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Membrane-bound *Dictyostelium* myosin heavy chain kinase: A developmentally regulated substrate-specific member of the protein kinase C family

SHOSHANA RAVID* AND JAMES A. SPUDICH

Departments of Cell Biology and Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305

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ABSTRACT **A cDNA clone corresponding to the *Dictyostelium* myosin heavy chain kinase (MHCK) gene was isolated using antibodies specific to the purified enzyme. Sequence analysis of the cDNA revealed that the *Dictyostelium* MHCK possesses all of the domains characteristic of members of the protein kinase C family. The amino-terminal region of the MHCK contains the cysteine-rich motif with an internal duplication that is present in all known protein kinase C species. This domain precedes sequences that are highly homologous to protein kinase catalytic domains. The carboxyl-terminal region contains a cluster of 23 serine and threonine residues that may represent the autophosphorylation domain of the *Dictyostelium* MHCK. These results, along with previous studies that indicate that this enzyme has very restrictive substrate specificity, incorporates approximately 20 mol of phosphate per mol of kinase through an autophosphorylation reaction, and is expressed only during development, suggest that the *Dictyostelium* MHCK is a distinct member of the protein kinase C family and imply that this kinase family, which may include members with very specific cellular functions, may be even more heterogeneous than previously thought.**

The protein kinase C (PKC) family plays a crucial role in the signal transduction system in eukaryotic cells. When a ligand binds to certain receptors on the cell surface, phosphatidylinositol 4,5-bisphosphate is hydrolyzed to diacylglycerol and inositol 1,4,5-trisphosphate (1), which are thought to act as second messengers. The primary effect of diacylglycerol is to activate PKC, which in turn phosphorylates a range of cellular proteins (2, 3). Inositol trisphosphate functions to mobilize Ca^{2+} from intracellular stores (4). PKC appears to be the receptor protein for tumor-promoting phorbol ester. The evidence available to date strongly suggests that some, if not all, of the pleiotropic actions of tumor promoters are mediated through this protein kinase family (5). Furthermore, PKC shows a broad substrate specificity when tested *in vitro*, suggesting that PKC possesses multifunctional catalytic activity (2, 3, 5). Although significant progress has been made toward understanding PKC activation, the *in vivo* substrates and the steps between the activation and subsequent cellular events remain obscure.

When cells of *Dictyostelium* are starved, they acquire the ability to bind cAMP to specific cell surface receptors and to respond to this signal by chemotaxis. Binding of cAMP induces formation of inositol trisphosphate and diacylglycerol (6). Reorganization of myosin is involved in the process of chemotaxis. Yumura and Fukui (7) have shown that myosin in *Dictyostelium* exists as thick filaments that translocate to the cortex in response to cAMP. This translocation is correlated with the *in vivo* increases in phosphorylation on both the heavy chain and the 18-kDa light chain of myosin (8, 9). We have purified a membrane-associated myosin heavy chain kinase (MHCK) that is implicated in the increase in myosin heavy chain phosphorylation during chemotaxis (10). This kinase phosphorylates the *Dictyostelium* myosin heavy chain specifically, which results in inhibition of myosin thick filament formation (10, 11). These studies suggest a regulatory role for the MHCK in the assembly process of myosin. Here we show that the *Dictyostelium* MHCK is a member of the PKC family and may provide a link between the extracellular chemotactic signal and subsequent intracellular events.†

MATERIALS AND METHODS

Growth and development of *Dictyostelium* AX3 was as described (8). Preparation of the *Dictyostelium* cells for immunoblot analysis was as described by De Lozanne and Spudich (12).

