Identification of a Protein Kinase from *Dictyostelium* with Homology to the Novel Catalytic Domain of Myosin Heavy Chain Kinase A*

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Myosin II assembly and localization into the cytoskeleton is regulated by heavy chain phosphorylation in *Dictyostelium*. The enzyme myosin heavy chain kinase A (MHCK A) has been shown previously to drive myosin filament disassembly *in vitro* and *in vivo*. MHCK A is noteworthy in that its catalytic domain is unrelated to the conventional families of eukaryotic protein kinases. We report here the cloning and initial biochemical characterization of another kinase from *Dictyostelium* that is related to MHCK A. When the segment of this protein that is similar to the MHCK A catalytic domain was expressed in bacteria, the resultant protein displayed efficient autophosphorylation, phosphorylated *Dictyostelium* myosin II, and also phosphorylated a peptide substrate corresponding to a portion of the myosin II tail. We have therefore named this gene myosin heavy chain kinase B. These results provide the first confirmation that sequences in other proteins that are related to the MHCK A catalytic domain can also encode protein kinase activity. It is likely that the related segments of homology present in rat eukaryotic elongation factor-2 kinase and a putative nematode eukaryotic elongation factor-2 kinase also encode the catalytic domains of those enzymes.

Myosin II has central roles in a variety of cellular motility events, including cytokinesis, cell locomotion, and morphological changes that occur during development. In *Dictyostelium*, myosin heavy chain (MHC) phosphorylation on threonine residues in the tail seems to be a mechanism regulating bipolar myosin filament assembly and localization into the cytoskeleton (1).

Two distinct MHC kinases have been previously implicated in myosin filament regulation. *In vitro* each of these enzymes is capable of driving myosin filament disassembly. A kinase that has been named MHC-PKC was purified from developed *Dictyostelium* cells (2) and subsequently cloned (3). This enzyme has a catalytic domain similar to members of the protein kinase C family and also contains a distinct domain with significant similarity to the catalytic domain of diacylglycerol kinases (4). This enzyme is expressed in a developmental-specific manner and seems to be involved in regulating myosin assembly/disassembly during chemotactic cell migration (5, 6). The enzyme MHCK A was first purified from growth-phase cells (7) and is expressed during both growth and development.

Molecular analysis of the MHCK A gene has revealed a highly novel three-domain structure (8). MHCK has an amino-terminal domain of ~70 kDa that has a predicted coiled-coil structure, a central domain of ~30 kDa, and a carboxy-terminal domain of ~30 kDa that consists of 7 WD or G,like repeats (9, 10). The deduced polypeptide sequence of MHCK A displays no detectable similarity to conventional eukaryotic protein kinases or to members of the histidine protein kinase family (11). Truncation analysis, coupled to expression in *Escherichia coli*, was used to demonstrate that the central domain of MHCK A (residues 550–841) contains all of the catalytic protein kinase functions (12). This segment of MHCK A displays substantial identity (~42%) to a *Caenorhabditis elegans* cDNA (yk3f11) that has been sequenced as part of the nematode genome project (GenBank® accession numbers U10414 and D27775). More recently, a cDNA sequence has been reported (13) for rat skeletal muscle eukaryotic elongation factor-2 (eEF-2) kinase (also known as calcium/calmodulin-dependent kinase III). The deduced protein sequence of the eEF-2 kinase also displays a high degree of identity to the catalytic domain of MHCK A (43%; see Ref. 12). Based upon similarity in the flanking regions, it seems that the *C. elegans* yk3f11 cDNA may encode a nematode homologue of eEF-2 kinase. As with MHCK A, neither rat eEF-2 kinase nor the nematode gene displays any significant similarity to conventional eukaryotic protein kinases.

The presence of sequences related to the novel MHCK A catalytic domain in this diverse set of organisms suggests a possibly widespread new family of protein kinases. We have performed polymerase chain reaction (PCR) experiments using primers matching regions conserved between MHCK A and the putative *C. elegans* eEF-2 kinase to search for additional genes in *Dictyostelium* that may encode members of this novel group of proteins. We report here the molecular cloning and primary biochemical characterization of an additional member of the MHCK A-related protein kinase family.

**MATERIALS AND METHODS**

PCR and Cloning—Several oligonucleotide primers were designed based on sequences conserved between MHCK A and the *C. elegans*...
putative eEF-2 kinase. The pair used for successful amplification was:

(a) KinU2 (5'-CAGAATTCCACNCNCAGCCTTTCG, in which N = (G,

A, T, and C), R = (G and A), and Y = (T and C)); and

(b) KinD2

(5'-CAGAATTCTGRTGDATYTGNGGGTG, in which D = (G, A, and

T)). The KinU2 oligonucleotide corresponds to the translated sequence

TPQAFS (single-letter amino acid code) of MHCK A, and the KinD2

oligonucleotide corresponds to the translated sequence DPQIHT of

MHCK A. PCR was performed using 100 ng of genomic DNA from a

MHCK A- Dictyostelium cell line in which the entire gene for MHCK A

was deleted (14). Amplification was performed as follows: 94 °C dena-
turation (30 s), 37 °C annealing (30 s), and 72 °C extension (1 min) for

3 cycles, and then 94 °C denaturation (30 s), 37 °C annealing (30 s), and

72 °C extension (1 min) for 30 cycles. A single product band of the

expected size (120 bp) was obtained and cloned into vector pGEM7

(Promega). Sequence analysis revealed that this product was related to

MHCK A.

