WD Repeat Domains Target Dictyostelium Myosin Heavy Chain Kinases by Binding Directly to Myosin Filaments

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Steimle, P.A., Licate, L., Naismith, T., Egelhoff, T.T. (2000) WD-Repeat domains target *Dictyostelium* myosin heavy chain kinase activities by binding directly to myosin II. *Journal of Biological Chemistry*. 276:6853-6860. doi: 10.1074/jbc.M008992200

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Abstract:

Myosin heavy chain kinase (MHCK) A phosphorylates mapped sites at the C-terminal tail of *Dictyostelium* myosin II heavy chain, driving disassembly of myosin filaments both *in vitro* and *in vivo*. MHCK A is organized into three functional domains that include an N-terminal coiled-coil region, a central kinase catalytic domain unrelated to conventional protein kinases, and a WD repeat domain at the C terminus. MHCK B is a homologue of MHCK A that possesses structurally related catalytic and WD repeat domains. In the current study, we explored the role of the WD repeat domains in defining the activities of both MHCK A and MHCK B using recombinant bacterially expressed truncations of these kinases either with or without their WD repeat domains. We demonstrate that substrate targeting is a conserved function of the WD repeat domains of both MHCK A and MHCK B and that this targeting is specific for*Dictyostelium* myosin II filaments. We also show that the mechanism of targeting involves direct binding of the WD repeat domains to the myosin substrate. To our knowledge, this is the first report of WD repeat domains physically targeting attached kinase domains to their substrates. The examples presented here may serve as a paradigm for enzyme targeting in other systems.

Article:

Complex cellular processes such as cytokinesis, cell migration, and receptor capping depend on the ability of myosin II (conventional myosin) to bring about contraction at specific locations within the cell (1). In *Dictyostelium*, myosin II undergoes a dynamic assembly/disassembly process that permits its specific spatial and temporal localization within the cell (2). In response to extracellular chemoattractants such as cAMP or folic acid, myosin II is recruited from the cytoplasm to the cell cortex in a process that involves myosin II bipolar filament assembly (3-5).

MHC[1] phosphorylation is the major route by which myosin filament assembly is controlled in*Dictyostelium*. Phosphorylation of mapped sites at threonine residues 1823, 1833, and 2029 of MHC leads to disassembly of filaments both *in vitro* and *in vivo* (6-9). The importance of these target sites in controlling myosin filament assembly has been demonstrated from studies showing that conversion of these threonines to aspartic acid residues creates a “pseudophosphorylated” myosin that is unable to assemble into the cytoskeleton. Conversely, myosin overassembles into the cytoskeleton if these regulatory sites are either removed by truncation of MHC (10) or converted to nonphosphorylatable alanine residues (9).

MHC phosphorylation appears to be regulated in *Dictyostelium*through the combined activities of at least one MHC phosphatase (11) and a group of MHC kinases (2, 12). *Dictyostelium* MHC kinase (MHCK) A was the first of the heavy chain kinases to be purified. Subsequent characterization of purified MHCK A revealed that its catalytic activity can drive myosin filament disassembly *in vitro* by phosphorylating MHC (13). Cloning and sequence analysis of the MHCK A gene has revealed that the catalytic domain of MHCK A is unrelated to any of the conventional protein kinases. As a result, MHCK A has become the prototype defining a new family of protein kinases (14, 15). A structurally related kinase, MHCK B, has been identified and cloned from

Dictyostelium via sequence homology to the catalytic domain of MHCK A. Although native MHCK B has not yet been purified, a bacterially expressed truncation of MHCK B phosphorylates MHC preferentially at the mapped regulatory sites discussed above (16). Another *Dictyostelium* homologue of MHCK A that has been identified by cDNA and genomic sequencing has been named MHCK C, although it has not yet been characterized at the protein level (GenBank™ accession number AF079447).

The MHCK A group of kinases in *Dictyostelium* share a conserved domain structure consisting of the novel kinase catalytic domain and a C-terminal domain containing seven tandem copies of the WD repeat motif. The WD repeat is a conserved amino acid motif that recurs four to sixteen times and is found in a large and heterogeneous family of proteins. The general makeup of the repeating unit, although not absolutely conserved among WD repeats, begins with a pair of GH residues followed by a variable core of about 36–46 amino acids and ends with the WD residues (17). The crystal structure of the β-subunit of the heterotrimeric G-protein complex (G_β) has revealed that its seven WD repeats fold into a ring-like β-propeller structure consisting of seven "blades" with each blade folded into a four-stranded anti-parallel β-sheet structure (18). Although no structural information is available for the WD repeat domains of MHCK A, B, and C, the conservation of their seven repeats with those of the G_β subunit indicates that the seven-bladed β-propeller structure is also likely to occur in these*Dictyostelium* proteins.

Functionally, the WD repeat class of proteins has been implicated in the regulation of a number of cellular functions such as signal transduction, RNA processing, gene regulation, vesicular traffic, and cytoskeletal assembly; however, no common function or interaction has been attributed to the WD repeat domain specifically (17). Insight on the function of the WD repeat domain of MHCK A has been gained from a previous study showing that a *Dictyostelium*-expressed truncation of MHCK A lacking its WD repeat domain (MHCK A-ΔWD) was unable to phosphorylate myosin II *in vitro* and *in vivo*. In contrast, MHCK A-ΔWD phosphorylated a soluble myosin-based peptide (MH-1) just as well as full-length MHCK A (19).

