Specific Phosphorylation of Threonine by theDictyostelium Myosin II Heavy Chain Kinase Family*

By: Xia Luo, Scott W. Crawley, Paul A. Steimle, Tom T. Egelhoff, and Graham P. Côté

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Abstract:

Dictyostelium myosin II heavy chain kinase A (MHCK A), MHCK B, and MHCK C contain a novel type of protein kinase catalytic domain that displays no sequence identity to the catalytic domain present in conventional serine, threonine, and/or tyrosine protein kinases. Several proteins, including myelin basic protein, myosin regulatory light chain, caldesmon, and casein were phosphorylated by the bacterially expressed MHCK A, MHCK B, and MHCK C catalytic domains. Phosphoamino acid analyses of the proteins showed that 91 to 99% of the phosphate was incorporated into threonine with the remainder into serine. Acceptor amino acid specificity was further examined using a synthetic peptide library (MA*XXXX(*S/T)*XXXX*AKKK; where *X* is any amino acid except cysteine, tryptophan, serine, and threonine and position 7 contains serine and threonine in a 1.7:1 ratio). Phosphorylation of the peptide library with the three MHCK catalytic domains resulted in 97 to 99% of the phosphate being incorporated into threonine, while phosphorylation with a conventional serine/threonine protein kinase, the p21-activated kinase, resulted in 80% of the phosphate being incorporated into serine. The acceptor amino acid specificity of MHCK A was tested directly by substituting serine for threonine in a synthetic peptide and a glutathione*S*-transferase fusion peptide substrate. The serine-containing substrates were phosphorylated at a 25-fold lower rate than the threonine-containing substrates. The results indicate that the MHCKs are specific for the phosphorylation of threonine.

Article:

Dictyostelium discoideum myosin II heavy chain kinase A (MHCK A)[1] phosphorylates threonine residues at positions 1823, 1833, and 2029 in the α-helical coiled-coil tail of myosin II (1, 2). Phosphorylation of the threonine residues drives myosin II from a filamentous to a monomeric state and plays a central role in inhibiting the cellular activity of myosin II (3-6). Sequence analysis of the 130-kDa MHCK A shows that it is composed of an ~50-kDa N-terminal α-helical coiled-coil domain, a 35-kDa central domain, and a C-terminal WD-repeat domain (7). Native MHCK A is a multimer with an apparent molecular mass in excess of 1000 kDa by gel filtration analysis and, when visualized by electron microscopy, appears as a highly asymmetric molecule with several globular domains clustered at one end of a 50-nm long rod (8).

Truncation analysis of MHCK A shows that the central 35-kDa domain, in the absence of both the coiled-coil domain and WD-repeat domain, is a highly active protein kinase, with the ability to catalyze phosphoryl transfer from ATP to both peptide and protein substrates (9). Of considerable interest is the finding that the MHCK A catalytic domain shares no significant sequence similarity to the conserved catalytic domain that distinguishes the extremely large family of "conventional" serine, threonine, and/or tyrosine protein kinases or to the catalytic domain present in the prokaryotic and eukaryotic histidine kinases (9-11).

A domain homologous to the MHCK A catalytic domain has now been recognized in several additional proteins, including two*Dictyostelium* proteins: MHCK B (12) and MHCK C, the product of the *mhkC* gene

(GenBank™ accession number 3420749). MHCK B and MHCK C are structurally similar to MHCK A, consisting of a catalytic domain followed by a WD-repeat domain. However, neither MHCK B nor MHCK C has the extended N-terminal coiled-coil domain found in MHCK A. *In vitro* studies show that bacterially expressed MHCK B is an active protein kinase and that it phosphorylates the same sites in the *Dictyostelium* myosin II tail as MHCK A (12). Although the *in vivo* role of MHCK B remains to be determined, these studies suggest that MHCK B may cooperate with MHCK A to regulate myosin II filament formation. No information is available concerning the enzymatic activity or potential substrates of MHCK C.

A number of mammalian proteins with a domain homologous to the MHCK A catalytic domain have also been identified (13, 14). The best characterized of these proteins is the eukaryotic elongation factor-2 (eEF-2) kinase, which inhibits the elongation phase of protein synthesis by phosphorylating two threonine residues in eEF-2 (15). Site-directed mutagenesis and truncation analysis of the eEF-2 kinase shows that a pair of conserved cysteine residues within the MHCK A-related kinase domain as well as sequences C-terminal to this domain are required for activity (16).

It is now clear that MHCK A represents the founding member of a novel family of protein kinases. Studies on the MHCK A family of protein kinases have so far failed to reveal any unusual or distinct catalytic properties that would serve to distinguish them from the superfamily of conventional serine/threonine protein kinases. In this paper we show that the MHCK A, MHCK B, and MHCK C catalytic domains are remarkably specific for the phosphorylation of threonine residues in both peptide and protein substrates.

MATERIALS AND METHODS

Reagents

ATP, myelin basic protein (MBP), and bovine casein were from Sigma and [γ-^{32}P]ATP was from PerkinElmer Life Sciences. Caldesmon and myosin regulatory light chain (RLC) were purified from chicken gizzard as described (17, 18). A plasmid expressing the full-length, constitutively active mouse p21-activated kinase (PAK) as a GST fusion protein was a gift of Dr. S. Bagrodia and has been previously described (19).

Plasmid Constructs

All DNA manipulations were carried out using standard methods. The MHCK A (GenBank™ accession number 1170675) catalytic domain used in these studies, designated A-CAT, comprises residues 552–841 of MHCK A and has a C-terminal hexahistidine tag. A-CAT is identical to the previously described T-5 (9). A vector expressing the MHCK B (GenBank™ accession number 3122317) catalytic domain was made by excising a 1.4-kilobase insert fragment from pMM3.1, which contains the full-length cDNA coding region of MHCK B, using an internal*Eco*RV site (nucleotides 1391) and an *Xba*I site in the upstream polylinker of pMM3.1. The *Xba*I site was blunted by treatment with Klenow polymerase and then ligated into pGEX-2T vector that had been digested with *Eco*RI and made blunt. Digestion of this intermediate with *Eco*RI and subsequent recircularization yielded a vector that expressed residues 13–459 of MHCK B, designated B-CAT, fused in-frame to the C terminus of GST. The genomic clone of MHCK C (GenBank™ accession number 3420749) contains introns, so a cDNA clone corresponding to MHCK C was isolated from a 4-h developed *Dictyostelium* λGT11 library using a polymerase chain reaction-generated probe. The catalytic domain was amplified from the cDNA clone by polymerase chain reaction using primers that contained *Bam*HI restriction sites upstream and downstream of the predicted catalytic domain. The polymerase chain reaction fragment was cloned into the vector pRSET-A (Invitrogen) and a stop codon was placed downstream by subsequent addition of a 3′-oligonucleotide. The resultant plasmid, pRSET-DG2, expresses a fusion protein containing residues 19–283 of MHCK C, designated C-CAT, with 36 vector-derived residues at the N terminus (including the hexahistidine tag) and 5 vector-derived amino acids at the C terminus of the protein. Two GST fusion peptide expression vectors were prepared by cloning annealed complementary oligonucleotides encoding the desired peptides into the*Bam*HI/*Xho*I site of pGEX-4T-3 (Amersham Pharmacia Biotech). The expressed proteins consist of GST followed by either YAYDTRYRR or YAYDSRYRR and then 7 vector-derived residues (LERPHRD). All constructs were confirmed by restriction digest and DNA sequencing.