The cloned PCR fragment was labeled with 32P and used as a probe
for library screening. A 4-h developed cDNA library (Clontech) was
probed using standard conditions (15). Two overlapping clones were
isolated that upon sequence analysis seemed to encode the entire
MHCK B open reading frame. The compiled sequence was determined
from both clones, using a combination of automated fluorescent se-
quencing and manual dideoxy sequencing. All portions of the gene were
sequenced at least once on both strands.

Fusion Protein Expression and Phosphorylation Assays—A segment
of the cDNA clone spanning predicted amino acid residues 31–387 was
cloned into an E. coli expression vector that attaches a carboxyl-termi-
nal 6X histidine tag (pET21c; Novagen). The resultant protein has a
predicted mass of 44 kDa. A protein of approximately this size was
found at high levels in the inclusion bodies of cells expressing this
construct when isopropyl-1-thio- b-D-galactopyranoside inductions were
performed at 37 °C. Isopropyl-1-thio- b-D-galactopyranoside inductions
were therefore performed at room temperature, which resulted in lower
yield, but a substantial portion of the protein product remained soluble
under these conditions. Lysis and Ni-affinity chromatography were
performed with a kit, according to the manufacturer’s instructions
(Novagen). The final protein was stored at

280 °C in 10 mM Hepes, pH

7.5, 50 mM KCl, and 30% glycerol. Final protein concentration was
determined by performing SDS-polyacrylamide gel electrophoresis and
Coomassie staining of the expressed protein in parallel with a standard
concentration series of bovine serum albumin (BSA; Pierce). Densitom-
etry was performed on the resulting gels to determine the concentration

FIG. 1. A, amino acid sequence of MHCK B. The proposed nucleotide-binding loop is double underlined (residues 298–301), and the partially
conserved D-X5-WD motifs of the WD repeat domain are underlined. The full nucleotide sequence of MHCK B cDNA is available from GenBank
(accession number U90946). B, schematic alignment of the domain structure of MHCK A, MHCK B, rat eEF-2 kinase, and the C. elegans
putative eEF-2 kinase (C.e. ORF). Structure of the C. elegans protein is predicted from the genomic and cDNA data base entries, using predicted splice
junctions indicated in the genomic DNA data base entry. MHCK A has a large coiled-coil domain (circle-filled box) that is absent in MHCK B and
the eEF-2 kinases. All sequences display substantial identity over a region of ~250 amino acids corresponding to the demonstrated catalytic
domain of MHCK A (■). MHCK A and MHCK B each contain a 7-fold repeat of the WD repeat motif at the carboxyl terminus (■). MHCK A and
MHCK B each seem to have a nonconserved linker region between the catalytic domain and the WD repeat domain (○), corresponding to residues
355–381 of MHCK B). The C. elegans gene and rat eEF-2 kinase contain a conserved carboxyl-terminal domain (checkered box) that displays no
similarity to MHCK A or MHCK B.
of the purified protein.

Myosin phosphorylation assays were performed in a 50-μl reaction volume containing 2 μg of Dictyostelium myosin, either 30 or 300 ng of the MHCK B fusion protein, and typically 1 μg of BSA as a negative internal control. Omission of BSA has no effect on autophosphorylation or phosphorylation of myosin. Myosin phosphorylation reactions were performed in 10 mM Tris, pH 7.0, 20 mM KCl, 2 mM MgCl2, 0.5 mM ATP, and 5 μCi of [γ-32P]ATP at 23 °C for 60 min. Peptide phosphorylations were performed with the peptide MH-1 (RKPGGEKETKFEKL-smide; (16)), which corresponds to the major MHCK A target site on the myosin heavy chain at residue 2029 (underlined T in the peptide). These reactions were performed in 10 mM Hepes, pH 7.5, 2 mM MgCl2, 1 mM ATP, 1 mM dithiothreitol, 50 μM MH-1 peptide, and 0.25 μCi/μl [γ-32P]ATP. The recombinant MHCK B protein was allowed to autophosphorylate in the presence of nonradioactive ATP for 25 min before initiation of the peptide phosphorylation reaction. Omission of this autophosphorylation step resulted in a lower activity toward the peptide during the peptide phosphorylation reaction. Omission of BSA has no effect on autophosphorylation or phosphorylation of myosin. Myosin phosphorylation reactions were also performed using 30 or 300 ng of the expressed MHCK B (Fig. 2), in which the 3 mapped target sites for MHCK A (at positions 1823, 1833, and 2029 of MHC) were converted to alanine residues (3X ALA MHC) (1). With this substrate, the MHCK B fusion protein transferred 0.6 mol of phosphate/mol of MHC. In experiments similar to that shown, we have observed MHC phosphorylation stoichiometry as high as 1.2 mol of P/ mol of MHC by the MHCK B fusion protein. Phosphorylation reactions were also performed using Dictyostelium myosin in which the 3 mapped target sites for MHCK A (at positions 1823, 1833, and 2029 of MHC) were converted to alanine residues (3X ALA MHC). With this substrate, the MHCK B fusion protein reproducibly incorporated less phosphate into the MHC (0.12 mol of P/ mol of MHC in the presented experiment). The MHCK B fusion protein autophosphorylated to a level of 1.2 mol of P/mol of fusion protein under tested conditions. Although the expressed segment of MHCK B phosphorylated MHC, the activity of the protein toward MHC was significantly lower than the activity observed previously with a similar recombinant segment of the MHCK A protein (12). Approximately 300 ng of the expressed MHCK B was required to achieve transfer of 1 mol of P/mol of MHC in reactions containing 2 μg of myosin.

