Dictyostelium Myosin II Heavy-Chain Kinase A Is Activated by Autophosphorylation: Studies with *Dictyostelium* Myosin II and Synthetic Peptides†

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ABSTRACT: One of the major sites phosphorylated on the *Dictyostelium* myosin II heavy chain by the *Dictyostelium* myosin II heavy-chain kinase A (MHCK A) is Thr-2029. Two synthetic peptides based on the sequence of the *Dictyostelium* myosin II heavy chain around Thr-2029 have been synthesized: MH-1 (residues 2020–2035; RKKFGESEKTKTKEFL-amide) and MH-2 (residues 2024–2035). Both peptides are substrates for MHCK A and are phosphorylated to a level of 1 mol of phosphate/mol. Tryptic digests indicate that the peptides are phosphorylated on the threonine corresponding to Thr-2029. When assays are initiated by the addition of MHCK A, the rate of phosphate incorporation into the peptides increases progressively for 4–6 min. The increasing activity of MHCK A over this time period is a result of autophosphorylation. Although each 130-kDa subunit of MHCK A can incorporate up to 10 phosphate molecules, 3 molecules of phosphate per subunit are sufficient to completely activate the kinase. Autophosphorylated MHCK A displays V_{max} values of 2.2 and 0.6 μmol·min^{-1}·mg^{-1} and K_m values of 100 and 1200 μM with peptides MH-1 and MH-2, respectively. Unphosphorylated MHCK A displays a 50-fold lower V_{max} with MH-1 but only a 2-fold greater K_m. In the presence of *Dictyostelium* myosin II, the rate of autophosphorylation of MHCK A is increased 4-fold. If assays are performed at 4 °C (to slow the rate of MHCK A autophosphorylation), autophosphorylation can be shown to increase the activity of MHCK A with myosin II.

Two different types of myosin have been identified in the highly motile lower eukaryote *Dictyostelium discoideum*: a myosin II enzyme similar in structure to conventional muscle myosins and composed of two heavy chains and two pairs of light chains (Clarke & Spudich, 1974; Warrick et al., 1986) and a small, monomeric, single-headed myosin I enzyme (Côté et al., 1985; Jung et al., 1989; Titus et al., 1989). Through the use of antisense RNA (Knecht & Loomis, 1987) and homologous recombination (De Lozanne & Spudich, 1987), *Dictyostelium* cells that lack myosin II have been produced. These cells are defective in cell division and the capping of Con A bound surface receptors and do not develop past the initial stage of aggregation [reviewed in Spudich (1989)]. *Dictyostelium* cells which express a truncated HMM fragment of myosin II (De Lozanne & Spudich, 1987; Fukui et al., 1990) display a similar phenotype to cells completely lacking myosin II (Manstein et al., 1989), demonstrating the importance of the tail region, and filament formation, for the contractile activities of *Dictyostelium* myosin II.

The actin-activated ATPase activity and filament-forming properties of *Dictyostelium* myosin II can be regulated by the phosphorylation of sites within the tail region of the myosin (Kuczmarski & Spudich, 1980; Peltz et al., 1981; Pagh et al., 1984; Côté & McCrea, 1987). Two different *Dictyostelium* myosin II heavy-chain kinases capable of phosphorylating these sites have been purified. One of these, termed myosin II heavy-chain kinase A (MHCK A),[1] is isolated from the cytoplasmic fraction of growth-phase *Dictyostelium* and electrophoreses as a single band of apparent M_r 130 000 on SDS–polyacrylamide gels (Côté & Bukiejko, 1987). On gel filtration columns, MHCK A elutes with an apparent molecular weight greater than 700 000, suggesting that the native kinase consists of multiple identical subunits. The second *Dictyostelium* myosin II heavy-chain kinase, which is isolated from the cytoskeletal fraction of starved, developing cells, electrophoreses as a single band of apparent M_r 85 000 on SDS–polyacrylamide gels and elutes from gel filtration columns with an apparent molecular weight of 250 000 (Ravid & Spudich, 1989).

