Cloning and Characterization of a Dictyostelium Myosin I Heavy Chain Kinase Activated by Cdc42 and Rac*

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The motile activities of the small, single-headed class I myosins (myosin I) from the lower eukaryotes Acanthamoeba and Dictyostelium are activated by phosphorylation of a single serine or threonine residue in the head domain of the heavy chain. Recently, we purified a myosin I heavy chain kinase (MIHCK) from Dictyostelium based on its ability to activate the Dictyostelium myosin ID isozyme (Lee, S.-F., and Côté, G. P. (1995) J. Biol. Chem. 270, 11776–11782). The complete sequence of the Dictyostelium MIHCK has now been determined, revealing a protein of 98 kDa that is composed of an amino-terminal domain rich in proline, glutamine, and serine, a putative Cdc42/Rac binding motif, and a carboxy-terminal kinase catalytic domain. MIHCK shares significant sequence identity with the Saccharomyces cerevisiae Ste20p kinase and the mammalian p21-activated kinase. Gel overlay assays and affinity chromatography experiments showed that MIHCK interacted with GTPγS (guanosine 5'-3-O-(thiotriphosphate))-labeled Cdc42 and Rac1 but not RhoA. In the presence of GTPγS, Rac1 MIHCK autophosphorylation increased from 1 to 9 mol of phosphate/mol, and the rate of Dictyostelium myosin ID phosphorylation was stimulated 10-fold. MIHCK may therefore provide a direct link between Cdc42/Rac signaling pathways and motile processes driven by myosin I molecules.

The class I myosins (myosin I) are small, single-headed, non-filament-forming myosins present in organisms ranging from protozoans such as Acanthamoeba and Dictyostelium to mammalian cells (1–3). Myosin I molecules are comprised of a 110–140-kDa heavy chain and one or more light chains, with the amino-terminal ~80 kDa of the heavy chain forming a motor domain, or head, that displays actin-activated MgATPase activity and drives actin-based motile processes. The carboxy-terminal portion of the myosin I heavy chain forms a globular tail domain that incorporates an acidic phospholipid-binding site and, in some isoforms, a nucleotide-insensitive actin filament-binding site, and a Src homology 3 (SH3) domain. Detailed biochemical studies on the Acanthamoeba myosin I isoforms have shown that phosphorylation of a single site in the head region of the heavy chain is required for these myosins to express maximal actin-activated MgATPase activity, to contract actin gels, and to move actin filaments (4–6). Evidence is available to indicate that at least two of the five Dictyostelium myosin I isoforms (myosin IB and ID) are regulated in an analogous manner (7–9).

Recently, we isolated a Dictyostelium MIHCK based on its ability to activate the Dictyostelium myosin ID isoform (9). We now report the complete cDNA sequence encoding the Dictyostelium MIHCK and show that it is a member of a protein kinase family that includes the Saccharomyces cerevisiae Ste20p and the mammalian p21-activated kinase (PAK). Members of the PAK kinase family bind to, and in some cases are activated by, Cdc42 and Rac (10–12), two members of the p21-Ras-related GTP-binding proteins (13). Evidence is presented to show that GTP-labeled Cdc42 and Rac bind to the Dictyostelium MIHCK and stimulate its activity.

EXPERIMENTAL PROCEDURES

Preparation of Proteins and Antibodies—Dictyostelium myosin ID and MIHCK were purified as described (8, 9). MIHCK was digested with trypsin and peptides were isolated and sequenced as described (8). Human Cdc42, Rac1, and RhoA were expressed as glutathione S-transferase (GST) fusion proteins in bacteria (kindly provided by Dr. Alan Hall, Medical Research Council Laboratory for Molecular Cell Biology, London) and purified over glutathione-Sepharose (Pharmacia Biotech Inc.) (14). Antibodies were raised in a New Zealand White rabbit using MIHCK excised from a Coomassie Blue-stained SDS gel and were affinity-purified using Affi-Gel 15 (Bio-Rad) to which MIHCK was coupled (8). Immunoblot analysis of proteins transferred to Immobilon-P (Millipore) was performed as described (8).