Catalytic activity was also assessed using the peptide substrate MH-1 (16), which corresponds to the previously characterized target site for MHCK A at residue 2029 of the Dictyostelium myosin tail. Minimal activity against this peptide was observed when the purified MHCK B fusion protein was added directly to peptide phosphorylation reactions. However, if a 25-min autophosphorylation step was performed first, in the

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RESULTS AND DISCUSSION

In a search for new genes related to MHCK A, PCR studies were performed using primers to regions highly conserved between MHCK A and the related C. elegans eEF-2 kinase (C. elegans putative eEF-2 kinase (C. elegans putative eEF-2 kinase (C. elegans putative eEF-2 kinase (C. elegans putative eEF-2 kinase). Positions of alignment: MHCK A, residues 563–805; MHCK B, residues 121–325; rat eEF-2 kinase, residues 112–322; C. elegans eEF-2 kinase, residues 99–306 (based upon the predicted translation in GenBank entry)). Residues that are identical in all four sequences are boxed.

Fig. 2. Clustal alignment of related portions of MHCK A, MHCK B, rat eEF-2 kinase (rat EEF2k), and the C. elegans putative eEF-2 kinase (C. elegans OFB).
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suggest that this enzyme has at least partial specificity to the same target sites phosphorylated by MHCK A, because the 3X ALA MHC is a poorer substrate than wild-type myosin. Given
the demonstrated role of MHCK A in regulating myosin localization in vivo (1, 14), these results imply that MHCK B may also participate in control of myosin localization.

Alignment of the conserved portions of MHCK A, MHCK B, rat eEF-2 kinase, and the putative C. elegans eEF-2 kinase (Fig. 2) reveals a series of conserved regions, including a possible nucleotide-binding motif (GXGXXG at residues 298–303 in MHCK B), and a conserved pair of cysteines (residues 316 and 320 in MHCK B). The presence of a GXGXXG motif at the extreme carboxyl-terminal end of the conserved portion of each sequence further suggests that these proteins may have a three-dimensional structure unrelated to the conventional eukaryotic protein kinases, in which the GXGXXG nucleotide-binding motif is located near the amino-terminal portion of the catalytic domain (17).

Although Redpath and colleagues (13) have suggested that the catalytic domain of rat eEF-2 kinase lies in the carboxyl-terminal portion of that protein, our biochemical analysis of MHCK A (12) and MHCK B (reported here) strongly suggests that the amino-terminal segment of eEF-2 kinase, which is approximately 50% identical to the catalytic domains of MHCK A and MHCK B, encodes the catalytic functions of rat eEF-2 kinase.

Further biochemical mapping of the catalytic sequences of MHCK B and other members of this novel family of protein kinases will clearly be important. Additional studies are in progress to determine the substrate specificity of MHCK B and to determine whether this enzyme plays a physiological role in the regulation of myosin function in Dictyostelium and/or whether MHCK B has other in vivo substrates of importance.

Note Added in Proof—Dr. Alexy Ryazanov and colleagues have recently demonstrated that the C. elegans gene discussed in this publication encodes a bona fide eEF-2 kinase and that closely related genes are present in mice and humans (Proc. Natl. Acad. Sci. U. S. A., in press).

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Fig. 3. MHCK B phosphorylation analysis. A, Coomassie-stained gel of phosphorylation reactions after SDS-polyacrylamide gel electrophoresis. B, autoradiogram of same sample set. Lane 1, 30 ng of MHCK B; lane 2, 30 ng of MHCK B, 2 μg of myosin; lane 3, 30 ng of MHCK B, 2 μg of 3X ALA myosin; lane 4, 300 ng of MHCK B; lane 5, 300 ng of MHCK B, 2 μg of myosin; lane 6, 300 ng of MHCK B, 2 μg of 3X ALA myosin. Each reaction contains 1 μg of BSA as an internal negative control. Positions of the MHC, BSA, and recombinant MHCK B fusion protein (rMHCK B) are indicated to the left. The faint band in B, lane 4, at approximately 70 kDa, does not coincide with the position of BSA when the autoradiogram is aligned to the dried gel. BSA was not detectably phosphorylated in any sample. C, activity of the expressed MHCK B segment tested with varying concentrations of the peptide substrate MH-1. The experiment was performed in triplicate. Error bars represent S.D. and are smaller than the symbols for most points.
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