Mapping of the Novel Protein Kinase Catalytic Domain of Dictyostelium Myosin II Heavy Chain Kinase A*

(Received for publication, November 14, 1996, and in revised form, December 23, 1996)

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Myosin heavy chain kinase A (MHCK A) in Dictyostelium was identified as a biochemical activity that phosphorylates threonine residues in the myosin II tail domain and regulates myosin filament assembly. The catalytic domain of MHCK A has now been mapped through the functional characterization of a series of MHCK A truncation mutants expressed in Escherichia coli. A recombinant protein comprising the central nonrepetitive domain of MHCK A (residues 552–841) was isolated in a soluble form and shown to phosphorylate Dictyostelium myosin II, myelin basic protein, and a synthetic peptide substrate. The functionally mapped catalytic domain of MHCK A shows no detectable sequence similarity to known classes of eukaryotic protein kinases but shares significant sequence similarity with the mammalian elongation factor-2 kinase (calcium/calmodulin-dependent protein kinase III). We suggest that MHCK A represents the prototype for a novel, widely occurring protein kinase family.

Assembly of Dictyostelium myosin-II into the cytoskeleton in vivo is regulated by myosin heavy chain phosphorylation (1, 2). The biochemically identified 130-kDa myosin heavy chain kinase (MHCK A) has a demonstrated role in regulating myosin II assembly both in vitro (3) and in vivo (4). A surprising feature of the primary sequence of MHCK A is that it displays no detectable similarity to the conserved catalytic domains found in conventional eukaryotic protein kinases (5). MHCK A also displays no detectable similarity to “histidine protein kinases” (6) or related proteins such as α-ketoacid dehydrogenase kinase (7, 8). MHCK A consists of an amino-terminal domain with probable coiled-coil structure, a central nonrepetitive domain, and a carboxyl-terminal domain consisting of seven WD repeats (9). The central nonrepetitive domain of MHCK A does contain a GXGXXG motif similar to that present in the nucleotide-binding site of conventional protein kinases, but this motif is located only about 75 residues distant from the start of the first WD repeat. In conventional protein kinases the GXGXXG motif is located in the first of the 12 distinct kinase subdomains and is followed by about 250 residues of conserved sequence that comprises the rest of the catalytic domain (10).

In this paper we define the location and extent of the novel MHCK A protein kinase catalytic domain by expressing in Escherichia coli and functionally characterizing a series of MHCK A truncation mutants. The results show that neither the MHCK A coiled-coil domain nor the WD repeat domain are required for kinase activity and identify the catalytic activity as residing solely in the 40-kDa MHCK A central domain. Although the central domain of MHCK A exhibits no homology with conventional protein kinases, it does share significant sequence similarity with the rat eukaryotic elongation factor-2 (eEF-2) kinase and with a possible eEF-2 kinase homolog from Caenorhabditis elegans. The results indicate that the novel kinase catalytic domain of MHCK A is evolutionarily conserved and thus may represent the prototype for a novel family of protein kinases.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Protein Purification—A plasmid that expresses “full-length” MHCK A (residues 8–1132) in E. coli with a hexahistidine (His6) tag at the carboxyl terminus was constructed using the pET21d vector and has been described (5). The first seven codons of the MHCK A coding region are absent in this construct, replaced by four codons from the pET21 vector. At the COOH terminus the last 14 codons of MHCK A are removed at the fusion site to the vector His6 tag. Subsequent constructs were generated using standard methods to create the set of truncations presented in Fig. 1. Each recombinant protein contains several polylinker-derived amino acids at the NH2 terminus and a His6 tag at the COOH terminus. Both wild type and IX ALA Dictyostelium myosin IIA were purified as described in Ref. 11, with the addition of a final Sephacryl S300 chromatography step to remove residual contaminating proteins.

Expression and Purification of Truncated Forms of MHCK A—Transformed E. coli strain BL21 was grown at either 37°C (for purification of proteins from inclusion bodies) or 24°C (for isolation of soluble protein). Induction, lysis, and purification on nickel chelation chromatography resin was performed according to standard protocols provided with the His-Bind resin (Novagen). For purification of insoluble protein from inclusion bodies, 8 ml urea was included during binding and elution steps. Recombinant proteins were eluted with 0.1 M NaCl, 0.2 M imidazole, 5 mM Tris, pH 8.0, dialyzed overnight against 20% glycerol, 20 mM NaCl, 1 mM dithiothreitol, 20 mM Hepes, pH 7.0, and stored at −80°C. Proteins stored in this manner retained activity for several months.