Protein Expression and Purification

A-CAT, B-CAT, and C-CAT were expressed in *Escherichia coli* BL21(DE3). Bacteria were grown overnight at 37 °C to an A_{600} of 0.6–1.0 and cooled to 25 °C prior to induction with isopropyl-β-D-thiogalactosidase (1 mM final concentration). A-CAT and C-CAT were isolated from the 15,000 ×*g* supernatant fraction of sonicated cell lysates using nickel chelation chromatography according to standard protocols provided with the His-Bind resin (Novagen). C-CAT recovered from the His-Bind resin was not further purified and was immediately stored in aliquots at −80 °C. A-CAT was dialyzed against 50 mMNaCl, 20 mM Tris, pH 7.5, and applied to a DE-53 (Whatman) column equilibrated in the same buffer. The flow-through was collected and passed over an SP Sepharose Fast Flow (Amersham Pharmacia Biotech) column equilibrated in 50 mM NaCl, 20 mM Tris, pH 7.0. The flow-through was collected, concentrated using a 10,000 molecular weight cut-off Ultrafree-4 Centrifugal Filtration Unit (Millipore), and stored at −80 °C. B-CAT, expressed as a GST fusion protein, was isolated from the 15,000 × *g* supernatant of sonicated cell lysates using glutathione-Sepharose beads (Amersham Pharmacia Biotech). Following elution with buffer containing 30 mM glutathione B-CAT was dialyzed against 12.5 mM Tris, pH 7.5, in 40% glycerol and stored at −80 °C. GST-peptide fusion proteins were expressed at 37 °C in *E. coli* DH5α and recovered using glutathione-Sepharose beads.

Peptide Synthesis

MH-3S, a variant of the previously described 16-residue peptide MH-3 (20), was synthesized using the HBTU/HObt coupling protocols on an Applied Biosystems 431A peptide synthesizer in the Department of Biochemistry, Queen's University. The same coupling protocols were used to construct a degenerate peptide library with the sequence MA*XXXX(*S/T)*XXXX*AKKK, where S/T designates a position containing a mixture of serine and threonine and *X* indicates a position containing all the common amino acids except tryptophan, cysteine, serine, and threonine (21). Position seven was synthesized using equal moles ofN^{α}-FMOC-blocked serine and threonine at 4-fold excess to the coupling resin while positions denoted by an *X*were synthesized using equal moles of a mixture of 16N^{α}-FMOC-blocked amino acids at 4-fold excess to the coupling resin. Tryptophan and cysteine were omitted from the degenerate positions to avoid problems with sequencing and oxidation while threonine and serine were omitted to ensure that each peptide has only a single potential site of phosphorylation (21, 22). Sequence analysis of the library, performed at the Alberta Peptide Institute (Edmonton, Alberta), showed that the amount of each of the 16 amino acids at each degenerate position was consistent from position to position and varied by no more than a factor of 2 from the expected value of 6.6% of the total amino acids. Isoleucine and lysine were present in the lowest amounts (3.0–3.5% of total amino acids) while proline and glycine were present in the highest amounts (9.5–10.5% of total amino acids).

Kinase Reactions

In vitro kinase assays for A-CAT, B-CAT, and C-CAT were carried out at 25 °C in kinase buffer (2 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 mM [γ-^{32}P]ATP (2–5 × 10^2 cpm/pmol) and 20 mM TES, pH 7.0). Assays were initiated by addition of the substrate (concentrations are provided in Table I and figure legends) followed by 1–5 μg/ml kinase. In some cases the kinases were preincubated in kinase buffer for 30 min at 25 °C prior to use. Phosphorylation rates were determined by removing 40-μl aliquots of the reaction mixture at time points from 30 s to 6 min. Incorporation of ^{32}P into MH-3, MH-3S, or MBP was determined by spotting aliquots onto 2 × 2-cm squares of P81 phosphocellulose paper (Whatman) (23). After washing in 1% phosphoric acid, the paper squares were placed into liquid scintillation fluid and counted using a Beckman LS 7500 scintillation counter. Assays with caldesmon, casein, and RLC were stopped by the addition of a one-fifth volume of SDS gel sample buffer (final concentration 1% SDS, 60 mM Tris-HCl, pH 6.8, 0.2% β-mercaptoethanol, and a trace of bromphenol blue) followed by boiling for 5 min. Assays with GST peptide fusion proteins were terminated by addition of 10 μl of glutathione-Sepharose. The resin was pelleted by centrifugation, washed with phosphate-buffered saline, and boiled for 5 min in SDS gel sample buffer. Radiolabeled proteins were separated on SDS-polyacrylamide gel electrophoresis and visualized and quantified using a storage phosphor screen and a Bio-

Rad Personal Molecular Imager FX. In some cases the protein band of interest was excised from the gel, placed in liquid scintillation fluid, and counted in a scintillation counter.

Table I
Substrate phosphorylation and phosphoamino acid analysis

Kinase	Substrate	Substrate concentration	Rate	Phosphate incorporated	Fraction Thr(P)
			$s^{-1} \times 10^3$	mol/mol	%
A-CAT	MH-3	200 μM	790		100
B-CAT	MH-3	200 μM	550		100
C-CAT	MH-3	200 μM	10		100
A-CAT	MBP	200 μM	300	3.5 [1-a]	96
B-CAT	MBP	200 μM	830	3.5 [1-a]	91
C-CAT	MBP	200 μM	360	4.8 [1-a]	95
A-CAT	Caldesmon	1.5 μM	5	5.0	98
B-CAT	Caldesmon	1.5 μM	81	7.6	97
C-CAT	Caldesmon	1.5 μM	28	5.7	99
A-CAT	RLC	5 μM	1.8	2.0	98
B-CAT	RLC	5 μM	0.8	0.2	96
C-CAT	RLC	5 μM	2.0	1.9	98
A-CAT	Casein	100 μg/ml	6	2.3	98
B-CAT	Casein	100 μg/ml	13	1.2	98
C-CAT	Casein	100 μg/ml	12	1.2	99
A-CAT	Peptide library	3.3 mg/ml		0.01	99
B-CAT	Peptide library	3.3 mg/ml		0.01	98
C-CAT	Peptide library	3.3 mg/ml		0.01	97
PAK	Peptide library	3.3 mg/ml		0.01	20

- 1-a The amount of phosphate incorporated was determined using 5 μM MBP.

Phosphopeptide Separation and Sequence Analysis
The peptide library (3.3 mg/ml) was incubated in 300 μl of kinase buffer with A-CAT, B-CAT, or C-CAT. The reaction was stopped by addition of 300 μl of acetic acid and the phosphorylated peptides isolated as described (21, 22). Briefly, the mixture was passed through a 10,000 molecular weight cut-off Ultrafree-4 Centrifugal Filtration Unit to remove the kinase and chromatographed on a DE-53 column equilibrated in 30% acetic acid to remove [γ-^{32}P]ATP. Fractions containing peptides were lyophilized, dissolved in 1 MNaCl, 50 mM MES, pH 5.5, and applied to a 1-ml column of ferric iminodiacetic acid beads (Pierce) equilibrated in the same buffer. After washes with equilibration buffer and 2 mMMES, pH 6.0, the phosphopeptides were eluted with 0.5 MNH_4HCO_3, pH 8.0. A control experiment was performed in which the peptide library was incubated in the absence of kinase and subjected to the same isolation protocol. The peptide mixtures recovered from the ferric column were sequenced at the Alberta Peptide Institute. The abundance of each amino acid at each degenerate position in the phosphopeptide fractions was first corrected for the presence of non-phosphorylated peptides, the level of which was estimated by the amount of serine and threonine recovered at cycle 7. The non-phosphorylated peptide control mixture was enriched in aspartic acid and glutamic acid, and so the major effect of this correction was to reduce the levels of these two residues (21). The corrected phosphopeptide data was then divided by the relative abundance of each amino acid at each degenerate position in the starting mixture.