Phosphorylation of the *Dictyostelium* myosin II tail by either of the myosin II heavy-chain kinases inhibits the actin-activated ATPase of the myosin and favors disassembly of bipolar filaments (Côté & Bukiejko, 1987; Côté & McCrea, 1987; Ravid & Spudich, 1989). In vivo studies indicate that the levels of phosphate on the heavy chains of *Dictyostelium* myosin II change rapidly in response to chemoattractants (Malchow et al., 1981; Berlot et al., 1985, 1987) and that these changes correlate with the translocation of the myosin II from the cytoplasm to the submembranous cell cortex (Yumura & Fukui, 1985; Nachmias et al., 1989). While these studies suggest that the activities of the myosin II heavy-chain kinases are regulated in vivo, in their purified state, both *Dictyostelium* myosin II heavy-chain kinases require only Mg^{2+} and ATP to display high activity. Second messengers or other factors that regulate the activities of these kinases have not been found, although both kinases do autophosphorylate (Côté & Bukiejko,

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[1] Abbreviations: MHCK A, *Dictyostelium* myosin II heavy-chain kinase A; SDS, sodium dodecyl sulfate; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; kDa, kilodalton(s).

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1987; Ravid & Spudich, 1989).

In previous studies, the activites of the *Dictyostelium* myosin II heavy-chain kinases have been assayed by using intact *Dictyostelium* myosin II or fragments of the myosin II tail. These substrates are phosphorylated under ionic conditions where they assemble into insoluble filaments (Côté & Bukiejko, 1987: Ravid & Spudich, 1989; O'Halloran et al., 1990). In this paper, we examine the activity of *Dictyostelium* MHCK A using small, soluble synthetic peptide substrates. The peptides have sequences analogous to the sequence around one of the major phosphorylation sites (Thr-2029) identified in the *Dictyostelium* myosin II heavy chain (Vaillancourt et al., 1988). We show that autophosphorylation of MHCK A greatly increases the rate at which these peptides are phosphorylated and also increases the rate of phosphorylation of *Dictyostelium* myosin II.

Experimental Procedures

[γ-^{32}P]ATP was purchased from New England Nuclear; ATP (grade I) and TES were from Sigma.

Protein Purification. Myosin II and MHCK A were purified from *Dictyostelium discoideum* strain Ax-3 as previsously described (Côté & Bukiejko, 1987). MHCK A fractions pooled from the aminohexyl-Sepharose 4B column (in 10 mM TES, pH 7.0, 300 mM KCl, 20% sucrose, and 1 mM dithiothreitol) were immediately divided into 100-μL aliquots and frozen in liquid nitrogen until use. Newly thawed aliquots of kinase were used for each experiment. Protein concentrations were determined by using the method of Bradford (1976) with bovine serum albumin as the standard. The values determined for purified MHCK A by using the Bradford assay were 20% lower than values determined by using the modification of the Lowry method of Markwell et al. (1978) with bovine serum albumin as standard.

Peptide Synthesis and Purification. Solid-phase synthesis of peptides MH-1 and MH-2 was performed on an Applied Biosystems Model 430 automatic synthesizer by the procedures of Erickson and Merrifield (1976). Peptides were cleaved from the resin by HF, lyophilized, redissolved in 0.1% trifluoroacetic acid, and then purified by high-performance liquid chromatography over a Waters μBondapak C-18 column (0.78 × 30 cm). Peptides were eluted by using a gradient of acetonitrile in 0.1% trifluoroacetic acid and detected on the basis of their absorption at 215 and 260 nm. The purified peptides were divided into aliquots and stored lyophilized. Concentrations of the peptides were determined by amino acid analysis on a Beckman 6300 analyzer.

MHCK A Assays. Assays for MHCK A activity were performed at 25 °C in a buffer consisting of 10 mM TES, pH 7.0, 2 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM [γ-^{32}P]ATP (100–200 cpm/pmol). Peptides (redissolved in 20 mM TES, pH 7.0) and *Dictyostelium* myosin II (in 10 mM TES, pH 7.0, and 1 mM dithiothreitol) were diluted 5–10-fold into this assay buffer and incubated for 1 min at 25 °C prior to addition of kinase. Phosphorylation reactions were initiated by diluting MHCK A (40–60 μg/mL) from 3- to 100-fold into the assay. For some experiments, MHCK A was preincubated at 25 °C in 10 mM TES, pH 7, 1 mM dithiothreitol, and 0.5 mg/mL ovalbumin with or without 2 mM $MgCl_2$ and/or 0.5 mM ATP before being assayed for activity. The presence of ovalbumin at concentrations of up to 0.5 mg/mL in the assay buffer was found not to affect the rates of peptides or myosin II phosphorylation. Incorporation of ^{32}P into the peptides and myosin II was determined by using squares of phosphocellulose paper as described by Roskoski (1983). For most experiments, the assays were terminated by spotting 20–40-μL aliquots of the assay mixture onto the phosphocellulose paper squares and dropping the squares into dilute phosphoric acid. For the experiments in Figure 7, MHCK A activity was stopped immediately by adding aliquots of the assay mixture to an equal volume of 0.5 M KCl, 0.35 M EDTA, and 1.0 mg/mL ovalbumin. Samples were then spotted onto phosphocellulose paper to determine ^{32}P incorporation.