Isolation and Sequencing of cDNA Clones—DNA manipulations were done according to standard procedures (15). A Dictyostelium Agt11 library prepared from 4-h starved cells (Clonetech) was plated at a density of ~2.5 × 10⁶ plaques/150-mm LB agar plate, grown for 4 h at 37 °C, and induced for 6 h using nitrocellulose filters (Schleicher & Schuell) impregnated with 1 mM isopropyl-β-D-thiogalactoside (Promega). The filters were probed with the affinity-purified anti-MIHCK polyclonal antibodies and positive clones detected using a goat anti-rabbit secondary antibody conjugated to alkaline phosphatase and chromogenic substrates (Bio-Rad). Positive λ phage clones were purified over a second round of screening, isolated from plate lysates using the Wizard DNA kit (Promega), and subcloned into pGEM7Z. Plasmid templates prepared using Qiagen columns (Qiagen Inc.) were sequenced on both strands using Sequenase (version 2.0, U.S. Biochemical Corp.) and/or an Applied Biosystems model 373A automated sequencer (Core Facility for Protein and DNA Chemistry, Queen’s University, Kingston, Ontario, Canada). Two independent, overlapping clones were identified that contained sequences coding for MIHCK tryptic peptides. The Dictyostelium cDNA library was rescanned with these clones or with polymerase chain reaction-amplified DNA fragments prepared from the clones to obtain clones containing the entire MIHCK cDNA sequence (complete details of the cloning strategy are available upon request). Nucleotide sequences were analyzed and aligned to form the full-length DNA sequence using the DNAStar program. Data bank searches were performed using the BLAST algo-
P motifs are aminoacid sequence (tides from MIHCK yielded sequences that exactly matched the deduced codon 222 base pairs upstream. Microsequencing of three tryptic peptides from MIHCK were subjected to SDS-gel electrophoresis and visualized by staining with Coomassie Blue.

The nucleotide sequence of MIHCK has been deposited in the GenBank™ data base (accession number U67716).

Other proteins (2 μg/ml) were tested in kinase buffer (2 mM MgCl₂, 0.1 mM dithiothreitol, 0.25 mM ATP, 0.1 mg/ml bovine serum albumin, 20 mM Tes, pH 7.0) with or without 0.2 μg/ml GTP-S·Rac. [γ-32P]ATP (Du-Pont NEN) at a specific activity of 1000 Ci/mmol was included in assays where phosphate incorporation into MIHCK was directly measured, but was absent when MIHCK was used to phosphorylate myosin ID. Phosphorylation of myosin ID (90 μg/ml) was performed in kinase buffer containing 1000 Ci/mmol [γ-32P]ATP with 0.6 μg/ml MIHCK. Reactions were stopped at the indicated times by taking aliquots of 10–20 μl from the assays and immediately adding them to a one-fifth volume of boiling hot SDS sample buffer (9). Following SDS-gel electrophoresis the gel was stained with Coomassie Blue and dried for autoradiography. To quantify 32P incorporation the appropriate protein bands were excised from the gel and counted in a scintillation counter.

RESULTS AND DISCUSSION

The affinity-purified anti-Dicyostelium MIHCK polyclonal antibody was found to recognize a single major band of ~110 kDa when used to probe crude Dicyostelium cell extracts and to react strongly with purified Dicyostelium MIHCK (Fig. 1A). An initial screen of a Agt11 Dicyostelium cDNA expression library with the affinity-purified anti-MIHCK antibody yielded two overlapping cDNA clones, which were then used to reprobe the same cDNA library. Several additional overlapping cDNA clones were isolated that could be aligned to yield a single open reading frame of 2,685 nucleotides encoding a predicted polypeptide of 895 amino acids (Fig. 1B). The sequences of three peptides obtained from tryptic digests of MIHCK were all found within the deduced amino acid sequence (Fig. 1B). The predicted molecular mass of MIHCK (98 kDa) is in good agreement with the molecular mass of 110 kDa estimated from SDS gels (9) and is comparable with results obtained with Ste20p, a kinase related to MIHCK (see below). Ste20p has a molecular mass of 110 kDa on SDS gels and a predicted size of 102 kDa (17, 18).