The abundance ratios were added and normalized to 16 (the number of amino acids at each degenerate position) to obtain the selectivity values. Selectivity values above 1 are obtained when an amino acid at a given position is enriched in the phosphopeptide fraction relative to the starting mixture and indicates that peptides containing this residue are preferentially phosphorylated by the kinase (21).

Phosphoamino Acid Analysis

Radiolabeled proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P membrane (Millipore). After staining with Amido Black, the piece of membrane containing the protein band was excised, washed with water and methanol, and hydrolyzed in 6 N HCl at 110 °C for 2 h under vacuum (24). Peptides were separated from kinase using a 10,000 molecular weight cut-off Ultrafree-4 Centrifugal Filtration Unit, chromatographed over DE-53 cellulose equilibrated in 30% acetic acid to remove [γ-^{32}P]ATP, dried in a Speed-Vac vacuum concentrator, and hydrolyzed as described above. Hydrolysates were resuspended in 1 ml of water and dried in a Speed-Vac vacuum concentrator. This process was repeated twice, then each hydrolysate was resuspended in 5–10 μl of pH 1.9 buffer (2.5% formic acid and 7.8% acetic acid) containing 0.1 mg/ml each of unlabeled Ser(P), Thr(P), and Tyr(P) standards (Sigma) and applied to a thin-layer cellulose chromatography plate (20 × 20 cm, Eastman). Electrophoresis was performed using the pH 1.9 buffer and, if necessary, in a second dimension with pH 3.5 buffer (5% acetic acid and 0.5% pyridine), using an HTLE-7000 thin layer electrophoresis unit (C.B.S. Scientific Co.) (25, 26). Phosphoamino standards were located by reaction with 0.25% (w/v) ninhydrin in acetone, while ^{32}P-labeled Ser(P) and Thr(P) were detected and quantified using a storage phosphor screen and a Bio-Rad Personal Molecular Imager FX.

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RESULTS

Expression of the Catalytic Domains of MHCK A, MHCK B, and MHCK C

The atypical protein kinase catalytic domain first defined in *Dictyostelium* MHCK A is present in two related *Dictyostelium* proteins: MHCK B and MHCK C (Fig.1 *A*). For the studies reported here the isolated catalytic domains of MHCK A, MHCK B, and MHCK C, designated here as A-CAT, B-CAT, and C-CAT, respectively, were expressed in bacteria. A-CAT comprises residues 552–841 of MHCK A, B-CAT comprises residues 13–459 of MHCK B, and C-CAT comprises residues 19–283 of MHCK C. A-CAT, B-CAT, and C-CAT were insoluble if expressed at 37 °C, but soluble if expressed at 25 °C. A-CAT and C-CAT, which were expressed with a hexahistidine tag, could be purified with yields of 1–2 mg/liter of culture while B-CAT, which was expressed as a GST fusion protein, was obtained with a yield of ~0.1 mg/liter of culture (Fig. 1 *B*).

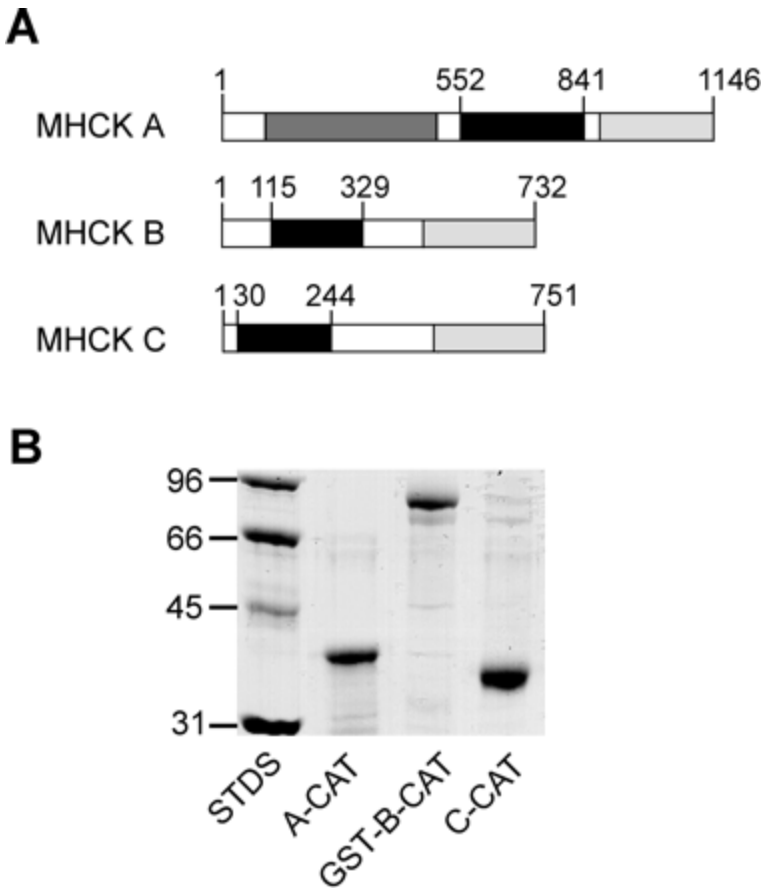

Figure 1
Expression of the MHCK catalytic domains. *A,* a schematic diagram showing the domain structure of MHCK A (GenBank™ accession number 1170675), MHCK B (GenBank™ accession number 3122317), and MHCK C (GenBank™ accession number 3420749). MHCK A consists of an α-helical coiled-coil domain (*dark gray*), a novel type of protein kinase catalytic domain (*black*), and a WD-repeat domain (*light gray*). MHCK B and MHCK C possess a catalytic domain and WD-repeat domain structurally related to those of MHCK A but lack an extended coiled-coil domain. *B,* a Coomassie Blue-stained SDS-polyacrylamide gel of the bacterially expressed MHCK catalytic domains used in this study. A-CAT and C-CAT were expressed with a hexahistidine tag while B-CAT was expressed as a GST fusion protein.

Phosphorylation of Peptide and Protein Substrates
The 16-residue peptide MH-3 (RKKFGEAEKTKAKEFL-amide) has been previously described (20) and is based on the MHCK A target site in the*Dictyostelium* myosin II tail at residue 2029 (underlined in peptide above). MH-3 (and its variants) are presently the only documented peptide substrates for MHCK A and MHCK B (12, 20, 27). Measurement of the initial rates of phosphorylation showed that MH-3 is a much better substrate for A-CAT and B-CAT than for C-CAT (TableI). MBP has previously been identified as a good substrate for A-CAT (9) and is also phosphorylated at a high rate by B-CAT and C-CAT. Several other protein substrates, including casein, RLC, and caldesmon were found to be substrates for A-CAT, B-CAT, and C-CAT, although they were phosphorylated at a considerably lower rate than MBP or MH-3 (Table I). When the kinase reactions were allowed to proceed to completion, the maximal amount of ^{32}P incorporated by A-CAT, B-CAT, and C-CAT into individual proteins was often quite different, suggesting that the sites targeted by each catalytic domain are not identical (Table I).

