. In all tested assays, the 3X ASP cells are phenotypically identical to MHC null cells (mhc−). These studies indicate that the MHCK A target sites play a critical role in regulated myosin II assembly in vivo and that filament assembly is required for myosin function in vivo.

Subsequent cellular analysis of Dictyostelium MHCK A null cells (mhc A−) and overexpressing cell lines (MHCK A+) indicated that MHCK A regulates the cytoskeletal myosin II assembly level during both growth and development (14). The mhc A− cells are viable, but display partial overassembly of myosin II in the cytoskeleton. MHCK A+ cells display reduced myosin II assembly in the cytoskeleton and reduced efficiency of growth in suspension and fail to complete development. The MHCK A+ cell phenotype resembles those of the mhc− cells and the 3X ASP cell lines, indicating that overexpressed MHCK A can drive myosin filament disassembly in vivo.

Molecular analysis of the MHCK A sequence (15) indicates that it has an amino-terminal domain that is predicted to have an α-helical coiled-coil structure, a catalytic domain that is not related to conventional protein kinases, and a carboxyl-terminal domain that contains the 7-fold WD repeat motif (16, 17) characteristic of β-subunits of heterotrimeric G proteins. MHCK A is activated 50-fold by autophosphorylation (18), and autophosphorylation is increased by polyamines such as DNA, heparin, phosphatidylserine, and phosphatidylinositol (19).

It has been demonstrated recently that the MHCK A catalytic domain is a prototype for a completely novel family of protein kinases unrelated to the conventional eukaryotic protein kinase superfamily (10, 15, 20–22). This novel group includes mammalian elongation factor-2 kinase, also known as calcium/calmodulin-dependent protein kinase III. Its novel structure and catalytic domain, we have performed a structure-function dissection of MHCK A to identify the biochemical and physiological roles of the domains that flank the
catalytic domain. The results reported here indicate that the amino-terminal coiled-coil domain is responsible for MHCK A oligomerization and that the WD repeats are important for full activity against native myosin II.

EXPERIMENTAL PROCEDURES

Dictyostelium Growth and Development—Cells were cultured axenically as described previously (14). All cell lines described here are derivatives of the parental line JH10 (23). For developmental studies, cells were harvested in log phase growth and plated on starvation plates containing 20 mM MES, 0.2 mM CaCl2, 2 mM MgSO4, and 1.5% agar, pH 6.8.

Plasmid Constructs and Transformations—DNA manipulations were done according to standard procedures (24). The plasmid pLMHCK has been previously described (14). This construct expresses residues 8–1146 of the mhck A DNA from the extrachromosomal vector pLittle, with constitutive expression driven by an actin-15 promoter. pLMHCK and other constructs were transfected into mhck A as described previously (14). Initial selections for these transfections were done using 10 μg/ml G418. Once transfected cell lines were established, G418 was raised to 50 μg/ml for cell line maintenance. Truncation constructs were generated as modifications of pLMHCK, in the same vector. In each case, inserts were fused to an amino-terminal 6-histidine tag derived from the vector pDNA-HC (25). The ΔWD-MHCK construct also bears a carboxyl-terminal 6-histidine tag. Neither 6-histidine tag proved reliable for purification from Dictyostelium. Nickel chelation chromatography was therefore not used for the purification reported here. The insert for pLΔCoil-MHCK expresses residues 499–1146 of mhck A and relies upon the native MHCK A stop codon for translation termination. The insert for pLΔWD-MHCK expresses residues 8–844 of mhck A and includes 19 residues of vector-derived coding region at the carboxyl terminus.