Production of Anti-*Dictyostelium* MHCK Antibody. Polyclonal antibodies against the *Dictyostelium* MHCK were prepared by BabCo Immunological (Berkeley, CA). A rabbit that had been prescreened to have no major reactions to *Dictyostelium* proteins was injected with MHCK purified as described (10). Affinity-purified antibodies were isolated as described (13). Immunoblot analysis was performed according to the method of Towbin *et al.* (14), using the affinity-purified MHCK antibodies diluted 1:1000 as the primary antibody and affinity-purified goat anti-rabbit IgG horseradish peroxidase conjugate (diluted 1:2000) as the secondary antibody (Bio-Rad). Antibody conjugates were visualized with 4-chloro-1-naphthol following the manufacturer's protocol (Bio-Rad).

Cloning and Sequence Analysis. A 2-h-developed *Dictyostelium* cDNA library in λZAP (generously provided by James Cardelli, University of Louisiana State University, Medical Center) was screened with the affinity-purified MHCK polyclonal antibodies. Nested deletions were constructed using the Erase-a-Base system following the manufacture's protocol (Promega). Sequencing was performed using the Sequenase system (United States Biochemical). Sequence analyses were done using the GCG sequence analysis software package (15).

Southern Blot Analysis. Genomic *Dictyostelium* DNA was prepared as described (16). The DNA was digested with restriction enzymes, fractionated on a 0.6% agarose gel, transferred to nitrocellulose, and probed with a fragment of the MHCK cDNA clone labeled with [α-^{32}P]dATP using a random-primed DNA labeling kit (Boehringer Mannheim).

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Abbreviations: MHCK, myosin heavy chain kinase; PKC, protein kinase C.

*Present address: Department of Membrane Research and Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M93393).

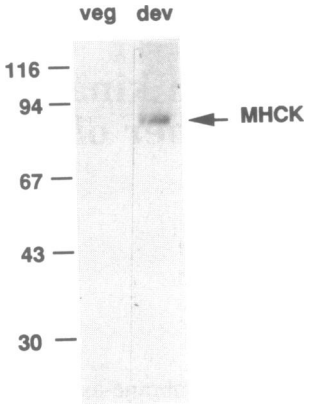

FIG. 1. Immunoblot analysis of vegetative (lane veg) and 4-h-developed (lane dev) *Dictyostelium* cells. Lysates of *Dictyostelium* cells were prepared, subjected to SDS/PAGE on 10% gels, and blotted to nitrocellulose. The immunoblots were stained with the affinity-purified anti-MHCK antibodies. Molecular mass marker standards are β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

The blots were washed at 68°C in 2× standard saline citrate/0.5% SDS.

RESULTS

We raised polyclonal antibodies against the MHCK purified from the membrane fraction of developed *Dictyostelium* cells. These antibodies inhibit MHCK activity (data not shown). In immunoblot analyses of developed *Dictyostelium* cells, the antibodies cross react strongly with a single band corresponding to a molecular mass of 84 kDa (Fig. 1), which is the molecular mass of the pure MHCK estimated by SDS/PAGE (10). In immunoblot analyses of vegetative *Dictyostelium* cells, the MHCK antibodies did not show any reaction (Fig. 1), indicating that the MHCK is expressed only during development. We used these antibodies to screen a *Dictyostelium* cDNA expression library and isolated a 2.5-kilobase (kb) clone that lacked the 5′ end. We therefore used a 250-base-pair fragment from the 5′ end of this clone to isolate a full-length clone from the cDNA library. A 2.6-kb clone (MHCK) was isolated and sequenced completely in both orientations (Fig. 2). The MHCK has a single open reading frame encoding a sequence of 783 amino acids with a predicted molecular weight of 84,564 (Fig. 2). This predicted molecular weight is consistent with that estimated for the purified MHCK (10). The open reading frame starts with a potential initiation codon of ATG. This codon is flanked by sequences that fulfill Kozak's criteria for initiation (17). A stop codon of TAA is found at nucleotides 2369–2371 in-frame downstream from the initiation codon.