Table I: Synthetic Peptide Substrates

peptide	location in *Dictyostelium* myosin II heavy chain	sequence[a]
MH-1	2020–2035	RKKFGESEKTKTKEFL-amide
MH-2	2024–2035	GESEKTKTKEFL-amide

[a] The underlined residue represents Thr-2029, the site phosphorylated by MHCK A in the *Dictyostelium* myosin II heavy chain.

Determination of the Peptide Phosphorylation Site. After incubation with MHCK A as described under MHCK A Assays, peptides MH-1 and MH-2 were loaded onto a high-performance liquid chromatography Waters μBondapack C-18 column. Elution of the column with an acetonitrile gradient in 0.1% trifluoroacetic acid separated the unphosphorylated and phosphorylated forms of the peptides. The peaks representing the ^{32}P-labeled peptides were pooled, lyophilized, and digested with 0.1 mg/mL trypsin in 50 mM ammonium bicarbonate. Digests were stopped by addition of 1 mM phenylmethanesulfonyl fluoride, lyophilized, and electrophoresed on thin-layer cellulose plates at pH 1.9 as described previously (Vaillancourt et al., 1988). Autoradiographs were prepared by exposing the sheets to Kodak X-Omat AR-2 X-ray film. To quantitate the amount of ^{32}P in each peptide, the area corresponding to the peptides was scraped from the plate and counted in scintillation fluid. For amino acid analyses, tryptic peptides were purified by high-performance liquid chromatography over a Waters μBondapack C-18 column and an LKB Ultropac TSK DEAE column (Vaillancourt et al., 1988).

Results

Phosphorylation of the Synthetic Peptides. Thr-2029 has been identified as a major site of phosphorylation by MHCK A within the *Dictyostelium* myosin II heavy chain (Vaillancourt et al., 1988). Two peptide analogues of the sequence around Thr-2029 in the *Dictyostelium* myosin II heavy chain (Warrick et al., 1986) were synthesized (Table I). Peptide MH-1 contains four additional amino-terminal residues not present in MH-2. Both peptides are substrates for MHCK A, and a maximum of 1 mol of phosphate/mol can be incorporated into each peptide.

When phosphate incorporation into the peptides is initiated by the addition of MHCK A, the rate of phosphorylation of the peptides increases progressively over the first few minutes of the assay (Figure 1). Maximum rates of phosphate incorporation are reached after 4–6 min and can then be maintained for periods of greater than an hour. The upward curvature of the time courses is observed with both peptides and at all peptide concentrations. Representative time courses obtained by using concentrations of MH-1 well above (320 μM) and well below (5 μM) its K_m for MHCK A are shown in Figure 1. Similar results have been obtained with all preparations of MHCK A tested.

The upward curvature of the time courses indicates that the activity of MHCK A is increasing during the initial stages of the assays. To test whether the increase in MHCK A activity is a result of autophosphorylation, MHCK A was preincubated in buffers containing Mg^{2+} and ATP, ATP alone, or Mg^{2+} alone, prior to being assayed for activity with the peptide

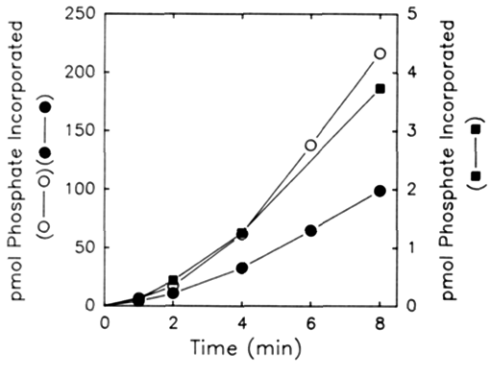

FIGURE 1: Initial rate of phosphate incorporation into the synthetic peptides. Peptide MH-1 was assayed at concentrations of 320 μM (O) and 5 μM (■) and peptide MH-2 at a concentration of 2.6 mM (●). ^{32}P incorporation into (O) and (●) is given on the left axis and into (■) on the right axis. ^{32}P incorporation was initiated in all cases by the addition of a final concentration of 1.8 μg/mL MHCK A to the assays. Assays were performed as described under Experimental Procedures with aliquots of 20 μL being removed at the indicated times to determine the incorporation of ^{32}P into the peptides.