Phosphorylation and Further Purification of T-4 and T-5—Soluble T-4 and T-5 recovered from E. coli grown at 24°C were dialyzed overnight against 20 mM NaCl, 1 mM dithiothreitol, and 20 mM Tris, pH 7.5, and dephosphorylated by addition of 2 mM MgCl2 and 10 μg/ml CIAP. After 1 h at room temperature samples were chromatographed over the His-Bind resin as described above or loaded onto a Mono Q column (Pharmacia Biotech Inc.) equilibrated in 20 mM NaCl, 1 mM dithiothreitol, 20 mM Tris, pH 7.5. T-4 and T-5 were eluted from the Mono Q column with an NaCl gradient to 1 M. Both procedures separated T-4 and T-5 from CIAP.

Phosphorylation Assays—In some cases recombinant proteins were assayed for the ability to autophosphorylate following SDS-PAGE and electrophoretic transfer to nitrocellulose filters (5). Briefly, filters were subjected to a denaturation/renaturation regime and then incubated in buffer containing 10 mM MgCl2, 2 mM MnCl2, 50 mM γ-[32P]ATP (1000 Ci/mmol), and 30 mM Tris, pH 7.5. The filters were then washed and exposed to x-ray film as described (5). Soluble proteins were assayed for the ability to autophosphorylate and phosphorylate exogenous sub-
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RESULTS AND DISCUSSION

A schematic representation of the MHCK A constructs used in this study is shown in Fig. 1A. The full-length and truncation mutants of MHCK A expressed in E. coli for this study are depicted schematically (A). The approximate boundaries for the three MHCK A domains are indicated: the α-helical coiled-coil domain (circle-filled bar), the central nonrepetitive domain (white bar), and the WD repeat domain (cross-hatched bar). The location of the GXGGXXG motif (residues 778–785) is indicated. Numbers at the ends of each construct denote the first and last residues of MHCK A present in the recombinant protein. The molecular masses shown are calculated based on the predicted amino acid sequence of the recombinant proteins. Autophosphorylation activity of all constructs purified from inclusion bodies was assessed using a filter renaturation assay described previously (5). Briefly, samples of each protein (2 μg) were subjected to SDS-PAGE and either stained with Coomassie Blue (B) or electrophoretically transferred to nitrocellulose, renatured, incubated with buffer containing [γ-32P]ATP, and subjected to autoradiography (C). Triplicate experiments were performed with each construct.

The full-length MHCK A and the T-4 recombinant protein were tested for autophosphorylation with [γ-32P]ATP and subjected to SDS gel electrophoresis. Shown are the Coomassie Blue-stained SDS gel (A) and the corresponding autoradiogram demonstrating autophosphorylation (B). Phosphorylation tests were also performed with the T-4 recombinant protein and wild type (WT) Dictyostelium myosin or Dictyostelium myosin bearing mutations in the mapped target sites for native MHCK A (3X ALA in figure). The Coomassie-stained gel (C) and corresponding autoradiogram (D) demonstrate substantially reduced phosphorylation of 3X ALA myosin relative to wild type myosin by both native MHCK A and the T-4 recombinant protein.

Previous studies have shown that bacterially expressed full-length MHCK A is able to autophosphorylate after being solubilized from inclusion bodies, subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with Mg2+, Mn2+, and [γ-32P]ATP (5). When subjected to the same procedure neither N-MHCK nor C-MHCK, which represent, respectively, the NH2- and COOH-terminal halves of MHCK A, displayed the ability to incorporate 32P into the amino terminus, because the ability of the recombinant proteins to interact with the His-Bind resin demonstrates the presence of the COOH-terminal His6 tag.

Strategies in a buffer containing 2 mM MgCl2, 1 mM dithiothreitol, 0.1 mM (γ-32P)ATP (0.1 Ci/mmol), and 10 mM HEPES, pH 7.0, unless noted otherwise. Incorporation of phosphate into peptide and protein substrates was quantitated using P81 phosphocellulose paper as described (12) or by excising the protein bands of interest following SDS-PAGE and Coomassie Blue staining and counting the bands in liquid scintillation fluid using a Beckman LS 7500 Scintillation Counter. Peptide phosphorylation assays were performed using MH-3 peptide (RRKF-GEAEKTKEFL) described previously (12). Wild type and 3X ALA myosin phosphorylation was performed in 10 mM HEPES, 0.2 mM MgCl2, 2 mM ATP, 1 mM DTT, pH 7.4, containing 0.3 μg/μl myosin and either 6 ng/μl MHCK A or 20 ng/μl T-4. Reactions were incubated 10 min at 22°C and then subjected to SDS-PAGE and autoradiography and phosphorimaging for quantitation.