In the current report, we describe results from experiments exploring the structure/function relationship between the conserved WD repeat domains of MHCK A and MHCK B and the kinase activity of each enzyme. For these studies, the catalytic domains of MHCK A and MHCK B, either with or without their WD repeat domains, were expressed in bacteria as recombinant glutathione *S*-transferase (GST) fusion proteins and then assayed for kinase activity toward several different substrates, including *Dictyostelium* myosin II. We found that the WD repeat domains of both MHCK A and MHCK B stimulate kinase activity toward myosin II substrate but not toward the other substrates tested. These results provide strong support for the hypothesis that MHCK B, like MHCK A, functions physiologically as an MHC kinase. Binding experiments presented here reveal that the mechanism by which the WD repeats promote kinase activity toward myosin substrate is likely to involve a direct interaction with myosin II. To our knowledge, this represents the first time a WD repeat domain has been shown to physically target an attached kinase domain to its substrate.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

All DNA manipulations were carried out using standard methods. For the MHCK A constructs, an intermediate plasmid (pMEX) containing the entire MHCK A DNA coding region was used to generate the inserts for the GST fusion vector. The numbers corresponding to the restriction sites are derived from the MHCK A DNA sequence (GenBank™ accession number P42527). For the pGST-A-CAT construct, pMEX was digested with *Hin*dIII (base pair 1493) and *Bst*XI (base pair 2529), and the resultant 1036 base pair fragment was treated with Klenow polymerase (New England Biolabs) to yield blunt ends. The pGST-A-CAT construct was completed by ligating the 1036-base pair fragment into pGEX-2T (Amersham Pharmacia Biotech) and then digesting with *Sma*I to generate an in phase fusion with GST. The pGST-A-CATWD construct was made in a similar manner, but the fragment was released from pMEX by digesting only with*Hin*dIII and then treating the fragment with Klenow polymerase to yield blunt ends. The upstream end is contained within a bacterial polylinker downstream of the native MHCK A termination codon. The 2.0-kilobase insert fragment was then ligated into pGEX-2T cut with*Sma*I.

MHCK B constructs were made from an intermediate plasmid, pMM3.1, which contains the full-length cDNA-coding region of MHCK B (GenBank™ accession number P90648). pMM3.1 contains the entire downstream end of MHCK B and has an *Eco*RI linker inserted next to codon 13 at the upstream end of the MHCK B coding region. For the pGST-B-CAT construct, a 1.4-kilobase insert fragment was isolated from pMM3.1 using an internal *Eco*RV site (nt1391) that lies 3′ of the catalytic domain of MHCK B and an*Xba*I site in the upstream polylinker of pMM3.1. The*Xba*I site was made blunt by treatment with Klenow polymerase and then ligated into pGEX-2T vector that had been digested with*Eco*RI and made blunt. This ligation recreated the upstream vector *Eco*RI site (junction of blunt *Eco*RI ligated with blunt *Xba*I). This plasmid insert also contains the *Eco*RI site that lies adjacent to codon 13 of the MHCK B coding region. Digestion of this intermediate with *Eco*RI was performed to cut at the upstream vector *Eco*RI site and at the internal *Eco*RI site. Subsequent recircularization yielded pGST-B-CAT, which has codon 13 of MHCK B fused directly to the upstream GST domain encoded by the vector and an artificial stop codon following residue 459 of MHCK B. This stop codon lies at a position ~110 amino acids downstream of the conserved catalytic domain portion of the native protein. The pGST-B-CATWD construct was prepared in a similar fashion. pMM3.1 was digested with *Eco*RI, releasing a 2.2-kilobase restriction fragment that has the entire downstream end of the MHCK B coding region and an upstream *Eco*RI site adjacent to codon 13 of MHCK B. This fragment was ligated into pGEX-2T to yield pGST-B-CATWD.

A GST fusion protein containing a 15-kDa segment of the*Dictyostelium* myosin II heavy chain was expressed in*Escherichia coli* from the plasmid pGST2029. Construction of pGST2029 involved the subcloning of a segment of myosin II tail centered on the MHCK A target site mapped to position 2029 (7) into the pGEX-3X (Amersham Pharmacia Biotech) expression plasmid. An 80-amino acid region of the myosin II gene spanning residues 1985–2064 was amplified using primers that introduced upstream and downstream*Eco*RI restriction sites. The polymerase chain reaction product was cut with *Eco*RI and ligated into pGEX-3X vector digested with *Eco*RI. The resulting plasmid, pGST2029, was then electroporated into *E. coli* cells (strain BL21-DE3, Novagen) for expression.

Protein Purification

The four GST fusion proteins were purified in a similar manner. A 100-ml culture of*E. coli* (BL21-DE3 strain, Novagen) containing each plasmid was grown overnight in a 37 °C shaker incubator. This culture was then used to inoculate (1:50 dilution) 1–2 liters of LB (Life Technologies, Inc.) containing ampicillin (50 μg/ml; Roche Molecular Biochemicals), and the cultures were grown at 37 °C to log phase (A_{600} of 0.6–1.0). At this time, cultures were cooled to 25 °C, and then 1 mMisopropyl-β-D-thiogalactoside was added to induce fusion protein synthesis. Induced cultures were incubated for 15–20 h at 25 °C on a rotating platform. Cells were then harvested by centrifugation at 6,000 × *g* for 5 min, and the resulting cell pellet was washed in 25 mM Tris buffer, pH 7.5, and then centrifuged again. The cell pellet was resuspended (5 ml/g of pellet weight) with Buffer A (25 mM Tris, pH 7.5, 150 mM KCl, 5 mM dithiothreitol, and 1 mM EDTA) containing 175 μg/ml phenylmethylsulfonyl fluoride and 2× concentration of the protease inhibitor mixtures, PIC-I and PIC-II. PIC-I was added from a 1,000 × stock containing 2 mg/ml antipain, 10 KIU/ml aprotinin, 10 mg/ml benzamidine, 1 mg/ml leupeptin, 5 mg/ml Pefabloc, and 10 mg/mlL-1-chloro-3[4-tosylamido]-7-amino-2-heptanone-HCl N-α-tosyl-L-lysine chloromethyl ketone in ddH_2O. PIC-II (1,000×) contains 1 mg/ml chymostatin, 1 mg/ml pepstatin, and 5 mg/mlL-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone tosyl-l-phenylalanine chloromethyl ketone in ethanol.