The MHCK sequence was compared with sequences in the National Biochemical Research Foundation data bases (December 1991). The proteins most closely related to the predicted *Dictyostelium* protein are all members of the PKC family. These PKC members all consist of a single polypeptide with four conserved (C_1–C_4) and five variable regions (18). The carboxyl-terminal half, containing two conserved regions C_3 and C_4, appears to be the protein kinase domain. The C_3 region possesses the ATP-binding sequence that is found in all protein kinases (19). The amino-terminal half, having conserved regions C_1 and C_2, resembles closely the regulatory domain of the mammalian PKCs that interacts with calcium, phospholipid, and diacylglycerol. The C_1 re-

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- 92                                                GAAGCTGGTATCGCCAAAAACGGTCAAACTCGTGAACACGCTCTCCTTGCTTACACTTTAGGTGTAAACAAATGATCGTTGCTATCAACAAG
   1  ATGGATCGAAAAAATCAAGCACTAACTCAGAAAGACCGTTACGACGTAAAATTCCCTAAAATTTCATACAACATCAAAATTCGAATTACAAAATTTTGTAATTATTGTAGGGAGACAACA
      M  D  R  K  N  Q  A  L  T  Q  K  D  R  Y  D  V  K  F  P  K  I  S  Y  N  I  K  I  R  I  T  K  F  C  N  Y  C  R  E  T  T    40
 121  TTTACAATCTCTGGTGAACCAGTGATGTGTAGTGAATGTAGATATATTGCACATGGACATTGTCAAACAAAGGTACCATTAAATTGTATTATACCCTATTCATCACCAATCAATACGATC
      F  T  I  S  G  E  P  V  M  C  S  E  C  R  Y  I  A  H  G  H  C  Q  T  K  V  P  L  N  C  I  I  P  Y  S  S  P  I  N  T  I    80
 241  AATGATGGTGGTAATGTTAGACATCATTGGGTTGAAGGTAATTTAAAAAAAGAGTAAAAAGTGTATACATTGTATGGAACCATGTGAAAAATCATTCTCTTTAGCTCATTATAAATGTTTA
      N  D  G  G  N  V  R  H  H  W  V  E  G  N  L  K  K  S  K  K  C  I  H  C  M  E  P  C  E  K  S  F  S  L  A  H  Y  K  C  L   120
 361  TGGTGTCATAAATATTTACATAGTTCATGTTTTGATAAACATAATCCAATTTGTGATTTTGGTTCATTATCTGATATGATTTTACCCACCTTCTTCAATAGATTATTATCAACAACAGCA
      W  C  H  K  Y  L  H  S  S  C  F  D  K  H  N  P  I  C  D  F  G  S  L  S  D  M  I  L  P  T  F  F  N  R  L  L  S  T  T  A   160
 481  ACTACTACAAATAAAATTGAAAAGTTAATAATTGAAGAACAACAACCACAATTATCATCATCACCATCTTCACCAAGATTAAATATTAATAATAATAATAATAATAATGAAATTATTGAA