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Fig. 1. Schematic of MHCK A truncation constructs (A). Coomassie-stained profile of each construct purified from inclusion bodies (B), and corresponding autoradiogram following denaturation/renaturation on nitrocellulose filters (C). The full-length and truncation mutants of MHCK A expressed in E. coli for this study are depicted schematically (A). The approximate boundaries for the three MHCK A domains are indicated: the α-helical coiled-coil domain (circle-filled bar), the central nonrepetitive domain (white bar), and the WD repeat domain (cross-hatched bar). The location of the GXGGXXG motif (residues 778–785) is indicated. Numbers at the ends of each construct denote the first and last residues of MHCK A present in the recombinant protein. The molecular masses shown are calculated based on the predicted amino acid sequence of the recombinant proteins. Autophosphorylation activity of all constructs purified from inclusion bodies was assessed using a filter renaturation assay described previously (5). Briefly, samples of each protein (2 μg) were subjected to SDS-PAGE and either stained with Coomassie Blue (B) or electrophoretically transferred to nitrocellulose, renatured, incubated with buffer containing [γ-32P]ATP, and subjected to autoradiography (C). Triplicate experiments were performed with each construct.
the NH2-terminal α-helical coiled-coil domain and the COOH-terminal WD repeat domain. Removal of 53 residues from the NH2 terminus of T-4 yielded a 35-kDa protein, T-5, with a reduced capacity to autophosphorylate (Fig. 1, B and C). The removal of 58 residues from the COOH terminus of T-4 yielded a 35-kDa protein, T-6, that displayed no ability to autophosphorylate (Fig. 1, B and C). The reduced autophosphorylation activity observed for T-5 and T-6 in the nitrocellulose renaturation assays could potentially result from the loss of intrinsic protein kinase activity, the elimination of key autophosphorylation sites, or an inability to renature on the nitrocellulose. Attempts were therefore made to isolate T-4, T-5, and T-6 in a soluble form so that their protein kinase activity could be analyzed in more detail. It was found that induction of expression in E. coli at 24 °C rather than 37 °C resulted in the recovery of T-4, T-5, and T-6 in the supernatant fraction of cell lysates, albeit with lower total yield. Chromatography on His-bind resin provided nearly homogenous purification (Fig. 2A).

When incubated with Mg2+ and [γ-32P]ATP, the soluble T-4 autophosphorylated to a significantly greater extent than soluble T-5, whereas no autophosphorylation of soluble T-6 was detected (Fig. 2B). The soluble form of the T-4 construct migrated with a higher apparent mass on SDS-PAGE than did T-4 isolated from inclusion bodies. This difference could be attributed to in vivo autophosphorylation of the soluble material in E. coli, because treatment of the soluble T-4 with CIAP shifted its mobility to the same position as the T-4 isolated from inclusion bodies. In contrast, soluble T-5 migrated with the same mobility on SDS-PAGE as T-5 from inclusion bodies and exhibited no mobility shift when treated with CIAP. Subsequent in vitro autophosphorylation of the CIAP-treated soluble T-4 protein resulted in an upwards mobility shift (data not shown) and the incorporation of 4 mol P/mole protein. This behavior is consistent with the shift in mobility displayed by MHCK A upon autophosphorylation (12). CIAP-treated T-5 protein incorporated only 1 mol P/mol protein when incubated with MgATP, confirming that several autophosphorylation sites are located between residues 499 and 551.

Native MHCK A and the recombinant soluble T-4 protein were assayed for their ability to phosphorylate wild type Dicyostelium myosin II and a mutant myosin II in which the major MHCK A phosphorylation sites in the tail (threonines 1823, 1833, and 2029) have been replaced with alanines (3X ALA myosin; Ref. 2). The 3X ALA myosin was phosphorylated to only about 10% of the level of wild type myosin by both T-4 and MHCK A (Fig. 2, C and D), indicating that T-4 retains the major determinants of substrate specificity.

A more detailed analysis of the kinase activity of T-4 and T-5 was performed using the synthetic peptide substrate MH-3, which is based on the sequence surrounding the Thr-2029 autophosphorylation site of intact MHCK A (12) and is based on the sequence surrounding the Thr-2029 MHCK A phosphorylation site in Dicyostelium myosin II. The autophosphorylated (CIAP-treated) forms of T-4 and T-5 phosphorylated MH-3 (Fig. 3, A and B, squares). Preincubation of the autophosphorylated T-4 and T-5 with MgATP to allow for autophosphorylation increased the activity of T-4 (Fig. 3, A, circles) but did not alter the activity of T-5 (Fig. 3B, circles). Although the activation by autophosphorylation observed with T-4 (∼3-fold) is small compared with that observed with intact MHCK A (∼50-fold), the results provide evidence that residues 499–551, which link the coiled-coil domain of MHCK A to the central domain, may function as an autoinhibitory sequence that can be regulated by autophosphorylation.