All subsequent purification steps were performed at 4 °C. Lysozyme (100 μg/ml, Sigma) was added to the resuspended cells. The cell mixture was sonicated in an ice water bath, and the resulting lysate was spun at 150,000 × *g* for 15 min to remove insoluble material. The cleared lysate was incubated with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 3 to 5 h at 4 °C. The beads were then washed several times

with buffer A to remove unbound material. For samples used in autophosphorylation experiments, a dephosphorylation step was added by incubating (12 h at 4 °C) bead-bound fusion protein in Buffer A containing 50 units of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals). After incubation, calf intestinal alkaline phosphatase was removed by washing the beads with Buffer A. Bound fusion protein was then eluted with buffer A containing 30 mM glutathione (Sigma), and the eluate was dialyzed against 12.5 mM Tris, pH 7.5, in 40% glycerol. Aliquots were frozen on dry ice and then stored at −80 °C until used in assays.

The GST-2029 fusion protein was prepared as described above but with the following modifications. A 1-liter culture of *E. coli*(BL21-DE3) cells containing the pGST2029 plasmid was grown to log phase at 37 °C, and then expression of the GST-2029 fusion protein was induced with 1 mMisopropyl-β-D-thiogalactoside. The culture was grown for 3 h at 37 °C, and then cells were harvested by centrifugation. The cell pellet was resuspended in Tris-buffered saline containing 175 μg/ml phenylmethylsulfonyl fluoride and 1 mg/ml pepstatin. The cell slurry was frozen in a dry ice/methanol bath and stored overnight in a −80 °C freezer. The following components were added after thawing the cells for purification of GST-2029: 1 mMdithiothreitol, 1 mM benzamidine, 100 μg/mlL-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone tosyl-l-phenylalanine chloromethyl ketone, 50 μg/mlL-1-chloro-3[4-tosylamido]-7-amino-2-heptanone-HCl*N*-α-tosyl-L-lysine chloromethyl ketone, 1 mM EDTA, and 100 μg/ml lysozyme. After a 10-min incubation on ice, Sarkosyl was added to 1.5%, and the cells were sonicated until fully lysed. Triton X-100 (2%) was added prior to centrifuging the sonicate and the cleared supernatant was incubated with glutathione-Sepharose beads for 45 min at 4 °C. Unbound material was removed by washing the beads with buffer containing 20 mM Tris, pH 7.5, 50 mM NaCl, and 1 mM EDTA. Fusion protein was eluted from the washed beads using 10 mM glutathione in wash buffer. The peak of eluted protein was determined by Bradford assay (Pierce), and pooled fractions were treated with aquacide (CalBiochem) to concentrate. The resulting concentrate was dialyzed into 10 mM TES, pH 7.0, 1 mM EDTA, and 1 mM dithiothreitol and then stored in aliquots at −80 °C until used.

Myosin was purified by a modification of the method of Aguado-Velasco,*et al.* (20), as has been described (11). Briefly, an initial actomyosin precipitate was subjected to several assembly/disassembly cycles followed by a final gel filtration step to remove residual actin.

Phosphorylation Assays

All kinase assays were performed at 25 °C in 10 mM TES, pH 7.0, 2 mM$MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM [γ-^{32}P]ATP (250–400 Ci/mol), using recombinant kinases at 20–80 nM, as noted for each experiment. The substrates used in this study were MH-1 peptide, GST-2029 (38 kDa), and myosin (243 kDa). The peptide substrate MH-1 (RKKFGESEKTKTKEFL-amide) has been described previously (21) and corresponds to the mapped MHCK A target site in the myosin II tail at residue 2029 (underlined in peptide above). For kinetic analysis of kinase activity, each of the substrates was used over a range of concentrations as noted for each experiment. For determination of MHC phosphorylation stoichiometry, myosin II was included in the reaction mix at a concentration of 0.42 μM. For myosin substrate specificity experiments, rabbit skeletal muscle myosin II (Sigma) and chicken gizzard myosin II (Sigma) were dialyzed against*Dictyostelium* myosin II storage buffer (5 mMTris pH 7.0 buffer containing 20% sucrose) prior to their addition to kinase reaction mixes.

Unless indicated otherwise, each kinase was allowed to auto-phosphorylate for 10 min at 25 °C before starting the kinase reaction with the addition of substrate. Kinase reactions containing MH-1 peptide substrate were terminated by blotting a fraction of the reaction mixture onto P-81 filter paper (Whatman) and then processing the filter for scintillation counting (22). For reactions in which the substrate was either GST-2029 or myosin II, the reaction was stopped by adding an equal volume of 2× SDS-PAGE sample buffer at each time point, boiling for 2 min, and then performing SDS-PAGE on the samples. The resulting gels were stained with Coomassie Blue, and protein bands of interest were excised for scintillation counting to quantify phosphate incorporation.

Analysis of fusion protein autophosphorylation was performed by incubating the kinase constructs (200 nM) in the kinase reaction described above and then blotting a fraction of the reaction mix to P-81 filter paper as described for the peptide assay.

Protein Concentration Determination

Purified kinase constructs were subjected to SDS-PAGE with a series of known amounts of BSA on the same gel. After Coomassie Blue staining, the protein bands were quantified by scanning densitometry, and the concentration of each purified fusion protein was determined by comparing the densitometry values for the purified kinases with those of the BSA standards in the same gel.

Myosin II Cosedimentation Assays

Centrifuge tubes used throughout these experiments were pretreated with Sigmacote (Sigma) and 3% BSA in 25 mM Tris, pH 7.5. Kinase fusion proteins used in cosedimentation assays were precleared by centrifugation at 95,000 × *g* for 10 min in a Beckman Ti Tabletop Ultracentrifuge in a TLA 100.3 rotor. The resulting supernatants were transferred to clean centrifuge tubes and then mixed with the other binding assay components to give 40-μl reaction mixes containing 0.3 μM kinase fusion protein, 1.0 μM *Dictyostelium* myosin II, 0.1% BSA, 2.5 mM TES, pH 7.0, 40 mM KCl, 2 mM $MgCl_2$, and 5% sucrose. Under these conditions, greater than 80% of the myosin II is in filaments (25). After incubation for 10 min at 4 °C, the binding reactions were centrifuged for 10 min at 95,000 ×*g*, and equal volumes of pellet and supernatant fractions were resolved by SDS-PAGE. The myosin II and BSA were visualized by Coomassie Blue staining of gels. The distribution of kinase fusion proteins in pellets and supernatants was determined from Western blots probed with anti-GST antibody (Upstate Biotechnology, NY). The relative amounts of kinase fusion protein in the pellet and supernatant fractions were quantified by densitometric analysis of the developed Western blots.