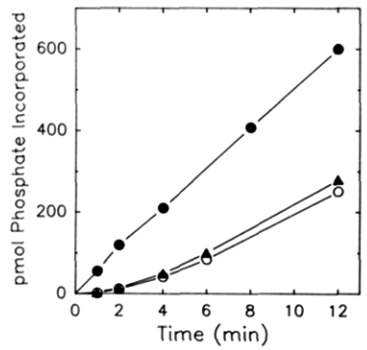

FIGURE 2: Phosphorylation of MH-1 by MHCK A preincubated in the presence and absence of Mg^{2+} and ATP. MHCK A (60 μg/mL) was diluted 3-fold into preincubation buffer containing either Mg^{2+} and ATP (●), ATP (O), or Mg^{2+} (▲) and preincubated for 20 min at 25 °C as described under Experimental Procedures. Phosphorylation of peptide MH-1 (0.32 mM) was initiated by diluting the preincubated kinase into the assay to give a final kinase concentration of 2 μg/mL. Aliquots of 20 μL were taken at the times indicated to determine ^{32}P incorporation into MH-1.

substrates. Following preincubation in the presence of Mg^{2+}ATP (conditions under which autophosphorylation occurs), MHCK A displayed a high initial activity in the assays, and linear rates of phosphate incorporation into the peptides were obtained (Figure 2). In contrast, preincubation of MHCK A in buffers which do not permit autophosphorylation (buffers lacking either ATP or Mg^{2+}) did not activate the kinase or shorten the extent of the initial lag phase (Figure 2). Similar results were obtained when MHCK A was assayed using peptide MH-2 or low concentrations (5 μM) of peptide MH-1 (data not shown).

Kinetic Parameters for the Synthetic Peptides. The kinetic parameters for the peptides were determined initially by using autophosphorylated MHCK A. Phosphate incorporation was linear over the first few minutes at all peptide concentrations, and the phosphorylation of the peptides obeyed Michaelis–Menten kinetics. The K_m and V_{max} values determined (Table II) demonstrate that MH-1 is a considerably better substrate for MHCK A than is MH-2. At attempt was also made to determine K_m and V_{max} values for MH-1 using the less active unphosphorylated form of MHCK A. To do this, phosphate incorporation into MH-1 was measured 30 and 60 s after the addition of MHCK A (Table II). Although the activity of MHCK A starts to increase as soon as the kinase is introduced into the Mg^{2+}ATP-containing assay, at 30–60 s the kinase is still relatively inactive (Figure 1). The short incubation times and low initial activity of MHCK A resulted in low levels of ^{32}P incorporation into MH-1, and thus somewhat inaccurate values of K_m and V_{max}. It is clear, though, that activation of MHCK A results in a large (approximtely, 50-fold) increase in V_{max} accompanied by only a small decrease in K_m (Table II).

Table II: Kinetic Constants for the Synthetic Peptide Substrates

peptide	MHCK A[a]	K_m(app)[b] (μM)	V_{max}[b] (μmol·min^{-1}·mg^{-1})
MH-1	autophosphorylated	105 ± 10	2.24 ± 0.05
MH-2	autophosphorylated	1160 ± 60	0.62 ± 0.02
MH-1	unphosphorylated (0–30 s)	195 ± 50	0.04 ± 0.01
MH-1	unphosphorylated (0–60 s)	230 ± 75	0.12 ± 0.02
MH-3	autophosphorylated	101 ± 15	2.20 ± 0.05

[a] Autophosphorylated MHCK A was preincubated in the presence of Mg^{2+}ATP as descrubed under Experimental Procedures before being assayed. Unphosphorylated MHCK A was not preincubated. Assays involving the unphosphorylated MHCK A were initiated by the addition of kinase (0 time) and carried out for the time indicated. [b] Kinetic constants (±SE) were estimated by fitting the data to the Hanes–Woolf plot by linear regression analysis. Final KCl and sucrose concentrations in the assays were 12 mM and 0.8%, respectively.