The deduced amino acid sequence of the Dicyostelium MIHCK predicts a protein composed of ~400-residue amino-terminal domain in which ~50% of the residues are proline, serine, or asparagine; a putative binding motif for Cdc42 and Rac; a linker region rich in proline, glutamine, and asparagine; and a carboxyl-terminal protein kinase catalytic domain (Fig. 1B). Data base searches using the program BLASTP revealed no significant similarity between the amino-terminal domain of MIHCK and other proteins; however, the presence of multiple proline-rich sequences containing the motif PXSP (where X is any amino acid; double underlined in Fig. 1B) suggests that this domain may mediate interactions with SH3 domain-containing proteins (19). Data base searches with residues 393–455 of MIHCK identified several protein kinases, including the S. cerevisiae Ste20p (17) and Cla4p (20) protein kinases and the mammalian PAK kinase (12), with homology to this region asparaginerepeatandtwopotentialSH3domain-bindingsites

The MIHCK Cdc42/Rac binding motif is separated from the kinase catalytic domain by a linker region rich in proline, serine, or asparagine; a putative binding motif for Cdc42 and Rac; and a carboxyl-terminal protein kinase catalytic domain (Fig. 1B). Data base searches using the program BLASTP revealed no significant similarity between the amino-terminal domain of MIHCK and other proteins; however, the presence of multiple proline-rich sequences containing the motif PXSP (where X is any amino acid; double underlined in Fig. 1B) suggests that this domain may mediate interactions with SH3 domain-containing proteins (19). Data base searches with residues 393–455 of MIHCK identified several protein kinases, including the S. cerevisiae Ste20p (17) and Cla4p (20) protein kinases and the mammalian PAK kinase (12), with homology to this region (Fig. 2A). This region includes a 14–16-residue motif that has been defined as the minimal sequence required for the GTP-dependent binding of the Ras-related GTP-binding proteins Cdc42 and Rac (10). MIHCK retains only four of the eight conserved residues. The Cdc42/Rac-activated Myosin I Heavy Chain Kinase (MIHCK) catalytic domain shares the structure with the catalytic domain of the yeast protein kinase, Ste20p, and the mammalian PAK kinase (12), with homology to this region asparaginerepeatandtwopotentialSH3domain-bindingsites

Phosphorylation Assays—MIHCK was autophosphorylated at a concentration of 30 μg/ml by incubation in kinase buffer (2 mM MgCl₂, 1 mM dithiothreitol, 0.25 mM ATP, 0.1 μg/ml bovine serum albumin, 20 mM Tes, pH 7.0) with or without 0.2 μg/ml GTP-S·Rac. [γ-32P]ATP (Du-Pont NEN) at a specific activity of 1000 Ci/mmol was included in assays where phosphate incorporation into MIHCK was directly measured, but was absent when MIHCK was used to phosphorylate myosin ID. Phosphorylation of myosin ID (90 μg/ml) was performed in kinase buffer containing 1000 Ci/mmol [γ-32P]ATP with 0.6 μg/ml MIHCK. Reactions were stopped at the indicated times by taking aliquots of 10–20 μl from the assays and immediately adding them to a one-fifth volume of boiling hot SDS sample buffer (9). Following SDS-gel electrophoresis the gel was stained with Coomassie Blue and dried for autoradiography. To quantify 32P incorporation the appropriate protein bands were excised from the gel and counted in a scintillation counter.