Proteins that had been maximally phosphorylated by A-CAT were subjected to acid hydrolysis and their phosphoamino acid content examined by two-dimensional electrophoresis on thin-layer cellulose plates. In all cases the phosphorylated proteins contained significantly greater amounts of Thr(P) than either Ser(P) or Tyr(P) (Fig.2). Measurement of the amount of radioactivity at the position corresponding to each of the phosphoamino acid standards indicated that Tyr(P) accounted for less than 1%, Ser(P) for 2 to 5%, and Thr(P) for 95 to 98% of the total radioactivity (Table I). Since the level of phosphate incorporated into tyrosine residues was negligible, further analysis focused on quantifying the relative amounts of Ser(P) and Thr(P). Experiments in which Ser(P)

and Thr(P) were separated by one-dimensional electrophoresis at pH 1.9 showed that B-CAT and C-CAT displayed a strong preference for the phosphorylation of threonine residues in caldesmon (Fig. 3 *A*) and other protein substrates (Table I).

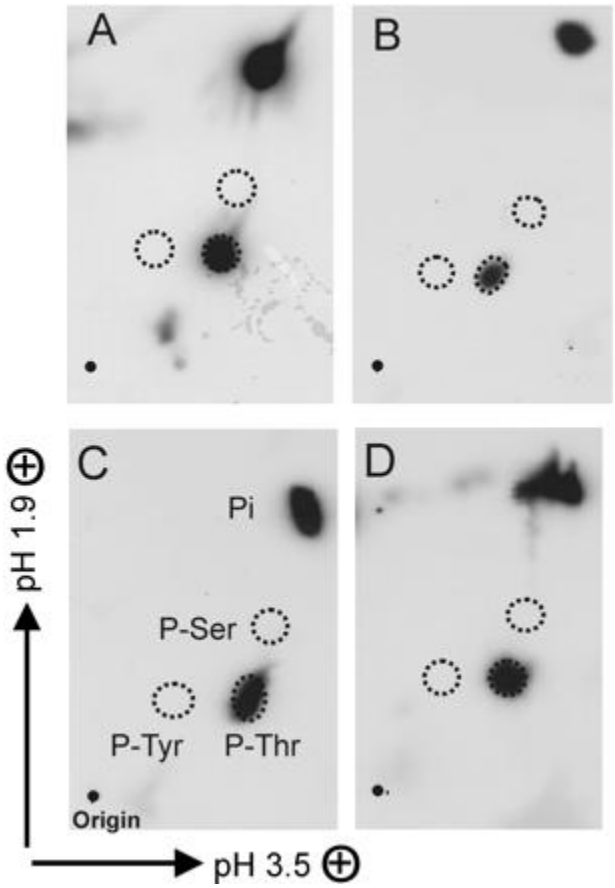

Figure 2

Phosphoamino acid analysis of proteins phosphorylated by A-CAT. The panels show autoradiographs of the phosphoamino acid content of: *A*, RLC; *B*, MBP;*C*, casein; and *D*, caldesmon, after being maximally phosphorylated by A-CAT. Following phosphorylation the proteins were subjected to SDS-gel electrophoresis, transferred to Immobilon-P, and hydrolyzed to their constituent amino acids. The hydrolysates were separated by electrophoresis at pH 1.9 and then at pH 3.5 in the directions indicated by the *arrows* for*panel C*. The positions of the unlabeled Ser(P), Thr(P), and Tyr(P) standards were visualized by ninhydrin staining and are shown as*dotted outlines*.

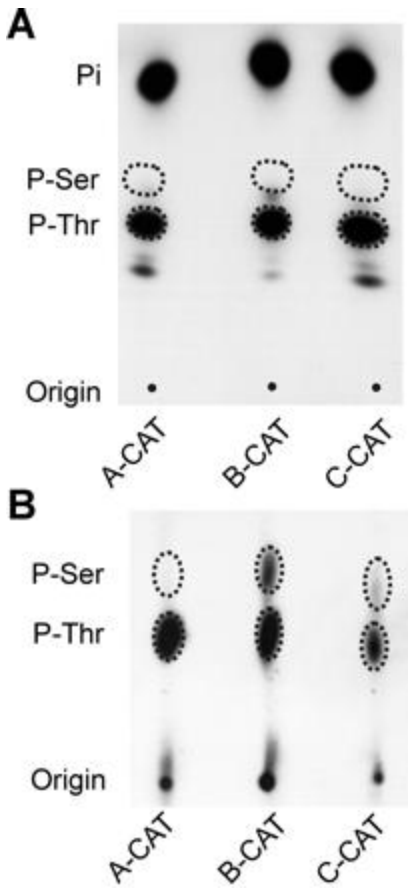

Figure 3
Phosphoamino acid analysis of caldesmon and autophosphorylated A-CAT , B-CAT , and C-CAT. The panels show autoradiographs of the phosphoamino acid content of: *A*, caldesmon maximally phosphorylated with A-CAT, B-CAT, and C-CAT; and*B*, autophosphorylated A-CAT, B-CAT, and C-CAT. Following phosphorylation the proteins were subjected to SDS-gel electrophoresis, transferred to Immobilon-P, and hydrolyzed to their constituent amino acids. Ser(P) and Thr(P) were separated by electrophoresis at pH 1.9. The *dotted circles* represent the positions of the unlabeled Ser(P) and Thr(P) standards detected by ninhydrin staining.

The incorporation of phosphate into A-CAT, B-CAT, and C-CAT as a result of autophosphorylation was also examined (9, 12). A-CAT incorporated 2 mol of phosphate/mol, B-CAT 10 mol of phosphate/mol, and C-CAT only 0.5 mol of phosphate/mol. Phosphoamino acid analysis showed that Thr(P) accounted for 98%, 80 and 90% of the total phosphate incorporated into A-CAT, B-CAT, and C-CAT, respectively (Fig. 3 *B*).

Phosphorylation of a Serine/Threonine Peptide Library
To further investigate the acceptor amino acid specificity of A-CAT, B-CAT, and C-CAT a degenerate serine/threonine peptide library was synthesized composed of peptides with the sequence MA*XXXX*(S/T)*XXXX*AKKK, where *X*represents a degenerate position containing all the amino acid except cysteine, tryptophan, serine, and threonine and position seven contains a mixture of serine and threonine. This peptide library provides a choice of more than 4×10^9 distinct peptides each with a single serine or threonine residue as a potential site of phosphorylation. Although the serine/threonine position was synthesized using equal moles of N^{α}-Fmoc-blocked serine and threonine, quantitative amino acid analysis of the library yielded values of 0.62 mol/mol for serine and 0.36 mol/mol for threonine (corrected for 90% recovery after acid hydrolysis). The low abundance of threonine in the peptide library may reflect the fact that the β-substituted threonine couples at a slower rate than serine during solid phase peptide synthesis (28).

The serine/threonine peptide library was phosphorylated using A-CAT, B-CAT, C-CAT, or the conventional serine/threonine protein kinase PAK. The reactions were terminated when 1% of the peptides were phosphorylated, so that the kinases would not be forced to phosphorylate suboptimal substrates (21).

Phosphoamino acid analysis of the peptide library showed that A-CAT, B-CAT, and C-CAT incorporated phosphate almost exclusively into threonine residues, while PAK predominately phosphorylated serine residues (Fig.4, *A* and *B*). Quantification of the results demonstrated, remarkably, that 97–99% of the total phosphate incorporated by A-CAT, B-CAT, and C-CAT was recovered as Thr(P) (Table I). In contrast, 80% of the phosphate incorporated by PAK was recovered as Ser(P).