RESULTS

Construction of MHCK A and MHCK B Truncations Expressed and Purified as GST Fusion Proteins

In earlier studies, domain truncations of MHCK A were generated for expression in*Dictyostelium* cells. Biochemical analysis of the purified constructs revealed that the WD repeat domain of MHCK A was necessary for efficient phosphorylation of intact myosin II but not for phosphorylation of a myosin-based peptide (MH-1) substrate (19). In the current study, we explored further the role of WD repeat domains in defining the kinase activities of both MHCK A and its recently identified homologue, MHCK B. For this study, truncated forms of both kinases were constructed, expressed, and purified as GST fusion proteins either lacking or containing their WD repeat domains (Fig.1). The GST-A-CAT fusion protein construct contains the minimal region of MHCK A that is necessary for basal kinase catalytic activity (23). The GST-A-CATWD fusion protein encompasses this segment and the C-terminal WD repeat domain of MHCK A. In contrast to MHCK A, the catalytic properties of MHCK B have not been studied thoroughly, and nothing is known of the WD repeat domain contribution to MHCK B kinase activity. Although a fine level mapping of the catalytic domain of MHCK B has not been performed, comparison with the MHCK A sequence suggests that the region of MHCK B necessary for basal kinase catalytic activity is probably contained within residues 100–340 (16). The GST-B-CAT fusion protein used in this study encompasses this region, and the GST-B-CATWD construct includes this region as well as the more distal WD repeat domain of native MHCK B. All of the constructs described above were expressed in *E. coli* and purified to near homogeneity (Fig. 1).

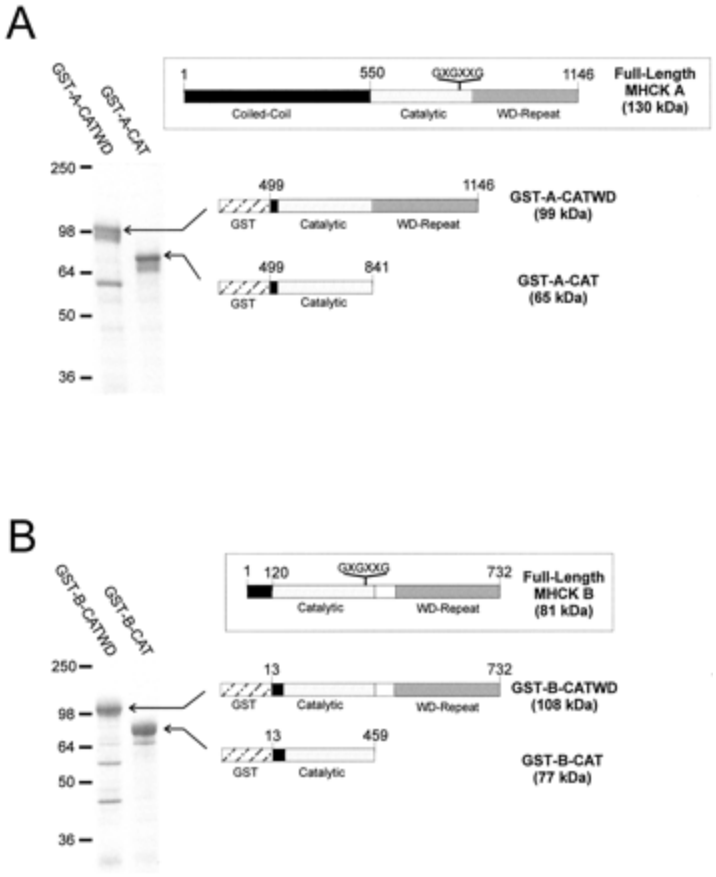

Figure 1
Recombinant MHCK fusion protein constructs. *A*, schematic diagrams of full-length MHCK A and the bacterially expressed truncations of MHCK A used in this study. Numbering indicates positions of included amino acids from MHCK A (GenBank™ accession number P42527). At *left* is an SDS-PAGE gel (Coomassie Blue-stained) of purified GST-A-CAT and GST-A-CATWD (2 μg each). *B*, schematic diagrams of full-length MHCK B and its bacterially expressed truncations used in this study. Numbering corresponds to included amino acids from MHCK B (GenBank™ accession number P90648). Coomassie Blue-stained SDS-PAGE gel at left was loaded with 2 μg of purified GST-B-CAT and GST-B-CATWD each.

The Role of the WD Repeat Domain in Defining MHCK A Catalytic Activity toward Various Substrates

The GST-A-CAT and GST-A-CATWD constructs were assayed for kinase activity toward intact myosin II. Initial experiments determining the time course of MHC phosphorylation by each of the GST fusion proteins revealed that only the GST-A-CATWD construct phosphorylated myosin efficiently (Fig.2 *A*). The GST-A-CAT construct exhibited kinase activity that was barely detectable above background levels. Similar results were obtained when GST-A-CAT and GST-A-CATWD activities were analyzed over a range of myosin II concentrations (Fig.2 *B*). Although kinetic parameters for MHC phosphorylation could not be determined because of the insolubility of myosin II substrate at higher concentrations (≥ 2 μM), these results clearly show that the presence of the WD repeat domain is required for efficient phosphorylation of myosin II by MHCK A.