Further studies with the synthetic peptide MH-3 showed that the activity of T-4 was dependent on Mn2+ or Mg2+ and was not supported by Ca2+ (Fig. 3C). T-4 activity was strongly inhibited at higher ionic strengths, with 80% loss of activity at 100 mM KCl. T-4 displayed a KM for ATP of ∼50 μM and was able to utilize GTP as a substrate only poorly (rate less than 1% of that of ATP). T-4 and T-5 displayed KM values for MH-3 that were about 4–5-fold higher than native MHCK A and Vmax.

### Table I

| Enzyme | Substrate | K\_m (μM) | V\_max (μmol/mg/min) | K\_cat (s\(^{-1}\)) |
|--------|-----------|-----------|----------------------|---------------------|
| MHCK A | MH-3      | 100       | 2.2                  | 4.8                 |
| T-4    | MH-3      | 370       | 2.9                  | 2.0                 |
| T-4    | MBP       | 380       | 3.8                  | 2.7                 |
| T-5    | MH-3      | 550       | 3.3                  | 1.9                 |

Fig. 3. Characterization of the protein kinase activity of T-4, A, the relative activities of dephosphorylated and autophosphorylated T-4 was assayed using peptide MH-3. The rate of phosphate incorporation is greater with autophosphorylated T-4 (○) than with dephosphorylated T-4 (□). B, the relative activities of dephosphorylated and autophosphorylated T-5 assayed using MH-3. The rate of phosphate incorporation is similar with autophosphorylated T-5 (○) or with dephosphorylated T-5 (□). C, the activity of T-4 at varying concentrations of Mn2+ (□), Mg2+ (○), or Ca2+ (△).

Phosphorylation assays were performed with autophosphorylated enzymes as described under “Experimental Procedures” using a range of concentrations of the indicated protein or peptide substrates that spanned the K\_m value.
values that were about 2–4 fold lower (Table I). Histone III-S (a lysine-rich fraction) and histone VIII-S (arginine-rich fraction) were poor substrates for T-4, but myelin basic protein was as good a substrate as MH-3 (Table I). T-6 displayed no activity when assayed under various conditions with MH-3 or myelin basic protein, and we conclude that this construct is inactive.

It is noteworthy that the V_max of native MHCK A with the peptide substrate MH3 (2.2 μmol/mg-min) is roughly similar to those of members of the conventional family of protein kinases such as cAMP-dependent protein kinase, phosphorylase kinase, and chicken gizzard myosin light chain kinase, which range from 10 to 15 μmol/mg-min (12, 13). In contrast, most reported “unconventional” protein kinases either have not been characterized genetically or display extremely low activity. For example, a publication reporting that topoisomerase I has protein kinase activity indicated a specific activity of 3 nmol/mg-min (14), approximately 1000 fold less than the catalytic domain of the eEF-2 kinase. The published sequence of eEF-2 kinase (15), also known as calcium/calmodulin-dependent protein kinase III. The published sequence in GenBank, which was deposited by the C. elegans genome project (genomic, accession number U10414; cDNA, accession number D27775). The second is the recently published sequence of eEF-2 kinase (15), also known as calcium/calmodulin-dependent protein kinase III. The published sequence of the eEF-2 kinase is highly similar to the C. elegans open reading frame (ORF) throughout its entire length, suggesting that the C. elegans ORF may encode an eEF-2 kinase, but the sequence similarity of these proteins to MHCK A is interesting that MHCK A, eEF-2 kinase, and the C. elegans ORF all display a highly conserved GXXG motif and is representative of the conserved GXGXXG motif as typical of conventional protein kinases (15). However, the authors comment on the surprisingly low degree of conservation of this region of the eEF-2 kinase with other serine/threonine protein kinases. For example, the highly conserved catalytic site sequence DXXXXX (residues 166–171 of protein kinase A) is missing in the eEF-2 kinase. We suggest instead that the eEF-2 kinase shares the novel kinase catalytic domain found in MHCK A, so that in fact the eEF-2 kinase catalytic domain lies to the amino-terminal side of the GXGXXG motif and is represented by the region that shares 42% identity with the catalytic domain of MHCK A. Functional mapping of the eEF-2 catalytic domain will be needed to resolve this issue. It will also be important to identify the key residues involved in nucleotide binding and catalysis within the MHCK A catalytic domain. The results that show complete conservation between the Dictyostelium, C. elegans, and mammalian kinases (Fig. 4) are obvious candidates for further investigation.

The detailed mapping of the catalytic domain of MHCK A presented here, together with the demonstration of highly related primary sequences in both C. elegans and mammalian eEF-2 kinase, suggests the presence of a novel and widespread family of eukaryotic protein kinases that is unrelated to the previously characterized conventional protein kinase superfamily.

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