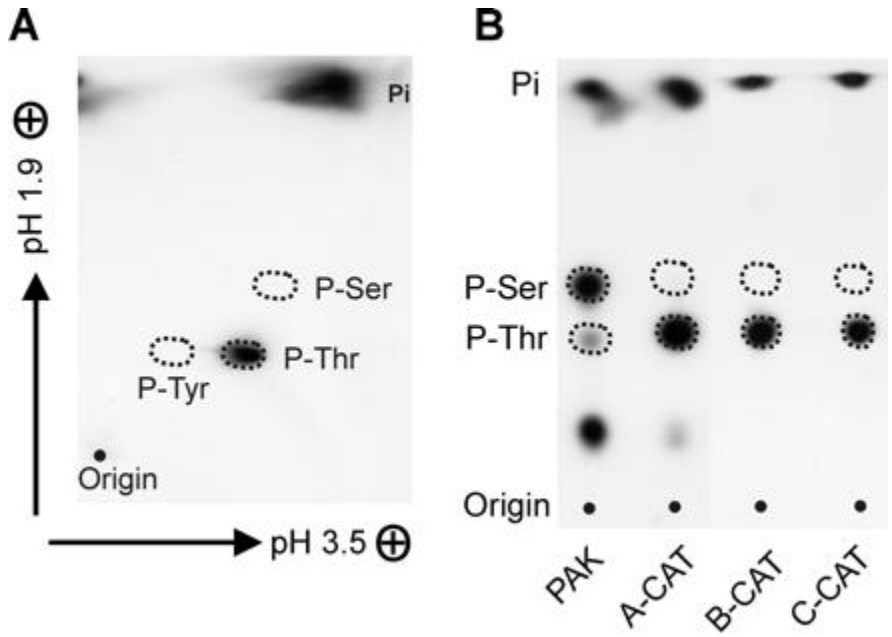

Figure 4
Phosphoamino acid analysis of the serine/threonine peptide library. *A,* an autoradiograph showing the phosphoamino acid content of the serine/threonine peptide library following phosphorylation with A-CAT. Peptides were hydrolyzed to yield free amino acids as described under "Materials and Methods" and the hydrolysate was subjected to electrophoresis at pH 1.9 and then at pH 3.5 on a thin-layer cellulose plate as indicated by the *arrows*. The *dotted circles* show the positions of the unlabeled Ser(P), Thr(P), and Tyr(P) standards detected by ninhydrin staining. *B,* an autoradiograph showing the phosphoamino acid content of the serine/threonine peptide library following phosphorylation with PAK, A-CAT, B-CAT, or C-CAT. Separation was performed in one dimension at pH 1.9. The *dotted circles*represent the positions of the unlabeled Ser(P) and Thr(P) standards detected by ninhydrin staining.

The phosphoamino acid analyses results reported above were obtained following 2 h of acid hydrolysis. The half-life of Thr(P) is greater than that of Ser(P) in 6 n HCl at 105 °C (more than 25 h as compared with 8 h) suggesting that shorter hydrolysis times might increase the ratio of Ser(P) to Thr(P) (29). To examine this possibility, the peptide library phosphorylated by A-CAT was subjected to acid hydrolysis times varying from 30 min to 4 h. At 30 min the amount of Ser(P) was still less than 4% that of Thr(P) (Fig. 5).

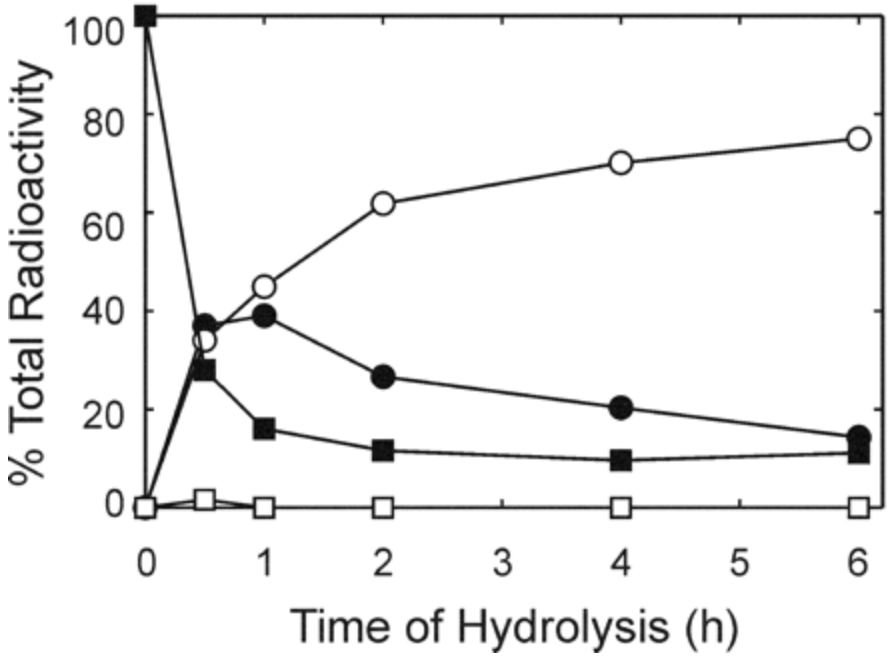

Figure 5
Acid hydrolysis time course. The serine/threonine peptide library was phosphorylated with A-CAT. Phosphoamino acid analysis was performed as described under "Materials and Methods" except that the duration of the acid hydrolysis was varied from 30 min to 6 h. Following electrophoresis on thin-layer cellulose plates at pH 1.9 the amount of radioactivity in the P_i (○), Thr(P) (●), and Ser(P) (□) spots and at the origin (▪) was quantified using a Bio-Rad Personal Molecular Imager FX.

Consensus Phosphorylation Sequences for A-CAT, B-CAT, and C-CAT

The peptide library was phosphorylated to a low level (~1% of the peptides phosphorylated) using A-CAT, B-CAT, or C-CAT. The small fraction of phosphopeptides was isolated from the bulk of unphosphorylated peptides as described under "Materials and Methods" and subjected to amino acid sequence analysis to determine the amino acid composition at each degenerate position. The abundance of individual amino acids at the eight degenerate positions in the pool of phosphorylated peptides was then compared with the abundance of the same amino acid at the same position in the starting peptide library to give a selectivity value (Table II). A selectivity value above 1 indicates that peptides with that residue have been preferentially chosen by the kinase as substrates during the phosphorylation reaction (22). The results showed that A-CAT, B-CAT, and C-CAT all favor peptides with tyrosine at the −2 and −4 positions and basic residues at the +3 and +4 positions. In the +1 position A-CAT displayed a preference for a basic residue and B-CAT strongly selected for valine. C-CAT showed an unusual preference for aromatic, hydrophobic residues in all positions from −4 to +2.

Table II

Substrate specificities of the MHCK catalytic domains

Kinase	Selectivity value							
	−4	−3	−2	−1	0+1	+2	+3	+4
A-CAT	Y(1.8)		Y(1.7) E(1.5)	D(1.6)	TH(1.9) R(1.7)	**Y(2.1)** H(1.6)	**R(2.3)** **K(2.1)** K(1.5)	R(1.9) K(1.6)
B-CAT	**Y(2.1)**	**E(2.1)**	Y(1.5)	**R(2.3)** D(1.8)	**TV(2.4)**	R(1.7)	**R(2.0)** K(1.7)	R(1.8) K(1.6)
C-CAT	**Y(3.3)** F(1.8)	**Y(2.3)** F(1.8)	**Y(2.4)** F(1.8)	**Y(2.5)** E(1.9)	**TY(2.3)** E(1.8)	**Y(2.2)** F(1.6)	**K(2.4)** **R(2.1)**	**K(2.3)** R(1.9)

Kinase	Selectivity value							
	−4	−3	−2	−1	0+1	+2	+3	+4
				F(1.6)	F(1.6)		Y(1.9)	

Amino acids with selectivity values higher than 1.5 at a degenerate position are shown. Boldface indicates amino acids that were most preferentially selected (value > 2.0).