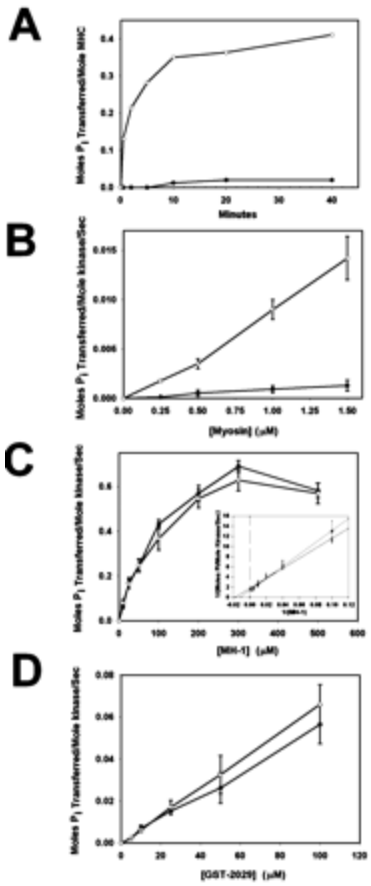

Figure 2
Phosphorylation of myosin II heavy chain, MH-1 peptide, and GST-2029 using MHCK A-derived fusion proteins either containing or lacking the WD repeat domain. *A*, the stoichiometry of *Dictyostelium* myosin II (0.42 μm) phosphorylation by GST-A-CAT and GST-A-CATWD (both at 20 nm final concentration) was assessed over time by subjecting kinase reactions to SDS-PAGE, Coomassie blue staining, and then scintillation counting of the excised MHC band (see "Experimental Procedures"). Plotted points represent the mean values from two separate experiments. *B*, GST-A-CAT and GST-A-CATWD constructs were assayed for kinase activity over a range of myosin II concentrations using the method described for the myosin stoichiometry experiment above. *C*, the MHCK A fusion proteins were assessed for kinase activity over a range of MH-1 peptide concentrations using the filter binding method described under "Experimental Procedures." The *inset* is a Lineweaver-Burk plot of the values obtained from this assay.*D*, GST-A-CAT and GST-A-CATWD activities were assessed over a range of GST-2029 concentrations by excising GST-2029 bands from Coomassie-stained SDS-PAGE gels followed by scintillation counting of those bands. For all of the plots in this figure, GST-A-CAT is represented by *closed circles*, and *open circles*represent the activity of the GST-A-CATWD fusion protein. The GST-A-CAT and GST-A-CATWD constructs used in all of these experiments were preautophosphorylated for 10 min prior to their addition to the reaction mix. For substrate concentration range experiments (*B–D*), both GST-A-CAT and GST-A-CATWD were included in the reaction mixes at a final concentration of 40 nm and incubated with substrate for 1 min at 25 °C. For *B–D*, the plotted values represent the mean activities determined from at least four independent experiments. The *vertical bars*indicate the standard error of each mean.

The recombinant GST-A-CAT and GST-A-CATWD fusion proteins were tested next for kinase activity toward the MH-1 peptide. MH-1 is a previously described (21) 16-residue peptide corresponding to the mapped MHCK A target site at residue 2029 of MHC. Both GST-A-CAT and GST-A-CATWD phosphorylated MH-1 substrate efficiently (Fig. 2 *C* and TableI) and exhibited specific activities within an order of magnitude to those reported previously for both native MHCK A (13) and recombinant MHCK A catalytic domain expressed as a His_6-tagged fusion protein (Table I) (23). Our results showing a lack of difference between the activities of GST-A-CAT and GST-A-CATWD toward MH-1 demonstrate that the presence or absence of the WD repeat domain does not affect the basic catalytic activity of MHCK A. Moreover, these results, together with the myosin II phosphorylation results described above, demonstrate unequivocally that the recombinant WD repeat domain is necessary and sufficient to stimulate MHCK A catalytic activity toward *Dictyostelium* myosin II.

Table I

MH-1 kinetics constants for GST fusion proteins

Kinase	K_m	V_{max}
	μM	*mol P_i/mol kinase/s*
Native MHCK A	105 ± 10	4.8
GST-A-CAT	107 ± 15	0.81 ± 0.04
GST-A-CATWD	59 ± 6.2	0.62 ± 0.09
GST-B-CAT	75 ± 6.5	0.49 ± 0.06
GST-B-CATWD	60.6 ± 6.5	0.42 ± 0.02

Kinetics constants (± S.E.) were estimated by fitting data of Lineweaver-Burk plots by linear regression analysis using the SigmaPlot program. The kinetics constants for native MHCK A were obtained from Ref. 21.

The fusion proteins of both MHCK A and MHCK B were also assessed for the ability to autophosphorylate. Previous studies have shown that autophosphorylation is a major mechanism regulating native MHCK A, resulting in a 50-fold increase in kinase activity (21). In the current study, fusion protein constructs derived from MHCK A consistently autophosphorylated to levels of 1–2 mol P_i/mol kinase (Fig.4 *A*). The level of autophosphorylation does not change if the WD repeat domain of MHCK A is absent. In comparison, the MHCK B-derived fusion proteins autophosphorylated to a level of 15–20 mol P_i/mol kinase (Fig. 4 *B*), with some preparation-to-preparation variability in autophosphorylation stoichiometry. For both the MHCK A and MHCK B-derived fusion proteins, the presence or absence of the WD repeat domain does not significantly affect autophosphorylation rate or stoichiometry.