      T  T  T  N  K  I  E  K  L  I  I  E  E  Q  Q  P  Q  L  S  S  S  P  S  S  P  R  L  N  I  N  N  N  N  N  N  E  I  I  E   200
 601  TGGGAATTAATTGAAAATTCAAATATGCCAGAAAAAGTTTTATTTGTATTTGTAAATTCAAAGAGTGGTGGTCAATTTGGTAGTACATTAATTAGAAAGTTATCATCATTATTGAATCCT
      W  E  L  I  E  N  S  N  M  P  E  K  V  L  F  V  F  V  N  S  K  S  G  G  Q  F  G  S  T  L  I  R  K  L  S  S  L  L  N  P   240
 721  TTACAAATTATAGATTTAATTAAATGTGGTGGACCAGATTCAACCTTACAAATGATTAATAGATATTTAGCAAAACATCCAGAACAAACCAATAGATTTAGAATTTTAGTTTGTGGTGGT
      L  Q  I  I  D  L  I  K  C  G  G  P  D  S  T  L  Q  M  I  N  R  Y  L  A  K  H  P  E  Q  T  N  R  F  R  I  L  V  C  G  G   280
 841  GATGGTAGCAGGTTGGTTGGTTATTTAAACAAAATGACAAAACATTTGGTTCCATCAACTATACCCATTGGTATTATACCATTGGTACTGGTAATGATTTGGCACGTTCGTTTAGGTTGG
      D  G  S  R  L  V  G  Y  L  N  K  M  T  K  H  L  V  P  S  T  I  P  I  G  I  I  P  L  V  L  V  M  I  W  H  V  R  L  G  W   320
 961  GGGATTGGATATGATGGTGAAAAGCTAATTGAAATCTTAAAGAGTATAAATGAAGCTAAAACAATTCAAATGGATACTTGGAGTATTGAAATGTGGGAGGATCATAAACCCGAACTAGCA
      G  I  G  Y  D  G  E  K  L  I  E  I  L  K  S  I  N  E  A  K  T  I  Q  M  D  T  W  S  I  E  M  W  E  D  H  K  P  E  L  A   360
1081  TCAATGATCTCGATAAATTTGGATAACATAACCGATCTAATCATCAATGATACTATTACATTGGTTGATGCAATGTGCATTGGTTTCATTAGCAAGGAAGCAAATCCTCAACTTTCACA
      S  M  I  S  I  N  L  D  N  I  T  D  L  I  I  N  D  T  I  T  L  V  D  A  M  C  I  G  F  I  S  K  E  A  N  P  Q  L  F  T   400
1201  GGTCGTACAGTTAATAAACTTTGGTATACAAAGATTGGTCTTGAGGAATTTGTTACAAAGAATTTCGTTAGTCTAGCTAGAATCGTTAAAATTAATGTGGGAACTCGGGAAATTAGAGTC
      G  R  T  V  N  K  L  W  Y  T  K  I  G  L  E  E  F  V  T  K  N  F  V  S  L  A  R  I  V  K  I  N  V  G  T  R  E  I  R  V   440
1321  GATATGATCCATGAGGGTATTATCATCCTCAATTTAGGTAGTTATCGTGGTGGTGTGGGACTTATGGGGTGCCAAATAGGAAAAGGAAGTTTTGGGAAAGTTCATAGTGATGGCACTGGT
      D  M  I  H  E  G  I  I  I  L  N  L  G  S  Y  R  G  G  V  G  L  M  G  C  Q  I  G  K  G  S  F  G  K  V  H  S  D  G  T  G   480
1441  AATCAGTTTATCGATGATCAAACTAAAGAGATTGTTGGTGTCACTGTTTTACCCCATTTAGATGATGTTTTATGTTCAATTGTTCTCCAATTAAAATGGGTCAAGCTGATGAAATTCGTA
      N  Q  F  I  D  D  Q  T  K  E  I  V  G  V  T  V  L  P  H  L  D  D  V  L  C  S  I  V  L  Q  L  K  W  V  K  L  M  K  F  V   520