Protein Purification and MHCK A Kinase Assay—MHCK A was purified from Dictyostelium mhck A+ cells as described previously (26) with modifications described below. The location of MHCK A activity in column eluants was determined using a peptide phosphorylation assay. Assays for MHCK A activity were performed at 23 °C in assay buffer containing 10 mM TES, pH 7.0, 2 mM MgCl2, 1 mM EDTA, 500 μM γ-32PATP (100–500 cpm/pmol), and 30 μM peptide MH-1. The previously described peptide MH-1 (RRKKFGESEKTKTEFL) (18), which corresponds to amino acids 2020–2035 in the Dictyostelium myosin II heavy chain, was dissolved in 10 mM TES, pH 7.0. Protein purification column elution fractions were assayed by the addition of 2 μl of each fraction to 48 μl of assay buffer. The tube was vortexed and incubated for 2 min at 25 °C. The reaction was terminated by spotting a 25-μl aliquot onto phosphocellulose P-51 paper and washing as described previously (14). Quantitation of expression of MHCK A+, ΔCoil-MHCK A+, and ΔWD-MHCK A+ in each cell line was performed by Western blot analysis using affinity-purified anti-MHCK A polyclonal antibodies and 125I-labeled goat anti-rabbit secondary antibody. The relative level of expression was determined by performing parallel Western blotting of cell extracts and a series of known amounts of each purified kinase construct to generate a standard linear curve for each cell line. All signals were then quantitated by a PhosphorImager. Calculation of the cellular concentrations of each protein was performed by making the estimate that a single Dictyostelium cell is a sphere 5 μm in diameter. The concentration of myosin in Dictyostelium was determined by densitometry on SDS-polyacrylamide gels of cell extracts using bovine serum albumin to generate a standard curve.

RESULTS

Expression of MHCK A Constructs

A schematic representation of full-length MHCK A and constructs in which the amino-terminal coiled-coil (ΔCoil-MHCK A) or the carboxyl-terminal WD repeat (ΔWD-MHCK A) domains were truncated are shown in Fig. 1. The three MHCK A constructs were overexpressed in the Dictyostelium mhck A cell line by fusing each cDNA segment to an actin-15 promoter in an extrachromosomal vector. Once established, transfected cell lines were maintained in a 50 μg/ml concentration of the selective antibiotic G418. This elevated G418 concentration centrifugation, and the supernatant was decanted. The DE52 supernatant was mixed with packed DE52 cellulose (2 ml of supernatant/ml of cellulose) and rotated at 4 °C for 60 min. The phosphocellulose was collected by centrifugation and washed two times with buffer A (10 mM TES, pH 7.0, 50 mM KCl, 20% sucrose, and 1 mM DTT). The resins were pooled into a 3 × 20-cm column and washed with buffer A until the absorbance (A550) of the eluant reached base line. MHCK A was eluted from phosphocellulose by applying buffer A adjusted to 300 mM KCl. The resulting activity pool was applied to a hydroxylapatite column and washed until the A550 of the eluant reached base line. Kinase activity was eluted with 200 mM KPO4, 20% sucrose, and 1 mM DTT, pH 7.0. This pool was loaded onto a 3 × 100-cm Sepheryl S-300 gel filtration column equilibrated with 10 mM TES, 0.3 mM KCl, 20% (w/v) sucrose, 1 mM EDTA, and 1 mM DTT, pH 7.0. ΔWD-MHCK A was purified as described above except an aminohexyl-Sepharose column step was added after gel filtration. The peak of activity off of gel filtration was diluted to 50 mM KCl, loaded onto a 1-ml aminohexyl-Sepharose column; and eluted with 300 mM KCl, 1 mM DTT, and 10 mM TES, pH 7.0. ΔCoil-MHCK A was purified as described above except that the peak of activity off of gel filtration was diluted to 50 mM KCl and loaded onto a 5-ml Mono-S column. Elution was performed with a 50–400 mM KCl gradient in 1 mM DTT and 10 mM TES, pH 7.0. ΔCoil-MHCK A eluted at ~150 mM KCl. Protein concentrations were determined throughout the purification using the method of Bradford (32) with bovine serum albumin as the standard. Purified MHCK A, ΔCoil-MHCK A, and ΔWD-MHCK A were concentrated by binding to a small volume of hydroxylapatite resin and eluting with 200 mM KPO4, 100 mM KCl, 20% sucrose, and 1 mM DTT. Eluants were immediately divided into 100-μl aliquots and frozen in liquid nitrogen until use. Purified proteins were evaluated for functional activity by determining kinase activity in an in vitro assay system using bovine serum albumin to generate a standard curve.