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The MIHCK Cdc42/Rac binding motif is separated from the kinase domain by a linker region that contains a 16-residue asparagine repeat and two potential SH3 domain-binding sites (Fig. 1B). The MIHCK kinase catalytic domain shares the conserved features of primary structure typical of protein Ser/Thr kinases, including all residues that have been recognized as invariant or nearly invariant (21). A BLASTP search of the data base using the MIHCK catalytic domain as a query yielded the highest similarity scores with the kinase domains of Ste20p, Cla4p, PAK, and other members of this family (Fig. 2B). The catalytic domains of the yeast and human kinases are

**Fig. 1.** Characterization of antibodies used for cDNA library screening and the complete deduced amino acid sequence of Dicyostelium MIHCK. *A*, samples of a Dicyostelium cell lysate or purified MIHCK were subjected to SDS-gel electrophoresis and visualized by staining with Coomassie Blue (*CB* stain). Identical samples were transferred to Immobilon-P and probed with an affinity-purified anti-MIHCK antibody as described under “Experimental Procedures.” Molecular mass standards (kDa) are indicated. *B*, schematic representation and predicted amino acid sequence of the Dicyostelium MIHCK. The Initiator methionine was identified on the basis of an in-frame stop codon 222 base pairs upstream. Microsequencing of three tryptic peptides from MIHCK yielded sequences that exactly matched the deduced amino acid sequence (*bold italics*). PXSP motifs are *double underlined*. The nucleotide sequence of MIHCK has been deposited in the Gen-Bank™ data base (accession number U67716).
Identical residues are Cla4p, and rat brain PAK (12, 17, 20). The top line interacted with GTP 

Topline Sepharose bead co-precipitation assay. No significant co-precipitation with native, soluble MIHCK using a glutathione-S-labeled Cdc42 and Rac1, but not with RhoA (Fig. 3A). MIHCK autophosphorylated to 1 mol of phosphate/mol were incubated with glutathione-Sepharose beads containing GDP- or GTP-γ-S-Rac1 GST fusion protein. The glutathione eluates were subjected to SDS-gel electrophoresis and stained with Coomassie Blue to visualize bound MIHCK.

Binding of MIHCK to Cdc42 and Rac1. A, Purified MIHCK (CB stain) was transferred to Immobilon-P, overlaid with the indicated [35S]GTP-γ-S-labeled GST fusion protein, washed, and exposed to x-ray film as described under “Experimental Procedures.” B, MIHCK or P-MIHCK (MIHCK autophosphorylated to 1 mol of phosphate/mol) were incubated with glutathione-Sepharose beads containing GDP- or GTP-γ-S-Rac1 GST fusion protein. The glutathione eluates were subjected to SDS-gel electrophoresis and stained with Coomassie Blue to visualize bound MIHCK.

Fig. 2. Amino acid sequence comparison of MIHCK with related protein kinases. A, Alignment of the putative Cdc42/Rac binding domains, B, the kinase domains of MIHCK, S. cerevisiae Ste20p, and Cla4p, and rat brain PAK (12, 17, 20). The top line in A displays the core consensus amino acids conserved in the Cdc42/Rac binding motif (10). Identical residues are boxed. C, a dendogram showing the relationship between the protein sequences of the MIHCK, Ste20p, Cla4p, PAK, and human MST1 (24) kinase domains. The scale at the bottom indicates percent sequence divergence. Sequences were aligned using the Clustal V method (DNASTAR).

Fig. 3. Binding of MIHCK to Cdc42 and Rac1. A, Purified MIHCK (CB stain) was transferred to Immobilon-P, overlaid with the indicated [35S]GTP-γ-S-labeled GST fusion protein, washed, and exposed to x-ray film as described under “Experimental Procedures.” B, MIHCK or P-MIHCK (MIHCK autophosphorylated to 1 mol of phosphate/mol) were incubated with glutathione-Sepharose beads containing GDP- or GTP-γ-S-Rac1 GST fusion protein. The glutathione eluates were subjected to SDS-gel electrophoresis and stained with Coomassie Blue to visualize bound MIHCK.