1561  TTCAAGTATCAATGCCTTCAATCATTTTTAAAGGATAAAAGGTTATATGAAATTGAAACTGCTTTTCAAGTCGATGGTGATCTCGAACCAATTGAAGTTCAAATTGGACTTTTTAAAATAT
      F  K  Y  Q  C  L  Q  S  F  L  K  D  K  R  L  Y  E  I  E  T  A  F  Q  V  D  G  D  L  E  P  I  E  V  Q  I  G  L  F  K  Y   560
1681  CCTTCTTTAAAAAGTTTTCGATTAACTTTAGAAATTATCTTATTTTTCCTGATTTTTGTTAAGATTACCTATAGACCTCTAAACCTTTACAACCCTACTCTATTTGGCTCTAGCATCTCA
      P  S  L  K  S  F  R  L  T  L  E  I  I  L  F  F  L  I  F  V  K  I  T  Y  R  P  L  N  L  Y  N  P  T  L  F  G  S  S  I  S   600
1801  GTAGCTTTACTTATTGCAGATTTCGGTACACCAACCTACGTTACCACGAATCCCAAAGTAAATTGCTTCCTTACGTTTAGAGTTAGAAAAGTGTCCAGCATGTCAATTATTTTGAACCAT
      V  A  L  L  I  A  D  F  G  T  P  T  Y  V  T  T  N  P  K  V  N  C  F  L  T  F  R  V  R  K  V  S  S  M  S  I  I  L  N  H   640
1921  ATGTCTAACCAGACTCCTAACATGTTCTTAAGAAGGGTATTAAAGTATTTTGTAGTTTTATTTAATGGTTTAAAAGATTTAATAACACTCTTCTGTTGTAAAATGTTTGAGACCTCTTTG
      M  S  N  Q  T  P  N  M  F  L  R  R  V  L  K  Y  F  V  V  L  F  N  G  L  K  D  L  I  T  L  F  C  C  K  M  F  E  T  S  L   680
2041  GTCACTTACACATCACTTACATCTATAAATAGCTGTACCCGCAACATGTTCCATGTATTACAAACATGGAAAGTATGGTAGTATGCCTAGTACTACACATACATCTGAGTACCACTACCTA
      V  T  Y  T  S  L  T  S  I  I  A  V  P  A  T  C  S  M  Y  Y  K  H  G  K  Y  G  S  M  P  S  T  T  H  T  S  E  Y  H  Y  L   720
2161  GATGAAATCATGATTCTGATTATCAACTTTCAATATCTCAATCACCAAATCTTTATTCTCCACCTTCTTTTAAAATCCACCACCAAATTTAAATCAATATCAACAACCACATCAACCACC
      D  E  I  M  I  L  I  I  N  F  Q  Y  L  N  H  Q  I  F  I  L  H  L  L  L  K  S  T  T  K  F  K  S  I  S  T  T  T  S  T  T   760
2281  TCAATCACAACCTCAACCTCAACCTCAACCTCAACAACTCACTTCAATCACCCCAATCACCAGAACCGATAAATAATAATACAATAATAATAATAATAATAATAACAATAATAATAATAA
      S  I  T  T  S  T  S  T  S  T  S  T  T  H  F  N  H  P  N  H  Q  N  R  *                                                783
2401  TAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATAATTTCAAA
2521  TGATCTCACGAATAATAGTGAAGTTAATCCTGAGAAAA
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FIG. 2. Nucleotide sequence (numbered on the left) and predicted amino acid sequence (numbered on the right) of the MHCK. The predicted amino acid sequence starts with the first methionine codon in the open reading frame.