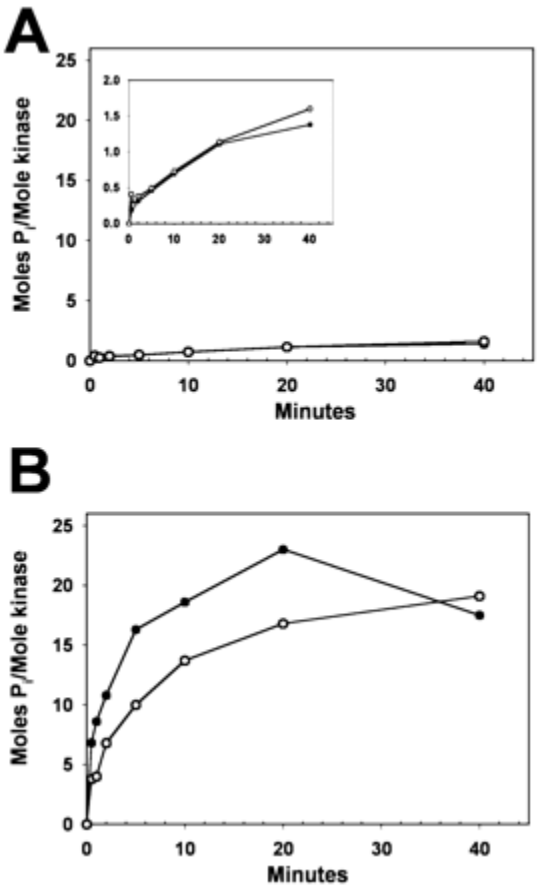

Figure 4
Autophosphorylation of MHCK A and MHCK B-derived fusion proteins. Autophosphorylation reactions were performed at 200 nm of each kinase construct, and the level of autophosphorylation was determined with a filter binding assay as described under

"Experimental Procedures." *A*, autophosphorylation of GST-A-CAT (●) and GST-A-CATWD (○)*versus* time. The *inset* represents the same data plotted to a smaller scale. *B*, autophosphorylation of GST-B-CAT (●) and GST-B-CATWD (○) *versus* time.

Studies Exploring the Mechanism of WD Repeat-mediated Enhancement of Myosin Heavy Chain Kinase Activities

The results presented thus far demonstrate that both MHCK A and MHCK B require their WD repeat domains to phosphorylate myosin II efficiently but do not depend on the presence of their WD repeat domains for basic catalytic activity toward other substrates. However, it is not clear whether the WD repeat domain interacts with myosin II to physically target the kinase catalytic domain to its substrate or if an interaction between the WD repeat domain and myosin II results in stimulation of the inherent catalytic activity of the kinase. As a means of testing the latter possibility, the GST-A-CAT and GST-A-CATWD constructs were assayed for kinase activity toward MH-1 peptide in the presence and absence of myosin II. Under these conditions, neither kinase construct was stimulated to phosphorylate MH-1 peptide above normal levels, thus indicating that the kinase catalytic activity is not affected by an interaction between the WD repeat and myosin II (Fig. 5). These findings further support a role for the WD repeat domain in physically targeting the kinase specifically to myosin substrate.

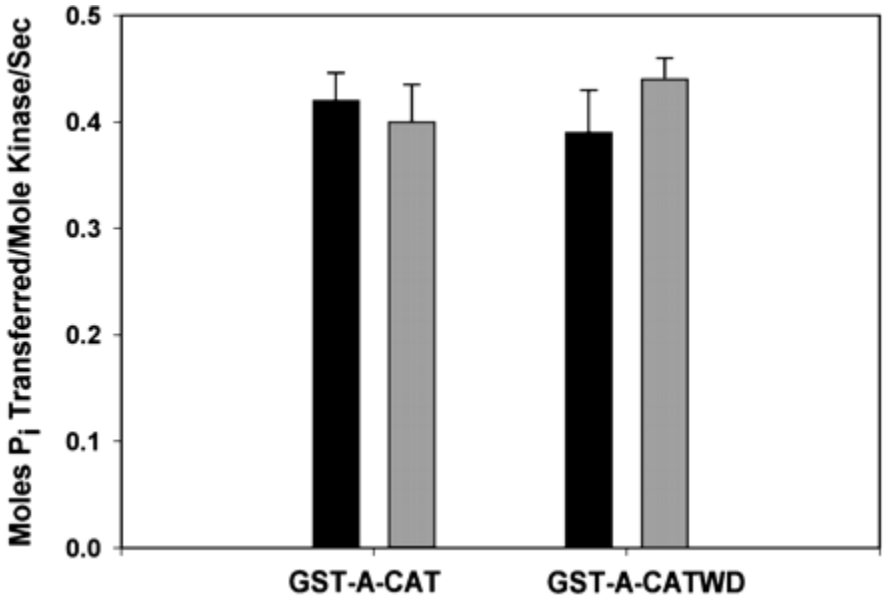

Figure 5

Phosphorylation of MH-1 peptide by GST-A-CAT and GST-A-CATWD in the presence and absence of myosin II. The MHCK A fusion proteins (40 nm) each were incubated (4 min at 25 °C) with 50 μm MH-1 substrate in the presence (*gray bars*) or absence (*black bars*) of 0.5 μm myosin II. Specific activity toward MH-1 peptide was measured using the filter binding method described under "Experimental Procedures." Values obtained from control reactions containing only kinase and myosin II were subtracted from experimental values to control for MHC phosphorylation in these reactions. The GST-A-CAT and GST-A-CATWD constructs were preautophosphorylated for 10 min prior to their addition to the reaction mix. The *bars*represent the mean activity from three independent experiments with the*error bars* representing the standard error of each mean.

To explore the possibility that WD repeat targeting of MHCK A and MHCK B involves a direct interaction with myosin, we assayed all four of the fusion proteins for cosedimentation with myosin filaments. Results from these assays revealed that up to 40% of the WD repeat containing constructs of both MHCK A and MHCK B bound directly to myosin II filaments under the conditions used in the cosedimentation assay (Fig.6, *A*–*C*). In contrast, the GST-A-CAT and GST-B-CAT fusion proteins, both of which lack WD repeat domains, exhibited significantly less cosedimentation with myosin II filaments. Sedimentation of GST alone was not detectable in the presence or absence of myosin (data not shown). A GST fusion protein containing only the WD repeat domain of MHCK A was also tested, but high basal sedimentation caused by aggregation made this construct unsuitable for these assays (data not shown). To determine whether the catalytic domain contributes to myosin II binding, we assayed GST-A-CATWD and GST-B-CATWD for cosedimentation with myosin II filaments in the presence of MH-1 peptide. Under these conditions, an interaction between the catalytic domain and myosin II should be inhibited by peptide binding to the substrate-binding site. Our results demonstrate that the presence

of MH-1 peptide at either 100 and 400 μM has no detectable effect on MHCK A or MHCK B binding to myosin II filaments and thus suggest that the interaction of the kinases with myosin II is mediated mainly through their WD repeat domains with little contribution from the catalytic domains. These results, showing that both MHCK A and MHCK B bind to myosin filaments in a manner that requires the presence of a WD repeat domain, indicate that the mechanism by which the WD repeat domain promotes myosin phosphorylation involves physically targeting the kinases to myosin substrate.