Western Blot Analysis and Quantitation of Expression—Total cell extracts from each of the cell lines were prepared for Western blot analysis as described previously (14). Quantitation of expression of MHCK A+, ΔCoil-MHCK A+, and ΔWD-MHCK A+ in each cell line was performed by Western blot analysis using affinity-purified anti-MHCK A polyclonal antibodies and 125I-labeled goat anti-rabbit secondary antibody. The relative level of expression was determined by performing parallel Western blotting of cell extracts and a series of known amounts of each purified kinase construct to generate a standard linear curve for each cell line. All signals were then quantitated by a PhosphorImager. Calculation of the cellular concentrations of each protein was performed by making the estimate that a single Dictyostelium cell is a sphere 5 μm in diameter. The concentration of myosin in Dictyostelium was determined by densitometry on SDS-polyacrylamide gels of cell extracts using bovine serum albumin to generate a standard linear curve.

MHCK A Subdomains: α-Helical Coiled-coil and WD Repeats

FIG. 1. Schematic of MHCK A truncation constructs. Full-length MHCK A and truncation constructs were expressed in Dictyostelium mhck A+ cells. The approximate locations of MHCK A domains are indicated, and numbers above the scale denote amino acids. The molecular masses were calculated based on the predicted amino acid sequence of each recombinant protein. The level of expression of MHCK A in wild-type cells and other cell lines used in this study was determined by phosphoimaging Western blots using MHCK A antibodies and 125I-labeled goat anti-rabbit antibody and using each purified kinase construct to generate a standard curve.
resulted in significantly higher kinase expression (3–4-fold) relative to the previously described overexpression of full-length MHCK A (14). The expression of each kinase was quantified by phosphoimaging of Western blots performed with MHCK A polyclonal antibodies and 125I-labeled anti-rabbit secondary antibody using each of the three purified proteins (described below) to generate a standard curve for each corresponding cell line, including wild-type cells. Calculations were performed using this Western blot analysis to generate estimates of the in vivo expression levels of each recombinant kinase construct. Based upon this analysis, recombinant full-length MHCK A is expressed at 27 µM, ∆Coil-MHCK A is expressed at 46 µM, and ∆WD-MHCK A is expressed at 90 µM. MHCK A expression in the parental cell line JH10 was calculated to be 0.3 µM. These expression levels represent 90- and 153-fold overexpression of recombinant full-length MHCK A and ∆Coil-MHCK A, respectively, and 300-fold overexpression of ∆WD-MHCK A relative to that of MHCK A in the parental cell line (Fig. 1). By comparison, we calculate myosin II in Dictyostelium to be ~6 µM.

Cytokinesis

Previous studies have demonstrated that myosin II is essential for cytokinesis as assayed by growth of cells in suspension cultures (1). mhck A− cells are unable to form a contractile ring, which results in the cells becoming large and multinucleated without any increase in cell number (1, 27). Similar phenotypes have been observed in 3X ASP cells and in MHCK A− cells (13, 14). We compared growth rates of MHCK A truncation cell lines in suspension cultures as an in vivo test of myosin function (Fig. 2a). The mhck A− cell line, which was previously shown to grow at a similar rate compared with control cells (14), was used as a control (filled circles). Cells that overexpress full-length MHCK A (filled squares) and ∆Coil-MHCK A in cells containing either wild-type MHC or 3X ALA MHC. Notice that cells expressing MHCK A in a wild-type myosin II background are large and multinucleated, whereas cells expressing MHCK A in a 3X ALA myosin II background are not affected by MHCK A overexpression.
MHCK A Subdomains: α-Helical Coiled-coil and WD Repeats

Development

Previous studies have shown that mhc− cells, 3X ASP cells, and MHCK A− cells are unable to complete development (13, 14, 27). We therefore used development as an in vivo test for myosin function in the MHCK A truncation cell lines (Fig. 2b). MHCK A− cells and ΔCoil-MHCK A− cells arrested at mound stage and were unable to complete development, whereas control (mhc A−) and ΔWD-MHCK A− cells completed development and formed spores at normal rates.