gion contains the putative pseudosubstrate domain and a tandem repeat of cysteine-rich sequence (18). Alignment of the MHCK sequence with bovine PKCγ (18), rat PKCβ (20), rabbit PKCδ (21), and rat PKCα (22) sequences reveals an overall homology in two domains (Fig. 3). The catalytic domain of the *Dictyostelium* MHCK shares 37% amino acid identity with its mammalian PKC counterparts (Fig. 3*B*) and includes hallmark sequences found only in the PKC family of protein kinases (19). The degree of overall similarity is increased substantially (67%) if conservative amino acid substitutions are considered. Although the regulatory domain is less conserved than the kinase catalytic domain, it contains the putative pseudosubstrate site and the two cysteine-rich sequence motifs that are characteristic of the PKC family (Fig. 3*A*). A modest degree of sequence similarity (10%; not shown in Fig. 3) also exists in the region that corresponds to the putative Ca^{2+} binding domain conserved among PKC subspecies (5).

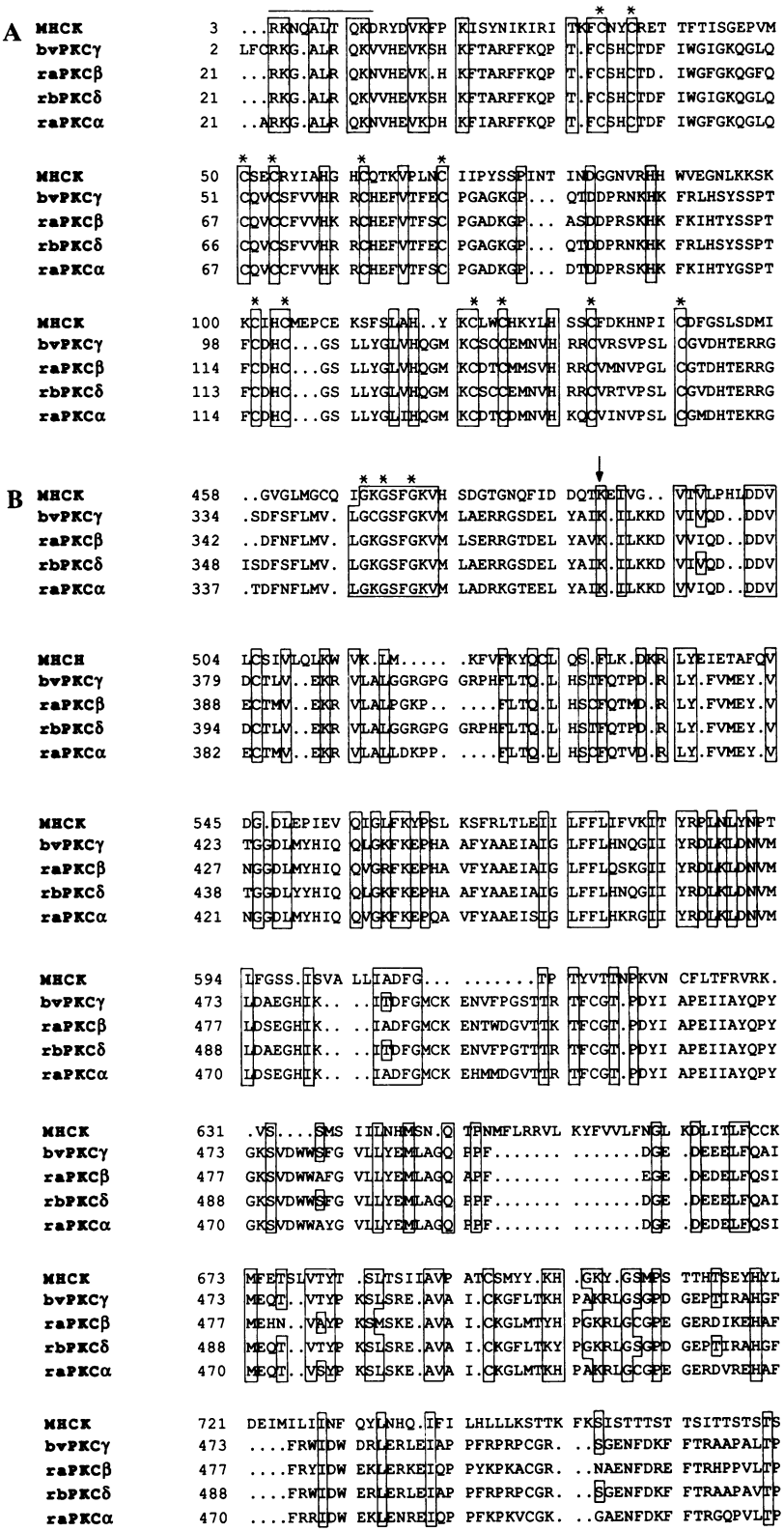

FIG. 3. Alignment of the predicted *Dictyostelium* MHCK amino acids with known mammalian isoenzymes of PKC; identical residues between *Dictyostelium* MHCK and mammalian PKC isoenzymes are boxed; dots indicate the gaps in alignment. (*A*) The region defined by Coussens *et al.* (18) as C_1 includes the putative pseudosubstrate site (overline) and the two intramolecular repeats rich in cysteine residues (asterisks). The spacings of the cysteine residues in the *Dictyostelium* MHCK are strictly conserved in the two repeats and are identical with those for all mammalian PKCs. (*B*) The catalytic domain. Asterisks indicate absolutely conserved residues within the ATP binding region and the arrow indicates the invariant lysine. Sequences are from the following sources: bvPKCγ (bovine) from Coussens *et al.* (18), raPKCβ (rat) from Knopf *et al.* (20), rbPKCδ (rabbit) from Ohno *et al.* (21), and raPKCα (rat) from Ono *et al.* (22).