The consensus recognition sequence determined for A-CAT (YAYDTRYRR) was synthesized and found to be a good A-CAT substrate. Kinetic analysis showed that A-CAT displayed a K_m for YAYDTRYRR of 550 μM and a k_{cat} of 14 s^{-1} (Fig. 6 *A*). With MH-3 as the substrate, A-CAT exhibited a somewhat lowerK_m (280 μM) but an 8-fold lowerk_{cat} (1.8 s^{-1}) (Fig.6 *B*). Similar kinetic constants for the phosphorylation of MH-3 by A-CAT have been reported previously (9).

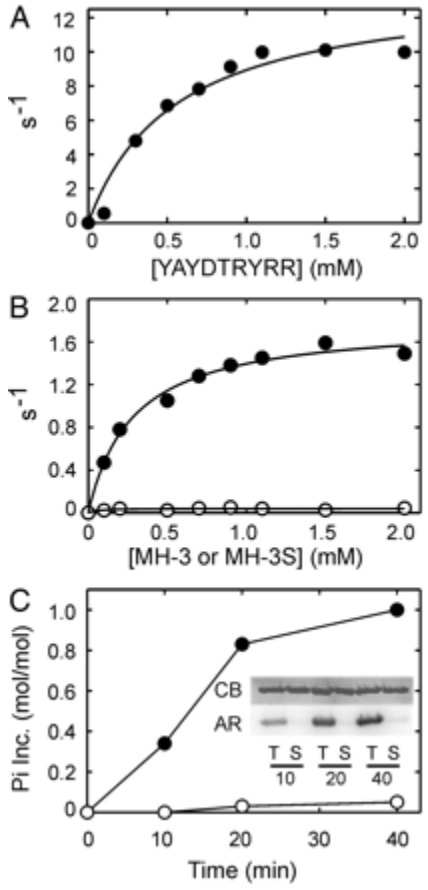

Figure 6

Phosphorylation of peptides and GST fusion peptides by A-CAT. *A*, phosphorylation of the peptide YAYDTRYRR by A-CAT. Rates of phosphorylation at each peptide concentration were determined by removing aliquots from the reaction at 1, 2, 3, 4, and 5 min and spotting them onto phosphocellulose paper as described under "Materials and Methods." Over this time period linear rates of phosphate incorporation were obtained at all peptide concentrations. The data were fit to a hyperbolic equation by regression analysis using the program SigmaPlot for Windows (SPSS Science Inc.). *B* **,** phosphorylation by A-CAT of the peptides MH-3 (●) and MH-3S (○). The sequence of MH-3S (RKKFGEAEKSKAKEFL-amide) is identical to that of MH-3 but with serine in place of threonine. Rates of phosphorylation and data analysis was performed as described above. *C* **,** phosphorylation by A-CAT of YAYDTRYRRL (●) or YAYDSRYRRL (○) fused to the C terminus of GST. The *inset* shows the Coomassie Blue-stained gel (*CB*) and the corresponding autoradiograph (*AR*) for the threonine (*T*) and serine (*S*) GST peptide fusions phosphorylated for 10, 20, and 40 min. Assays contained 1 mg/ml GST-peptide fusion protein and 5 μg/ml A-CAT. Phosphate incorporation into the substrates was quantified using a Bio-Rad Personal Molecular Imager FX.

Substitution of Serine for Threonine in Substrates

To directly examine the impact that a threonine or serine residue at the site of phosphorylation has on the activity of A-CAT, the serine-containing counterpart of MH-3 was synthesized (MH-3S:

RKKFGEAEKSKAKEFL-amide). In contrast to MH-3, MH-3S was not appreciably phosphorylated by A-CAT even at a concentration of 2 mM(Fig. 6 *B*). A second pair of serine/threonine substrates was prepared by fusing either YAYDTRYRR or YAYDSRYRR to the C terminus of GST. GST alone was not a substrate for A-CAT (data not shown) but the GST-YAYDTRYRR fusion peptide was readily phosphorylated by A-CAT to a level of 1 mol of phosphate/mol (Fig. 6 *C*). In contrast, the GST-YAYDSRYRR fusion peptide was a very poor substrate for A-CAT. Over the linear portion of the time course the threonine-containing GST fusion peptide was phosphorylated at a rate more than 25-fold higher than its serine-containing analogue (Fig. 6 *C*). These results directly demonstrate that A-CAT strongly prefers a threonine residue at the site of phosphorylation.

DISCUSSION

Dictyostelium MHCK A, MHCK B, and MHCK C contain a type of protein kinase catalytic domain whose primary sequence is very different from the catalytic domain found in conventional serine, threonine, and/or tyrosine protein kinases (9, 12). The goal of the present study was to examine the intrinsic substrate specificity of the MHCK catalytic domains. For this purpose the isolated catalytic domains of MHCK A, MHCK B, and MHCK C, designated A-CAT, B-CAT, and C-CAT, were expressed in bacteria and purified (Fig. 1). The use of all three catalytic domains, which share between 48 and 54% sequence similarity, provides a degree of confidence that the results obtained should be applicable to the entire family of MHCK A-related kinases.

A-CAT, B-CAT, and C-CAT phosphorylated a selection of structurally diverse proteins, including MBP, RLC, caldesmon, and casein, and in many instances incorporated more than 1 mol of phosphate/mol into these proteins (Table I). Phosphoamino acid analysis produced the striking observation that for all of the protein substrates more than 95% of the phosphate was incorporated into threonine residues with the remainder being incorporated into serine. No phosphorylation of tyrosine was observed. Experiments using a degenerate serine/threonine peptide library showed that when confronted with a choice of a serine or threonine phosphorylation sites, A-CAT, B-CAT, and C-CAT overwhelmingly chose peptides containing threonine. As judged by the distribution of radioactivity following phosphoamino acid analysis, 98–99% of the phosphate incorporated into the peptide library by A-CAT, B-CAT, and C-CAT was present on threonine residues.

The ability of A-CAT to select between a serine and threonine acceptor amino acid was further examined using two defined substrates: a synthetic peptide (MH-3) and a GST fusion peptide. Substitution of serine for the target threonine in MH-3 and the GST fusion peptide virtually eliminated phosphorylation by A-CAT (Fig. 6, *A* and*B*). MH-3S, the serine version of MH-3, was such a poor substrate for A-CAT that its kinetic constants could not be calculated; thus, it was not possible to determine whether the low rate of phosphorylation of MH-3S was due to its inability to bind to A-CAT or to a large decrease in k_{cat}. However, when added to phosphorylation reactions containing MH-3, MH-3S did not behave as a competitive inhibitor (data not shown). These results suggest that the replacement of threonine with serine substantially reduces the affinity of the peptide for A-CAT.