Overexpression of MHCK A and ΔCoil-MHCK A causes defects in myosin filament-dependent processes as assayed in vivo by cytokinesis and development, suggesting that these MHCK A constructs drive myosin filament disassembly in vivo. In contrast, no defects in cytokinesis or development were observed in the ΔWD-MHCK A cell line. Interestingly, ΔWD-MHCK A had the highest expression compared with the other MHCK A-expressing cell lines (Fig. 1), with 300-fold higher expression than wild-type cells. Lack of phenotypic defects in ΔWD-MHCK A− cells therefore is not due to lower expression of the kinase. As reported previously (14), elevated MHC phosphorylation in crude cell lysates was observed in MHCK A− cells. Similar behavior was observed in ΔCoil-MHCK A− cells, whereas no detectable increase in MHC phosphorylation was observed in lysates of ΔWD-MHCK A− cells (data not shown).

Elimination of Myosin-based Defects with 3X ALA Myosin

Further analysis was performed to confirm that the defects in cytokinesis and development in MHCK A− and ΔCoil-MHCK A− cell lines were due to phosphorylation of myosin II and disassembly of filaments. Cell lines were constructed in which either wild-type myosin or 3X ALA myosin was expressed in an mhc− background. The 3X ALA myosin bears mutations in the in vitro mapped target sites of MHCK A in the myosin II tail. This 3X ALA myosin is predicted to be resistant to disassembly in vivo by overexpressed MHCK A if the in vitro mapped target sites are also the physiologically relevant in vivo target sites. Clonal populations of these cells expressing either wild-type or 3X ALA MH were transfected with cDNA encoding full-length mhc A or Δcoil-mhc A to test whether the 3X ALA myosin mutation relieves the defects induced by MHCK A or ΔCoil-MHCK A overexpression. When cDNA expressing MHCK A or ΔCoil-MHCK A was transfected into cells expressing wild-type myosin, they failed to grow in suspension (Fig. 3a, filled and open squares, respectively), as expected based upon results presented above. This defect in cytokinesis caused by overexpression of MHCK A or ΔCoil-MHCK A was eliminated in cells expressing 3X ALA myosin (Fig. 3a, filled and open triangles, respectively). Overexpression of MHCK A or ΔCoil-MHCK A in wild-type myosin cells caused the cells to become large and multinucleated in suspension culture, whereas 3X ALA myosin cell lines expressing these kinase constructs remained small and displayed no increase in size (Fig. 3b) in suspension culture. In addition, wild-type MHC cells that overexpress either full-length MHCK A or ΔCoil-MHCK A constructs were not able to complete development and arrest at the mound stage, whereas 3X ALA MHC cells that overexpress either kinase construct were able to complete development (data not shown). These results are consistent with the hypothesis that full-length MHCK A and ΔCoil-MHCK A hyperphosphorylate myosin on residues 1823, 1833, and 2029 in vivo (or a subset of these residues), resulting in myosin disassembly and consequently an mhc− phenotype.

Purification of Full-length MHCK A, ΔCoil-MHCK A, and ΔWD-MHCK A

To determine the activity of each construct, the proteins were purified from Dictyostelium cultures. The previously reported purification method for MHCK A (26) was modified to allow purification of each MHCK A construct using four to five column steps. A summary of the purification of MHCK A constructs is found in Table I, and details on the purification of each MHCK A construct are given under "Experimental Procedures." The purification table is presented as a guide to show relative purification during the purification process. A detailed comparison of the activities of the proteins under equivalent conditions is presented below. Fig. 4 shows purified MHCK A, ΔWD-MHCK A, and ΔCoil-MHCK A after SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining (panel a) or Western blot analysis (panel b). MHCK A and ΔCoil-MHCK A ran at 130 and 73 kDa, respectively. ΔWD-MHCK A ran as a doublet at 96 and 90 kDa. This could represent autophosphorylation, heterogeneity or a partial proteolytic clip. Both forms

| MHCK A | Total extract | ml | 90 | 2160 | 0.03 | 65 |
|--------|---------------|----|----|------|------|----|
|        | Supernatant   |    | 80 | 1290 | 0.04 | 52 |
|        | DE52 supernatant | 80 | 640 | 0.6 | 384 |
|        | Phosphocellulose | 22 | 77 | 5 | 385 |
|        | Hydroxylapitite | 5 | 18 | 45 | 818 |
|        | Sepharyl S-300 | 23 | 4 | 60 | 238 |