To estimate the number of homologous MHCK genes within the *Dictyostelium* genome, we probed Southern blots of *Dictyostelium* genomic DNA with a portion of the MHCK under conditions of moderate stringency (Fig. 4). The probe used was a 0.9-kb cDNA fragment (Fig. 4*B*) that contains the coding sequence for 60% of the C_1 domain and the entire C_2 domain. A relatively simple pattern of hybridizing fragments was obtained when genomic DNA was digested with various enzymes (Fig. 4*A*). This pattern is consistent with a single genomic locus whose restriction enzyme map is shown in Fig. 4*B*. It is possible that *Dictyostelium* contains more than one MHCK and/or PKC species and that under lower stringency conditions other genes will hybridize with the MHCK probe.

DISCUSSION

The high homology between the *Dictyostelium* MHCK and mammalian PKC, particularly at the regions that are unique to the PKC family, indicates that the MHCK is a member of the PKC family. This conclusion is supported by biochemical properties of the *Dictyostelium* MHCK (10). MHCK is a membrane-associated protein like all known PKCs (2, 3). It has an apparent molecular mass (84 kDa; ref. 10) similar to the average molecular mass of PKCs (77 kDa; ref. 23), and like all PKCs it undergoes intramolecular autophosphorylation (10). PKCs phosphorylate serine and threonine residues but not tyrosine (24, 25), and MHCK phosphorylates only threonine residues on the myosin heavy chain (10). Biochemical evidence from other mammalian systems also suggests that those PKCs phosphorylate myosin heavy chains. Myosin heavy chain from several nonmuscle cell sources have been shown to undergo phosphorylation *in vitro* and/or *in vivo* (26–31).

Although the MHCK sequence contains the domains that are presumably involved in phospholipid, diacylglycerol, and Ca^{2+} binding, the purified MHCK unlike most mammalian PKCs is active without the addition of the above substances (10). It is possible that the purified MHCK lost its regulation during the purification and that *in vivo* this enzyme is regulated by the above substances. Several mammalian PKCs do not demonstrate Ca^{2+}, phospholipid, and diacylglycerol regulation after purification from cell extracts. It has been assumed that regulation was lost during purification. For example, PKC from human platelets is not sensitive to Ca^{2+} (32), and in other cases, neither Ca^{2+} nor phospholipid is needed for the enzymatic activity of PKC (33, 34). It is also possible that the mechanism of MHCK regulation is distinct. The cysteine-rich domain might be required only for binding of the MHCK to the membrane and not for diacylglycerol binding.

Each mole of *Dictyostelium* MHCK incorporates about 20 mol of phosphate by autophosphorylation (10). A cluster of 23 serine and threonine residues is located at the carboxyl-terminal end of the MHCK sequence (Fig. 2) that could represent the autophosphorylation domain. The *Dictyostelium* MHCK autophosphorylation may be a form of regulation. Indeed, autophosphorylation in a mammalian PKC has been shown to increase its rate of histone phosphorylation (35). The mechanism by which the autophosphorylation regulates PKC is unknown.

The PKC family plays a crucial role in signal transduction for the activation of multiple cellular functions by the phosphorylation of multiple substrates (2, 3, 5). This family contains several discrete species, yet they all possess a primary structure containing conserved structural motifs with a high degree of sequence homology. Our results indicate that the *Dictyostelium* MHCK contains the structural motif of PKC but is distinct from all known PKCs. Whereas PKCs are thought to be involved in multiple cellular functions and have multiple substrates (2, 3), the MHCK is known to phosphorylate only myosin heavy chain and thus may affect only myosin-related functions (10).