A-CAT, B-CAT, and C-CAT are not absolutely specific for threonine since detectable levels of Ser(P) were obtained following the phosphoamino acid analysis of protein and peptide substrates. The highest proportion of Ser(P), amounting to 20% of the total phosphoamino acids, was present in the autophosphorylated B-CAT (Table I). A possible explanation for this relatively high level of serine phosphorylation is that B-CAT autophosphorylates multiple sites via an intramolecular reaction. If this is the case, relatively unfavorable serine residues may be phosphorylated simply because they are presented to the active site of B-CAT at a very high effective concentration. Interestingly, it has been reported that the mutation of the target threonine residues in the *Dictyostelium* myosin II tail to serine residues does not prevent phosphorylation by partially purified MHCK A (2). This result seems unusual given the threonine specificity for the MHCK A catalytic domain documented here, but may possibly be explained by the finding that MHCK A is physically targeted to myosin II by the WD-repeat domain (8, 27). By tethering the MHCK A catalytic domain in close proximity to the serine residues, the WD-repeat domain may enhance their phosphorylation.

The strong preference to phosphorylate threonine residues in both peptides and proteins distinguishes the MHCK A family of protein kinases from conventional protein serine/threonine kinases. Indeed, many conventional serine/threonine kinases display a bias toward the phosphorylation of serine residues. A compilation of the sites phosphorylated in proteins by conventional kinases shows that serine residues are targeted about four times more frequently than threonine residues (30). A comparable distribution (80% serine and 20% threonine) was obtained in this study when PAK, a conventional protein kinase, was used to phosphorylate the serine/threonine peptide library (Table I). Peptides containing serine residues are more effective substrates than their threonine-containing counterparts for several conventional protein kinases, including the cAMP-dependent protein kinase (31-34). Assays with the cAMP-dependent protein kinase, for example, show that the replacement of serine in Kemptide (LRRASLG) with threonine produces a 5-fold decrease in k_{cat} and a 20-fold increase in K_m (31, 35).

At least for some substrates, the active site of conventional protein kinases does not seem to readily accommodate a methyl group on the β-carbon of the substrate side chain (36, 37). Perhaps because of this bias, it is rare to find a conventional protein kinase that phosphorylates any one protein exclusively on threonine, much less a whole set of proteins. To take one example, the RLC, which is phosphorylated by A-CAT and C-CAT primarily on threonine residues, is a substrate for multiple conventional protein kinases, including the Ca^{2+}-calmodulin-dependent protein kinase II, cAMP-dependent kinase, casein kinase I, mitogen-activated protein kinase-activated protein kinase-2, myosin light chain kinase, PAK, phosphorylase kinase, protein kinase C, Rho kinase, and ZIP kinase (38-45). All of these kinases, except casein kinase II, phosphorylates the RLC either on serine or on serine and threonine residues. Casein kinase II phosphorylates the RLC primarily on threonine (39), but phosphorylates many other substrates, including caldesmon, primarily on serine (46). We are unaware of any conventional serine/threonine protein kinase that exhibits a strong and consistent preference for the phosphorylation of threonine residues. The preference displayed by the MHCK A type kinases for the bulkier threonine side chain, together with the lack of sequence homology to conventional protein kinases, suggests that the architecture of their catalytic domain may be quite different from that of conventional protein kinases. Attempts are presently underway to produce crystals of A-CAT and C-CAT suitable for x-ray crystallographic analysis.

The catalytic domain of the human eEF-2 kinase exhibits 40–46% sequence similarity to the *Dictyostelium* MHCKs, suggesting that it is likely to display the same fundamental properties, including a specificity for threonine residues, as the MHCKs. Consistent with this proposal, the sites phosphorylated by the eEF-2 kinase in elongation factor-2 are Thr-56 and Thr-58 (15). The eEF-2 kinase autophosphorylates on serine residues (47, 48) but, as noted above, intramolecular autophosphorylation sites may not be representative of the sites phosphorylated on optimal exogenous substrates. Clearly, it will be important to directly test the acceptor amino acid specificity of the eEF-2 kinase and other mammalian members of the MHCK A family.

Conventional protein kinases recognize phosphorylation sites located within the context of a characteristic sequence of amino acids and it seems likely that the same is true for A-CAT, B-CAT, and C-CAT. Based on the observation that MHCK A phosphorylates sites in the α-helical coiled-coil myosin II tail and that the eEF-2 kinase phosphorylates sites located within an α-helix in eEF-2, it has been suggested these kinases are specialized to recognize phosphorylation sites located within α-helices (14). As a consequence, the name α-kinases has been proposed for this kinase family (14). However, the results reported here support the view that A-CAT, B-CAT, and C-CAT are capable of phosphorylating short synthetic peptides and proteins that have little or no α-helical structure in solution. MBP is a good substrate for all three catalytic domains, yet secondary structure prediction methods, circular dichroism data, and electron microscopic three-dimensional reconstructions indicate that it has very low α-helical content (49-51). Casein proteins also have little α-helical content and a low degree of structural organization in aqueous neutral solvents (52, 53), yet are substrates for A-CAT, B-CAT, and C-CAT. Moreover, the ability to MHCK A and MHCK B to efficiently phosphorylate the α-helical myosin II tail depends to a large extent on the targeting function of the WD-repeat domain and does not seem to be an intrinsic property of the catalytic domain (27).

The three MHCK catalytic domains phosphorylate individual substrates at significantly different rates and also incorporate significantly different amounts of phosphate, indicating that they have distinct substrate specificities (Table I). An initial attempt to define the consensus sequences recognized by A-CAT, B-CAT, and C-CAT was made using the degenerate peptide library method, which tends to select for peptide substrates that have low K_m values or highk_{cat}/K_m ratios (21, 22). The results indicate that all three catalytic domains share some common recognition elements, including a preference for tyrosine in the −4 and −2 positions and for basic residues in the +3 and +4 positions, but differ in their selectivity for residues in the −1 and +1 positions (Table II). As a test of the validity of these results, a synthetic peptide (YAYDTRYRR) corresponding to the predicted A-CAT consensus sequence was synthesized and assayed for its ability to function as an A-CAT substrate (Fig. 6 *A*). Kinetic analysis showed that A-CAT phosphorylated YAYDTRYRR with a specificity constant (k_{cat}/K_m) of 0.025 $\mu M^{-1} s^{-1}$, which is 4-fold higher than the specificity constant for MH-3 (0.006 $\mu M^{-1} s^{-1}$). By this criteria, YAYDTRYRR is the best A-CAT peptide substrate yet identified.

It is of interest to compare the consensus phosphorylation sequence predicted using the peptide library with the sequences of sites phosphorylated in protein substrates; however, no MHCK C protein target sites have been mapped, and the only identified sites for MHCK A and MHCK B are the three threonine residues in the *Dictyostelium*myosin II tail (1, 2, 12). A part of the predicted A-CAT consensus sequence (D*T*-basic-Y-basic) corresponds exactly to the sequence of the 1833 site (DTKYK) within the myosin II tail but is less similar to the 2029 site (KTKTK) and the 1823 site (ATKTQ) (phosphorylated residue is underlined). (1). The predicted consensus sequence for B-CAT (RTV-basic-basic) is most similar to the sequence of the 2029 site. The relative rates at which A-CAT and B-CAT phosphorylate the three sites in the myosin II tail is, however, not known.

In summary, the studies reported here provide strong evidence that the MHCK A family of protein kinases exhibit a specificity for threonine residues much greater than that usually associated with conventional serine/threonine protein kinases. Since protein kinases are classified based on the nature of the acceptor amino acid (54), we propose that the MHCK A-related kinases be classed as threonine kinases. The ability to target threonine residues provides a rationale for why the MHCK A catalytic domain has been conserved throughout evolution (albeit in a relatively small group of proteins) and is likely to be critical for understanding how these kinases function in signaling pathways. The unique acceptor amino acid specificity of the MHCK A-related kinases might also make them useful reagents in cases where the selective phosphorylation of threonine residues in peptides or proteins is desired.