ΔCoil-MHCK A

| Total extract | ml | 90 | 2070 | 0.03 | 62 |
| Supernatant   |    | 80 | 1560 | 0.03 | 47 |
| DE52 supernatant | 140 | 742 | 0.3 | 223 |
| Phosphocellulose | 19 | 72 | 1.6 | 115 |
| Hydroxylapitite | 5 | 31 | 3.6 | 112 |
| Sepharyl S-300 | 22 | 9 | 4.9 | 44 |
| Mono-S        | 7 | 1.2 | 14.4 | 17 |

ΔWD-MHCK A

| Total extract | ml | 120 | 2070 | 0.03 | 62 |
| Supernatant   |    | 100 | 1000 | 0.08 | 80 |
| DE52 supernatant | 170 | 425 | 1.4 | 595 |
| Phosphocellulose | 43 | 108 | 3.6 | 398 |
| Hydroxylapitite | 8 | 21 | 6.8 | 130 |
| Sepharyl S-300 | 18 | 1.35 | 18.3 | 11 |
| AH-Sepharose* | 2.5 | 0.25 | 30 | 7.5 |

* AH-Sepharose, aminohexyl-Sepharose.

TABLE I

Purification of MHCK A truncations from Dictyostelium

| Volume | Protein | Specific activity | Total activity |
|--------|---------|------------------|----------------|
| ml     | mg      | nmol/min/mg      | nmol/min       |
| MHCK A | Total extract | 90 | 2160 | 0.03 | 65 |
|        | Supernatant   | 80 | 1290 | 0.04 | 52 |
|        | DE52 supernatant | 80 | 640 | 0.6 | 384 |
|        | Phosphocellulose | 22 | 77 | 5 | 385 |
|        | Hydroxylapitite | 5 | 18 | 45 | 818 |
|        | Sepharyl S-300 | 23 | 4 | 60 | 238 |

ΔCoil-MHCK A

| Total extract | 90 | 2070 | 0.03 | 62 |
| Supernatant   | 80 | 1560 | 0.03 | 47 |
| DE52 supernatant | 140 | 742 | 0.3 | 223 |
| Phosphocellulose | 19 | 72 | 1.6 | 115 |
| Hydroxylapitite | 5 | 31 | 3.6 | 112 |
| Sepharyl S-300 | 22 | 9 | 4.9 | 44 |
| Mono-S        | 7 | 1.2 | 14.4 | 17 |

ΔWD-MHCK A

| Total extract | 120 | 2070 | 0.03 | 62 |
| Supernatant   | 100 | 1000 | 0.08 | 80 |
| DE52 supernatant | 170 | 425 | 1.4 | 595 |
| Phosphocellulose | 43 | 108 | 3.6 | 398 |
| Hydroxylapitite | 8 | 21 | 6.8 | 130 |
| Sepharyl S-300 | 18 | 1.35 | 18.3 | 11 |
| AH-Sepharose* | 2.5 | 0.25 | 30 | 7.5 |
Native MHCK A, but still resulted in gel filtration at a slightly smaller apparent mass than its predicted molecular size of 96 kDa. Elimination of the amino-terminal 500 amino acids caused one or more globular protein of equivalent size, so the number of monomers in each oligomer cannot be estimated accurately from this elution behavior.