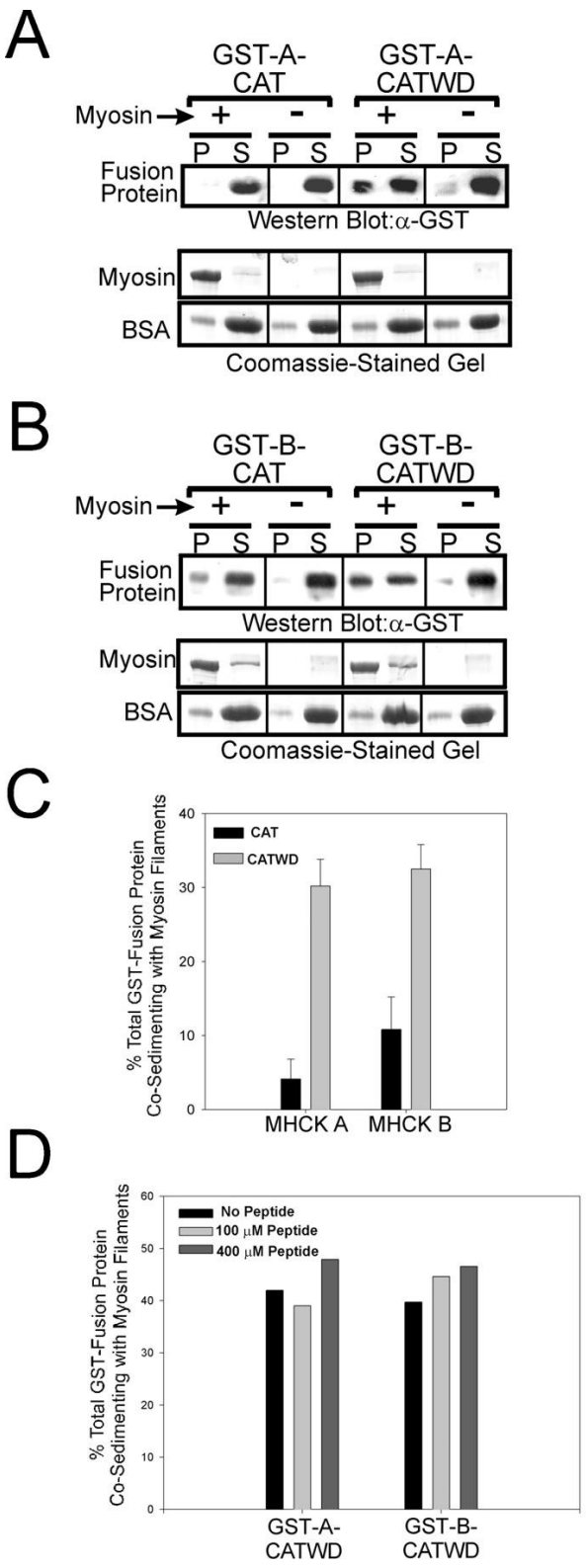

Figure 6

Cosedimentation assays of MHCK A and MHCK B-derived fusion proteins with myosin II filaments. Kinase fusion proteins (0.3 μm) were incubated with myosin filaments (1.0 μm) as described under "Experimental Procedures." Reaction mixes were centrifuged, and equal volumes of the resulting pellets (P) and supernatants (S) were subjected to SDS-PAGE. Myosin and BSA were visualized by Coomassie Blue staining of the gel, and the kinase fusion proteins were identified by Western blotting with anti-GST antibody. A, representative Western blot (top) and Coomassie-stained gel (bottom) from a myosin cosedimentation assay of GST-A-CAT and GST-A-CATWD. B, representative Western blot (top) and Coomassie-stained gel (bottom) from a myosin cosedimentation assay of GST-B-CAT and GST-B-CATWD.C, bar graph of kinase construct binding to myosin II. The values represent the means from three separate experiments performed in duplicate, and the error bars represent the standard error of each mean. D, bar graph of GST-A-

CATWD and GST-B-CATWD cosedimentation with myosin II in the presence and absence of MH-1 peptide. Cosedimentation results were quantified by densitometric analysis of Western blots, and the amount of fusion protein in the pellet fraction was divided by that in both the pellet and supernatant to yield the values indicated by the bars.

DISCUSSION

In the experiments presented here we have shown that the structurally related WD repeat domains of MHCK A and MHCK B share a conserved mechanism for targeting MHC kinase catalytic activity by binding directly to myosin substrate. This WD domain-mediated binding facilitates the phosphorylation of MHC. For MHCK A, the functional consequence of this phosphorylation is to promote myosin II filament disassembly and ultimately to inhibit myosin II-mediated contraction in cells (9, 19, 25). Further studies are needed to characterize the physiological roles of MHCK B and to determine whether MHCK B phosphorylation of MHC is restricted to the same or different sites phosphorylated by MHCK A.

Using recombinant, bacterially expressed GST fusion proteins of MHCK A and its homologue MHCK B, we have shown that both kinases are targeted by their WD repeat domains to phosphorylate myosin II substrate specifically. Assays of these constructs revealed that full kinase activity toward native myosin substrate is achieved only with MHCK A and MHCK B fusion protein constructs containing their corresponding WD repeat domains. MHCK A and MHCK B fusion proteins lacking their WD repeat domains exhibit reduced ability to phosphorylate myosin II. In contrast, activities of the kinases toward soluble substrates such as the MH-1 peptide or GST-2029 are not affected by the presence or absence of their WD repeat domains. This demonstrates that the basal kinase catalytic activities of both MHCK A and MHCK B are independent of their WD repeat domains. It is noteworthy that target recognition appears to involve an extended structural component because phosphorylation of GST-2029, a myosin-based substrate that is predicted to fold into a coiled-coil conformation but that lacks the region necessary for filament formation, is not stimulated by the presence of the WD repeat domain.