Although significant progress has been made toward elucidation of the pathways leading to PKC activation, the steps between this activation and subsequent cellular events remain uncharacterized. The finding that an enzyme that plays a regulatory role in the control mechanism of myosin assembly is a component of the signal transduction system provides a link between an external stimulus and directional movement by the signal transduction system. To study the role of PKC in signal transduction, it is useful to utilize systems such as *Dictyostelium* that are amenable to genetic analysis. The

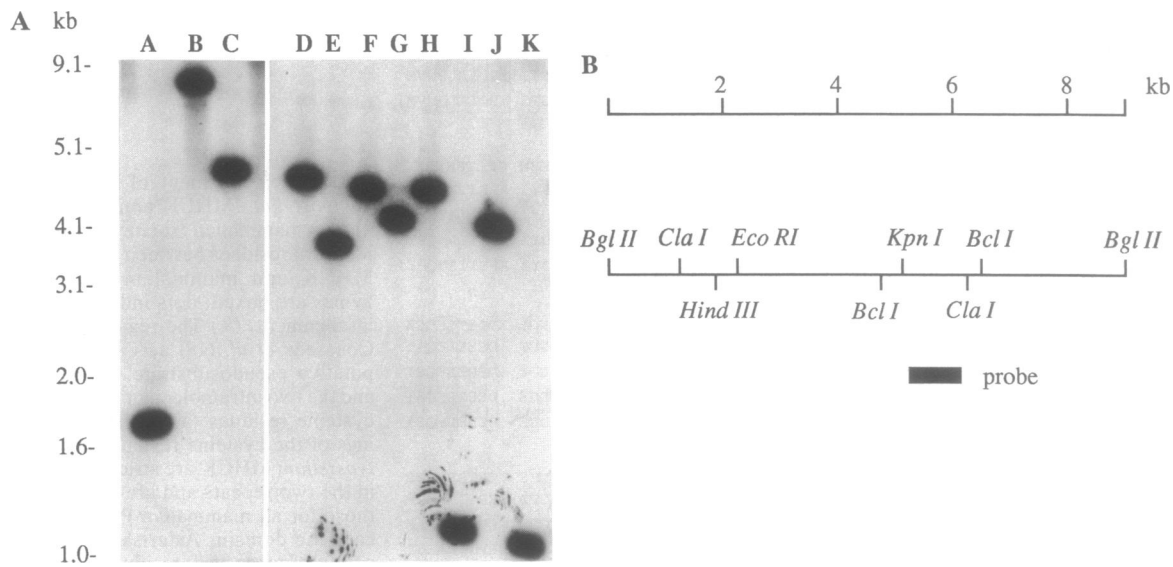

FIG. 4. Southern blot analysis of genomic *Dictyostelium* MHCK. (*A*) *Dictyostelium* genomic DNA was digested with restriction enzymes, fractionated on 0.6% agarose gel, transferred to nitrocellulose, and hybridized with the probe indicated in *B*. Lanes: A, *Bcl* I digest; B, *Bgl* II digest; C, *Cla* I digest; D, *Cla* I/*Bam*HI digest; E, *Cla* I/*Eco*RI digest; F, *Cla* I/*Eco*RV digest; G, *Cla* I/*Hin*dIII digest; H, *Cla* I/*Pst* I digest; I, *Kpn* I/*Bcl* I digest; J, *Kpn* I/*Bgl* II digest; K, *Kpn* I/*Cla* I digest. (*B*) Restriction enzyme map of the genomic locus (as deduced from the Southern blot patterns) and the relative position of the probe used in *A*. Molecular mass standards, which were electrophoresed in both the first and the last gel lanes (not shown), confirmed that the upper band in lanes C, D, F, and H is 5.0 kb in each case.

presence of only one gene of MHCK and the ability to target genes in *Dictyostelium* should enable the generation of mutants lacking this unique PKC. These studies should give a better understanding of the role of PKC and MHCK *in vivo*.

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