The ability of Dictyostelium MIHCK to interact directly with the Rho group of GTP-binding proteins was tested using an overlay assay. Human Cdc42, Rac1, and RhoA, expressed as GST fusion proteins, were labeled with the nonhydrolyzable GTP analog [35S]GTP-γ-S and overlaid onto filters containing immobilized MIHCK. Autoradiograms indicated that MIHCK interacted with GTP-γ-S-labeled Cdc42 and Rac1, but not with RhoA (Fig. 3A). Rac1, which consistently exhibited the highest levels of binding to immobilized MIHCK, was examined for its interaction with native, soluble MIHCK using a glutathione-Sepharose bead co-precipitation assay. No significant co-pre-

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to regions of the actin-rich cortex undergoing active movement, such as membrane ruffles, leading lamella, phagocytic cups, and neuronal growth cones (34–37), and have been implicated, largely through the analysis of knockout mutations in lower eukaryotes, in cellular processes such as endocytosis, phagocytosis, pinocytosis, secretion, pseudopod extension, and polarized cell growth (38–42). Such studies suggest that the myosin I molecules play a central role in actin-dependent membrane-based motile processes and thus represent attractive candidates to mediate some of the effects of Cdc42 and Rac.

The substrate specificity and regulatory properties of the Dictyostelium MIHCK are remarkably similar to those of the 97-kDa Acanthamoeba MIHCK (3). Both MIHCKs can activate Dictyostelium myosin I isozymes and require autophosphorylation for activity (7, 9, 43). In both cases autophosphorylation is catalyzed by a glutamate or aspartate residue, which, it has been argued, may relieve the requirement for phosphorylation (47). MIHCK reveals that, like Dictyostelium, it contains a catalytic domain related to Ste20p and PAK (50). This raises the question as to whether Ste20p and PAK, which share considerable sequence identity to the Dictyostelium MIHCK, both in the kinase and Cdc42/Rac binding domain, may function as myosin I-activating kinases. At present there is little direct evidence to show that myosin I molecules in organisms other than Acanthamoeba and Dictyostelium require phosphorylation of the heavy chain for activity, and indeed, sequence alignments indicate that the serine/threonine residue mapped as the site of heavy chain phosphorylation in the head domain of the Acanthamoeba myosin I isozymes (46) is replaced in the large majority of myosins, including all the known mammalian myosins I, by a glutamate or aspartate residue, which, it has been argued, may relieve the requirement for phosphorylation (47).

Myosins that retain the regulatory serine/threonine residue in the head domain and so are the most likely to require heavy chain phosphorylation for enzymatic and mechanoochemical activity include the S. cerevisiae and Aspergillus class I myosins and the Drosophila and mammalian class VI myosins (47). Interestingly, PAK and the Acanthamoeba MIHCK can activate smooth muscle myosin II by phosphorylating a site on the light chain, raising the intriguing possibility that members of this kinase family may coordinate the regulation of more than one class of myosins (48, 49).

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FIG. 4. Effect of GTPγS-Rac1 on MIHCK autophosphorylation and activity. A–C, MIHCK was autophosphorylated with (+) or without (−) GTPγS-Rac1 in the presence of γ-32P|ATP as described under “Experimental Procedures.” Aliquots were taken at the times indicated and subjected to SDS-gel electrophoresis. A shows the Coomassie Blue-stained gel, B the corresponding autoradiogram, C the amount of 32P incorporated into the MIHCK band determined by excising the MIHCK band from the SDS gel and counting it in a scintillation counter. D. Dictyostelium myosin I ID was phosphorylated with MIHCK autophosphorylated in the presence (+) or absence (−) of GTPγS-Rac1. Samples were taken at the times indicated, subjected to SDS-gel electrophoresis and autoradiography.
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