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

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*Dictyostelium discoideum* 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 (MAXXXX(S/T)XXXAKKK; 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.

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† The abbreviations used are: 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.
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-32P]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 552–541 of MHCK A and has a C-terminal hexahistidine tag. A-CAT is identical to the previously described T5-9 (20). 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 EcoRI site (nucleotides 1391) and an XhoI site in the upstream polylinker of pMM3.1. The XhoI site was blunted by treatment with Klenow polymerase and then ligated into pGEX fusion protein that had been digested with EcoRI and made blunt. Digestion of this intermediate with EcoRI 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 A GT11 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 BamHI 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. Two GST fusion peptide expression vectors were prepared by cloning annealed complementary oligonucleotides encoding the desired peptides into the BamHI/XhoI site of pGEX-4T-3 (Amersham Pharmacia Biotech). The expressed proteins comprised residues 552–841 of MHCK A and have a C-terminal hexahistidine tag. A-CAT is identical to the previously described T5-9 (9). A-CAT was isolated from the 15,000 molecular weight cut-off Ultrafree-4 Centrifugal Filtration Unit, chromatographed on 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 7.5, and, after elution with buffer containing 30 mM glutathione B-CAT was dialyzed against 12.5 mM Tris, pH 7.5, and 4% glycerol and stored at –80 °C. GST-phenotype fusion proteins were expressed at 37 °C in E. coli DH5α and recovered 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, and 40% glycerol and stored at –80 °C. GST-phenotype fusion proteins were expressed at 37 °C in E. coli DH5α and recovered using glutathione-Sepharose beads.

**Phosphoamino Acid Analysis**—Radiolabeled proteins were separated by 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. 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 6.5, 0.2% β-mercaptoethanol, and a trace of bromophenol blue) followed by boiling for 5 min. Assays with GST phenotype 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.

**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 kinase buffer and the phosphorylated proteins 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-32P]ATP. Fractions containing peptides were lyophilized, dissolved in 1 mM NaCl, 50 mM MES, pH 5.5, and applied to a 1-ml column of ferric inorganic acid beads (Pierce) equilibrated in the same buffer. The column was washed with 11 mM ferric ion saturation buffer and 2 ml MES, pH 6.0, and the phosphorylated peptides were eluted with 0.5 M NH4HCO3, 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 phosphorylated 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. The 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, chrom-
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 (black), a novel type of protein kinase catalytic domain (dark gray), 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.

matographed over DE-53 cellulose equilibrated in 30% acetic acid to remove [γ-32P]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 and 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 32P-labeled Ser(P) and Thr(P) were detected and quantified using a storage phosphor screen and a Bio-Rad Personal Molecular Imager FX.

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. 1A). 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 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. 1B).

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 (Table I). 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 32P 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 thinline-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. 3A) and other protein substrates (Table I).

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. 3B).

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 MAXXX(S/T)XXXXXAKKK, 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-Fmoc-blocked serine and threonine, quantitative amino acid analysis of the library yielded values of 0.82 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, 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
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 predominantly 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).

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 6N 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).

**TABLE I**

| Kinase | Substrate | Substrate concentration | Rate $a^{-1} \times 10^9$ | Phosphate incorporated | Fraction Thr(P) |
|--------|-----------|-------------------------|---------------------------|------------------------|-----------------|
| A-CAT  | MH-3      | 200 μM                  | 790                       | 100                    | 100             |
| B-CAT  | MH-3      | 200 μM                  | 550                       | 100                    | 100             |
| C-CAT  | MH-3      | 200 μM                  | 10                        | 100                    | 100             |
| A-CAT  | MBP       | 200 μM                  | 300                       | 3.5                    | 96              |
| B-CAT  | MBP       | 200 μM                  | 830                       | 3.5                    | 91              |
| C-CAT  | MBP       | 200 μM                  | 360                       | 4.8                    | 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                    | 0.5                       | 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 | 0.01/0.01 | 99/98 |
| B-CAT  | Peptide library | 3.3 mg/ml | 0.01 | 98 |
| C-CAT  | Peptide library | 3.3 mg/ml | 0.01/0.01 | 97/99 |
| PAK    | Peptide library | 3.3 mg/ml | 0.01 | 20 |

* The amount of phosphate incorporated was determined using 5 μM MBP.
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 $K_v$ for YAYDTRYRR of 550 $\mu$M and a $k_{cat}$ of 14 s$^{-1}$ (Fig. 6A). With MH-3 as the substrate, A-CAT exhibited a somewhat lower $K_v$ (280 $\mu$M) but an 8-fold lower $k_{cat}$ (1.8 s$^{-1}$) (Fig. 6B). Similar kinetic constants for the phosphorylation of MH-3 by A-CAT have been reported previously (9).

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. 6B). 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. 6C). 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. 6C). 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 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 $k_{cat}$ or to a large decrease in $K_m$. 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 $\beta$-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.
Threonine-specific Protein Kinases

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 high $k_{cat}/K_m$ 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. 6A). Kinetic analysis showed that A-CAT phosphorylated YAYDTRYRR with a specificity constant ($k_{cat}/K_m$) of 0.025 μM$^{-1}$ s$^{-1}$, which is 4-fold higher than the specificity constant for MH-3 (0.006 μM$^{-1}$ 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 (DTY-basic-Y-basic) corresponds exactly to the sequence of the 1833 site (DTKYYK) within the myosin II tail but is less similar to the 2029 site (KTQTKK) and the 1823 site (ATKQTQ) (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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