MHCK A Oligomerization and Rotary Shadowing

Several lines of evidence indicate that MHCK A is an oligomeric protein kinase, including gel filtration analysis of native protein (8), cross-linking studies (15), and coiled-coil predictive algorithms (15). Gel filtration chromatography performed as the final or penultimate step in each purification allowed us to test the hypothesis that the amino-terminal domain of MHCK A (residues 1–500) is responsible for oligomerization. The gel filtration analysis of full-length MHCK A and truncation constructs is presented in Fig. 5. Full-length MHCK A migrated at 130 kDa on SDS-polyacrylamide gel electrophoresis, but under native conditions, gel-filtered in the void volume with an estimated molecular mass >1000 kDa. This is consistent with the 130-kDa protein being oligomeric. Rod-shaped protein domains such as coiled-coil helices gel filter with larger apparent mass than globular protein of equivalent size, so the number of MHCK A monomers in each oligomer cannot be estimated accurately from this elution behavior.

Deletion of the carboxyl-terminal WD repeats (ΔWD-MHCK A) resulted in gel filtration at a slightly smaller apparent mass than native MHCK A, but still >670 kDa, which is much larger than its predicted molecular size of 96 kDa. Elimination of the amino-terminal 500 amino acids caused ΔCoil-MHCK A to gel filter with an apparent molecular mass of 80 kDa, which is consistent with the predicted mass of 73 kDa. These results indicate that the amino-terminal 500 residues of MHCK A, which are predicted to form a coiled-coil structure, are responsible for MHCK A oligomerization. The computer algorithm SCORER, created by Woolfson and Alber (28), predicts whether a sequence is expected to form a two- or three-stranded coiled-coil. According to this algorithm, MHCK A is predicted to form a three-stranded coiled-coil (data not shown).

Full-length MHCK A was visualized by platinum rotary shadowing (Fig. 6). The majority of the rotary-shadowed images appeared globular or aggregated, as in the lower right panel of Fig. 6. However, a number of images were also observed that appeared to reveal a globular domain associated with an extended rod-like structure (all other panels in Fig. 6). Measurements of these images indicated an average length for the rod-like segments of ~50 nm. The average calculated pitch of coiled-coil helices from proteins such as myosin tails is 6.7 residues/nm. If MHCK A residues 100–500 were contained entirely in a coiled-coil structure (15), a rod domain of ~60 nm would be predicted. This value is agreement with the ~50-nm length of the rod structures observed by rotary shadowing. The apparent globular segment of the images was always observed at only one end of the rod segment, suggesting that MHCK A complexes form via parallel oligomerization rather antiparallel oligomerization.

Biochemical Characterization of MHCK A Constructs

Autophosphorylation—Previous studies showed that MHCK A is activated by autophosphorylation, incorporating up to 10 mol of phosphate/mol of enzyme. Incorporation of the first 3 mol of phosphate was shown to be sufficient for maximum activation (18). Autophosphorylation behavior was assessed for MHCK A truncation constructs. Full-length MHCK A, ΔCoil-MHCK A, and ΔWD-MHCK A all autophosphorylated to a similar range of 8–10 mol of phosphate/mol of enzyme (Fig. 7a). The autophosphorylation rates of each construct were independent of concentration, suggesting that autophosphorylation is an intramolecular process (data not shown).

Activity of MHCK A Constructs toward Peptide—MHCK A has been shown to phosphorylate the myosin tail on threeines 1823, 1833, and 2029. We used a 16-residue peptide (MH-1) corresponding to the sequence around Thr-2029 in the myosin tail to test the enzymatic activity of each construct. Fig. 7b shows phosphorylation of the peptide by purified autophosphorylated MHCK A, ΔCoil-MHCK A, and ΔWD-MHCK A. The
were preincubated in 0.5 mM MgATP for 20 min. Incorporation of 32P by MHCK A truncation constructs. MHCK A truncation constructs 
counter. Symbols are an average of two samples.