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Abbreviations:

MHCK myosin II heavy chain kinase

A-CAT catalytic domain of MHCK A

B-CAT catalytic domain of MHCK B

C-CAT catalytic domain of MHCK C

eEF-2 eukaryotic elongation factor-2

GST glutathione *S*-transferase

MBP myelin basic protein

PAK p21-activated kinase

RLC myosin regulatory light chain

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

MES 4-morpholineethanesulfonic acid

REFERENCES

Vaillancourt J. P., Lyons C., Côté G. P.(1988) J. Biol. Chem. 263:10082–10087.

Luck-Vielmetter D., Schleicher M., Grabatin B., Wippler J., Gerisch G.(1990) FEBS Lett. 269:239–243.

Côté G. P., McCrea S. M.(1987) J. Biol. Chem. 262:13033–13038.

Egelhoff T. T., Lee R. J., Spudich J. A.(1993) Cell 75:363–371.

Kolman M. F., Futey L. M., Egelhoff T. T.(1996) J. Cell Biol. 132:101–109.

Côté G. P., Bukiejko U.(1987) J. Biol. Chem. 262:1065–1072.

Futey L. M., Medley Q. G., Côté G. P., Egelhoff T. T.(1995) J. Biol. Chem. 270:523–529.

Kolman M. F., Egelhoff T. T.(1997) J. Biol. Chem. 272:16904–16910.

Côté G. P., Luo X., Murphy M. B., Egelhoff T. T.(1997) J. Biol. Chem. 272:6846–6849.

Hanks S. K., Hunter T.(1995) FASEB J. 9:576–596.

Loomis W. F., Kuspa A., Shaulsky G.(1998) Curr. Opin. Microbiol. 1:643–648.

Clancy C. E., Mendoza M. G., Naismith T. V., Kolman M. F., Egelhoff T. T.(1997) J. Biol. Chem. 272:11812–11815.

Ryazanov A. G., Ward M. D., Mendola C. E., Pavur K. S., Dorovkov M. V., Wiedmann M., Erdjument, Bromage H., Tempst P., Parmer T. G., Prostko C. R., Germino F. J., Hait W. N.(1997) Proc. Natl. Acad. Sci. U. S. A. 94:4884–4889.

Ryazanov A. G., Pavur K. S., Dorovkov M. V.(1999) Curr. Biol. 9:R43–R45.

Price N. T., Redpath N. T., Severinov K. V., Campbell D. G., Russell J. M., Proud C. G.(1991) FEBS Lett. 282:253–258.

Diggle T. A., Seehra C. K., Hase S., Redpath N. T.(1999) FEBS Lett. 457:189–192.

Bretscher A.(1984) J. Biol. Chem. 259:12873–12880.

Hathaway D. R., Haeberle J. R.(1983) Anal. Biochem. 135:37–43.

Van Eyk J. E., Heinenon T., Arrel D. K., Foster B. D., Strauss J. D., Furmaniak-Kazmierczak E., Côté G. P., Mak A. S.(1998) J. Biol. Chem. 273:23433–23439.

Medley Q. G., Gariepy J., Côté G. P.(1990) Biochemistry 29:8992–8997.

Songyang Z., Blechner S., Hoagland N., Hoekstra M. F., Piwnica-Worms H., Cantley L. C.(1994) Curr. Biol. 4:973–982.

Nishikawa K., Toker A., Johannes F. J., Zhou S. Y., Cantley L. C.(1997) J. Biol. Chem. 272:952–960.

Casnellie J. E.(1991) Methods Enzymol. 200:115–120.

Kamps M. P.(1991) Methods Enzymol. 201:21–27.

Boyle W. J., Van der Geer P., Hunter T.(1991) Methods Enzymol. 201:110–149.

Duclos B., Marcandier S., Cozzone A. J.(1991) Methods Enzymol. 201:10–21.

Steimle P. A., Naismith T., Licate L., Egelhoff T. T.(2001) J. Biol. Chem. 276:6853–6860.

Gross E., Meienhofer J.Barany G., Merrifield R. B.(1979) in The Peptides, eds Gross E., Meienhofer J. (Academic Press, New York), pp 1–284.

Bitte L., Kabat D.(1974) Methods Enzymol. 30:563–590.

Pearson R. B., Kemp B. E.(1991) Methods Enzymol. 200:62–81.

Kemp B. E., Graves D. J., Benjamini E., Krebs E. G.(1977) J. Biol. Chem. 252:4888–4894.

House C., Wettenhall R. E. H., Kemp B. E.(1987) J. Biol. Chem. 262:772–777.

Kim S. T., Lim D. S., Canman C. E., Kastan M. B.(1999) J. Biol. Chem. 274:37538–37543.

Dale S., Wilson W. A., Edelman A. M., Hardie D. G.(1995) FEBS Lett. 361:191–195.

Prorok M., Lawrence D. S.(1989) Biochem. Biophys. Res. Commun. 158:136–140.

Kwon Y.-G., Mendelow M., Srinivasan J., Lee T. R., Pluskey S., Salerno A., Lawrence D. S.(1993) J. Biol. Chem. 268:10713–10716.

Kwon Y. G., Mendelow M., Lawrence D. S.(1994) J. Biol. Chem. 269:4839–4844.

Edelman A. M., Lin W.-H., Osterhout D. J., Bennett M. K., Kennedy M. B., Krebs E. G.(1990) Mol. Cell. Biochem. 97:87–98.

Singh T. J., Akatsuka A., Huang K. P.(1983) FEBS Lett. 159:217–220.

Komatsu S., Hosoya H.(1996) Biochem. Biophys. Res. Commun. 223:741–745.

Ikebe M., Hartshorne D. J., Elzinga M.(1986) J. Biol. Chem. 261:36–39.

Chew T.-L., Masaracchia R. A., Wysolmerski R. B.(1998) J. Muscle Res. Cell Motil. 19:839–854.

Ikebe M., Hartshorne D. J., Elzinga M.(1987) J. Biol. Chem. 262:9569–9573.

Feng J., Ito M., Kureishi Y., Ichikawa K., Amano M., Isaka N., Okawa K., Iwamatsu A., Kaibuchi K., Hartshorne D. J., Nakano T.(1999) J. Biol. Chem. 274:3744–3752.

Murata-Hori M., Suizu F., Iwasaki T., Kikuchi A., Hosoya H.(1999) FEBS Lett. 451:81–84.

Wawrzynow A., Collins J. H., Bogatcheva N. V., Vorotnikov A. V., Gusev N. B.(1991) FEBS Lett. 289:213–216.

Mitsui K., Brady M., Palfrey H. C., Nairn A. C.(1993) J. Biol. Chem. 268:13422–13433.

Redpath N. T., Proud C. G.(1993) Eur. J. Biochem. 212:511–520.

Ridsdale R. A., Beniac D. R., Tompkins T. A., Moscarello M. A., Harauz G.(1997) J. Biol. Chem. 272:4269–4275.

Ramwani J. J., Epand R. M., Moscarello M. A.(1989) Biochemistry 28:6538–6543.

Stone A. L., Park J. Y., Martenson R. E.(1985) Biochemistry 24:6666–6673.

Herskovits T. T.(1966) Biochemistry 5:1018–1026.

Creamer L. K., Richardson T., Parry D. A.(1981) Arch. Biochem. Biophys. 211:689–696.

Hunter T.(1991) Methods Enzymol. 200:3–37.