The mechanism by which the WD repeat domain targets the kinase to myosin II does not involve stimulation of the inherent activity of the catalytic domain because the ability of GST-A-CATWD to phosphorylate MH-1 peptide is not enhanced in the presence of myosin II (Fig. 5). Kinetic analysis of kinase activity over a range of myosin concentrations from 0.25 to 1.5 μM revealed no saturation of the reaction rate (Figs. 2 *B* and 3 *B*).

Solubility limitations preclude assays at fully physiological myosin II concentrations (~5 μM; Ref. 19); however, the lack of saturation or of an apparent V_{max} in these tests suggests that the WD repeat domains confer an elevated affinity of the kinases for myosin but do not lead to formation of a rigid substrate-enzyme complex. Further studies will be needed to determine whether there are cellular mechanisms that may dynamically regulate this affinity *in vivo* and whether substrate binding requires a fully assembled bipolar myosin filament or simply an extended region of the monomeric myosin tail.

Using myosin II cosedimentation assays we have determined that the conserved WD repeat domains of both MHCK A and MHCK B target MHC kinase catalytic activity by binding directly to myosin II substrate. To our knowledge, this represents the first report of a WD repeat domain physically targeting an attached kinase domain to its substrate. Although further examples are needed, the fact that the WD repeat domains of both MHCK A and MHCK B are substrate targeting domains may indicate that β-propeller domains present in other protein kinases play similar roles in substrate targeting. Potential candidates for testing this possibility include the *Dictyostelium* protein MHCK C, which possesses catalytic and WD repeat domains related to those of MHCK A, and a recently identified bacterial protein from*Thermomonospora curvata*, which is predicted to contain a conventional serine/threonine kinase domain coupled to a WD repeat containing region (26). Recently, the WD repeat domain of a receptor for activated protein kinase C (Rack1) has been shown to bind the cytoplasmic region of the integrin β-subunit and has been proposed to recruit activated protein kinase C to integrins (27). Thus, the WD repeat domain of Rack1, although not covalently attached to protein kinase C, may play a direct role in targeting protein kinase C activity to a substrate.

MHCK A, B, and C have a conserved domain structure that includes a catalytic domain unrelated to conventional protein kinases, with a WD repeat domain that lies C-terminal to the catalytic domain. Members of the emerging family of eEF-2 kinases, present throughout the animal kingdom (15, 28, 29), contain a catalytic domain highly related to that of MHCK A, B, and C but do not have detectable WD repeat motifs. However, these enzymes all contain a C-terminal domain of roughly similar size to the WD repeat domains observed in MHCK A, B, and C. Recent work from Pavur and colleagues (30) has revealed that the C-terminal segment of eEF-2 kinases also acts as a targeting domain toward the eEF-2 substrate. Moreover, these researchers identified a degenerate repeat motif present in the eEF-2 kinase targeting domain. This 36-amino acid repeat is intriguingly similar in size to the WD repeat motif (~40 amino acids) but is not detectably related to WD repeats at the primary sequence level.

The actin-fragmin kinase of *Physarum* may also display a related mode of targeting. This enzyme contains a highly divergent kinase catalytic domain with a fold related to conventional protein kinases (31). C-terminal to the catalytic domain, this enzyme contains a domain with a predicted β-propeller structure of the Kelch class (32). Notably, removal of the β-propeller domain was reported to cause a significant decrease in enzyme activity when assayed against the native actin-fragmin substrate (other substrates were not tested). We suggest that in this enzyme as well, the β-propeller domain may be serving a substrate targeting role.

Results from our studies of WD repeat domain targeting of MHC kinase activity provide strong evidence that MHCK B is a physiologically relevant myosin heavy chain kinase. Although the isolated catalytic domain of MHCK B has been shown to preferentially phosphorylate the mapped MHCK A target sites on MHC (16), we have found that the initial rate of activity of the MHCK B constructs toward myosin II and myosin-like substrates (MH-1 and GST-2029) is significantly lower than that observed for the MHCK A fusion proteins (Table I). This does not appear to be due to inherently lower kinase activity because the MHCK B fusion proteins phosphorylate myelin basic protein, a nonphysiological substrate, at a higher rate than do the MHCK A constructs. In comparison with MHCK A, the MHCK B fusion protein shows greater substrate promiscuity, exhibiting relatively higher initial rates of kinase activity toward skeletal muscle and smooth muscle myosin substrates. Although further studies of the activity and substrate specificity of native MHCK B will be important, the results presented here raise the possibility that myosin II may not be the only cellular substrate for MHCK B.

In summary, we have shown that the catalytic activities of MHCK A and MHCK B are targeted specifically to myosin II by a mechanism that involves direct binding via their associated WD repeat domains. We suggest that this targeting mechanism may be conserved among an array of identified protein kinases that have β-propeller domains adjacent to their catalytic domains. Although MHCK A and B fusion proteins share the property of binding to myosin filaments via their WD repeat domains, other aspects of their kinase activity clearly differ, suggesting different cellular roles for these enzymes.

ACKNOWLEDGEMENTS

We thank the Egelhoff Lab, Dr. Luke Szweda, and Dr. Graham Côté for helpful suggestions.

Footnotes

- * This work was supported by research Grant GM50009 (to T. T. E.) from the National Institutes of Health and by American Cancer Society Postdoctoral Fellowship PF-99-310-01-CSM (to P. A. S.).The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Abbreviations:

MHC myosin heavy chain

BSA bovine serum albumin

GST glutathione *S*-transferase

MHCK myosin heavy chain kinase

PIC protease inhibitor cocktail

PAGE polyacrylamide gel electrophoresis

TES 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]- amino} ethanesulfonic acid

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