Myosin bands were cut from the gel and counted in a scintillation 
SDS sample buffer and electrophoresed on an SDS-polyacrylamide gel. 
incubation for 4 min at 25 °C. Reactions were stopped by the addition 
m
MH-1 was initiated by the addition of kinase (2 M with a 
m
Medley et al. (18) (K_m = 105 μM and V_max = 2.2 

proteins; full-length recombinant MHCK A (circles), ΔCoil-MHCK A (squares), and ΔWD-
MHCK A (triangles). a, phosphate incorporation into MHCK A truncation constructs by autophosphorylation. Phosphate incorporation into 
MHCK A (final concentration of 200 nm) truncation constructs was 
performed in buffer containing 10 mM TES, 2 mM MgCl2, 1 mM DTT, and 
0.5 mM ATP (1000 cpm/mol). At the indicated times, aliquots (25 μl) 
were removed, and 32P incorporation was determined by phosphocellu-
lose filter paper assay. b, phosphate incorporation into peptide MH-1 by 
MHCK A truncation constructs. MHCK A truncation constructs were 
preincubated in 0.5 mM MgATP for 20 min. Phosphorylation of peptide 
MH-1 was initiated by the addition of kinase (2 μg/ml) and incubation 
for 1 min at 25 °C. Reactions were stopped by the addition of 50 mM 
EDTA, and 32P incorporation into peptide MH-1 was determined by 
phosphocellulose filter paper assay. Error bars represent S.D. for tri-
licate samples. c, phosphate incorporation into Dictyostelium myosin II 
by MHCK A truncation constructs. MHCK A truncation constructs were 
preincubated in 0.5 mM MgATP for 20 min. Incorporation of 32P 
into myosin II was initiated by the addition of kinase (2.5 μg/ml) and 
incubation for 4 min at 25 °C. Reactions were stopped by the addition 
SDS sample buffer and electrophoresed on an SDS-polyacrylamide gel. 
Myosin bands were cut from the gel and counted in a scintillation 
counter. Symbols are an average of two samples.

The phosphorylation of the peptide obeys Michaelis-Menten kinet-
cs; full-length recombinant MHCK A (circles) had a K_m of 92 
μM with a V_max of 3.6 μmol × min⁻¹ × mg⁻¹ for the peptide, in 
close agreement with previous values reported for native 
MHCK A by Medley et al. (18) (K_m = 105 μM and V_max = 2.2 
μmol × min⁻¹ × mg⁻¹). As indicated by the kinetic parameters 

(Table II), both truncation constructs displayed relatively sim-
lar activity against peptide MH-1 as compared with full-length 
MHCK A.

Activity of MHCK A Constructs toward Myosin II—Autophosphory-
lated MHCK A, ΔCoil-MHCK A, and ΔWD-MHCK A were 
assayed for their ability to phosphorylate wild-type Dictyoste-
lium myosin II (Fig. 7c). ΔCoil-MHCK A was able to phos-
phorylate myosin II at a rate similar to that of full-length MHCK 
A. In contrast, ΔWD-MHCK A showed a substantial decrease in 
activity against myosin as compared with MHCK A or ΔCoil-
MHCK. While it is difficult to obtain accurate kinetic constants 
for myosin II because it is filamentous under the assay condi-
tions, the specific activities of ΔWD-MHCK A measured with 
several concentrations of myosin were consistently 20-fold 
lower than those of MHCK A and ΔCoil-MHCK A. These 
results are consistent with the in vivo results and suggest that 
the WD repeat domain of MHCK A is required for efficient 
substrate recognition of myosin II both in vitro and in vivo.

DISCUSSION

In vitro and in vivo analyses of MHCK A truncation con-
structs have been used to identify the roles of the two domains 
that flank the catalytic domain of MHCK A. The earlier dem-
stration that full-length MHCK A overexpression leads to 
disassembly of cytoskeletal myosin II in vivo (14) provided a 
useful assay for in vivo activity of the expressed constructs.

Gel filtration analysis of the purified constructs demonstrated 
a loss of oligomerization in the ΔCoil-MHCK A con-
struct. An extended rod-like domain was also observed in the 
rotary-shadowed images of native MHCK A. These results pro-
vide experimental evidence that the segment of MHCK A span-
ning residues 1–500 contains the oligomerization functions 
and that the oligomer formation probably occurs via formation of a 
parallel coiled-coil rod structure.

Elimination of this domain did not appear to significantly 
impair in vitro kinase activity against native myosin II or 
peptide MH-1. Although ΔCoil-MHCK A displayed a slightly 
elevated K_m for peptide MH-1, its V_max and K_cat values were 
similar to those of full-length MHCK A. The autophosphoryla-
tion stoichiometry in this construct also appeared similar to 
that in full-length MHCK A. Furthermore, ΔCoil-MHCK A 
appeared active in vivo, as assessed by its ability to impede 
myosin-based contractile events in vivo in the presence of wild-
type myosin II. It can be speculated that the coiled-coil region 
of MHCK A could have other functions, such as annealing with 
 bipolar myosin filaments or serving as a pseudosubstrate or 
autoinhibitory domain. Preliminary results in our laboratory 
have not found evidence to support either of these theories, 
however.

Expression of either ΔCoil-MHCK A or full-length MHCK A 
completely eliminated myosin contractile function in vivo in 
cells containing wild-type MHC. However, when these kinase 
constructs were overexpressed in cells containing 3X ALA 
MHC, no inhibition of myosin-based contractile function was
observed. The relatively normal phenotype of these cells provides the first demonstration that the *in vitro* mapped target sites of MHCK A on the myosin tail are also the physiologically relevant *in vivo* target sites of MHCK A. The properties of these cell lines furthermore confirm the hypothesis that defects observed when MHCK A is overexpressed in wild-type MHC cells are due solely to the activity of the kinase against myosin II, as opposed to other *in vivo* substrates. All the phenotypic defects observed when MHCK A is overexpressed can be attributed to its activity toward residues 1823, 1833, and 2029 in the myosin II tail.

While overexpressing full-length MHCK A and ΔCol-MHCK A resulted in striking phenotypes, the elimination of amino acids 842–1146, corresponding to the WD repeat domain, resulted in no increase in MHC phosphorylation in Triton extracts and no impairment of myosin II function *in vivo* during development or growth in suspension. This phenotype is interesting since ΔWD-MHCK A is expressed at a 3-fold higher level than full-length MHCK A in MHCK A+ cells. Purified ΔWD-MHCK A autophosphorylated to a similar extent as MHCK A and ΔCol-MHCK A. More important, ΔWD-MHCK A also displayed similar kinetics as MHCK A and ΔCol-MHCK A toward the MH-1 peptide substrate, which corresponds to the target site at residue 2029 on the myosin II tail.

Full catalytic activity against peptide MH-1 by ΔWD-MHCK A indicates that the peptide substrate-binding regions of MHCK A and all core catalytic functions are intact in this construct. Despite this, a 95% drop in activity against native myosin II was observed *in vitro*, and the overexpressed kinase did not interfere with myosin II function *in vivo* despite its high expression level. These results support the hypothesis that the WD repeat domain of MHCK A plays a critical role in substrate recognition with the native myosin II substrate. Attempts to measure the *K*~m~ of each kinase construct for native myosin II are complicated by the filamentous nature of the substrate under assay conditions. Other methods are currently being explored to determine directly whether the WD repeats of MHCK A act as a myosin II-binding domain.

β-Subunits of heterotrimeric GTP-binding proteins serve as the prototype for members of the WD repeat family of proteins (16, 17). This prototype class consists of an amino-terminal segment of ~50 residues, which is followed by seven repeats of ~40 residues, with each repeat containing a conserved “WD” motif. Although the number of WD repeats varies in different members of the WD repeat family of proteins, most members probably fold into a structure similar to the seven-blade β-propeller structure identified for the human G protein β-subunit via x-ray crystallography (29, 30).

Although β-subunits of GTP-binding proteins have an established role in binding to and activating effector targets, the roles of WD repeat domains in other classes of WD repeat-containing proteins are not well characterized. To our knowl-

edge, the work presented here is the first biochemical evidence for a WD repeat domain serving to target an attached catalytic domain to its substrate.

Neer et al. (16, 17) have suggested that the conserved residues of the WD repeats provide a rigid scaffold and that the variable residues between may specify the interactions with different proteins. A more detailed analysis of the MHCK A WD repeat domain will be necessary to determine which residues dictate the specificity toward myosin II filaments.

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