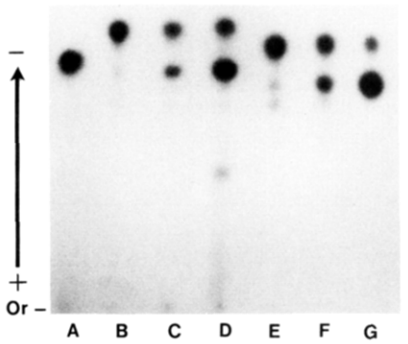

FIGURE 3: Autoradiogram of tryptic digest of ^{32}P-labeled MH-1 and MH-2 analyzed by electrophoresis at pH 1.9. Peptides MH-1 and MH-2 were phosphorylated and digested with trypsin as described under Experimental Procedures. Samples taken from the digest of MH-2 at (A) 0 time, (B) 10 min, (C) 3 h, and (D) 48 h and from the digest of MH-1 at (E) 10 min, (F) 3 h, and (G) 48 h were analyzed by electrophoresis for 45 min at 500 V in acetic acid/formic acid/water (8:2:90, pH 1.9) on cellulose-coated thin-layer sheets (20 × 20 cm, Eastman) using a Camag thin-layer electrophoresis cell. The peptides were spotted at the "Or" and electrophresed toward the cathode (–). Autoradiography was performed at –70 °C using a Du Pont Cronex Lightning Plus intensifying screen and Kodak X-Omat AR-2 X-ray film.

Identification of the Phosphorylation Site on the Synthetic Peptides. Both MH-1 and MH-2 contain three potential sites of phosphorylation: two threonines and one serine. Phosphoamino acid analysis indicates that MHCK A phosphorylates both peptides only on threonine (data not shown). To identify the phosphorylated threonine, MH-1 and MH-2 were phosphorylated by MHCK A using [γ-^{32}P]ATP and digested with trypsin. MH-1 and MH-2 are cleaved within a few minutes to yield one major phosphorylated peptide, which then is converted, over a period of days, to a phosphorylated peptide with a slightly lower mobility (Figure 3). Amino acid analyses of the purified ^{32}P-labeled tryptic peptides derived from MH-1 indicate that the initial phosphorylated peptide produced has the sequence F-G-E-S-E-K-T-K-T-K and the peptide with lower mobility the sequence F-G-E-S-E-K-T-K. This agrees with previous results showing that the same two peptides containing Thr-2029 are obtained from tryptic digests of *Dictyostelium* myosin II phosphorylated by MHCK A (Vaillancourt et al., 1988). Similarly, amino acid analyses

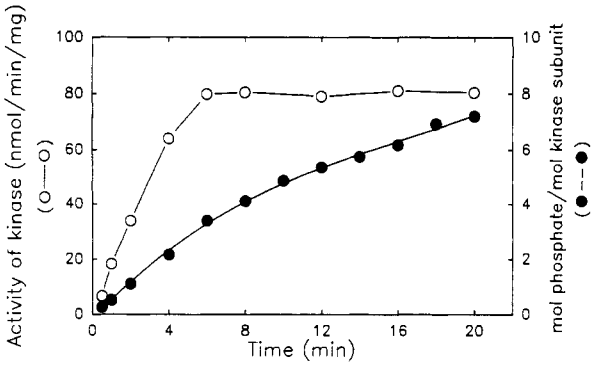

FIGURE 4: Correlation betwen the amount of phosphate incorporated into MHCK A and the activity of the kinase toward peptide MH-1. Phosphate incorporation into MHCK A (final concentration in the assay of 20 μg/mL) (●) was performed in a buffer containing 10 mM TES, pH 7.0, 0.3 mg/mL bovine serum albumin, 100 mM KCl, 7% sucrose, 1 mM dithiothreitol, 0.9 mM $MgCl_2$, and 0.23 mM ATP (940 cpm/pmol). At the indicated times, aliquots of 10 μL were removed, and the amount of ^{32}P incorporated per mole of the 130-kDa MHCK A band was determined by running the kinase on an SDS–polyacrylamide gel and excising and counting the kinase band in scintillation fluid. The activity of MHCK A with MH-1 (O) was determined under identical conditions except that the assay contained 0.16 mM MH-1 and the specific activity of the ATP was 180 cpm/pmol. Aliquots of 10 μL were withdrawn at intervals, and the amount of ^{32}P incorporated into the peptide was determined by the phosphocellulose filter paper assay (the amount of phosphate incorporated into the kinase is negligible compared to the amount incorporated into the peptide). The activity of MHCK A over each time interval was then calculated.

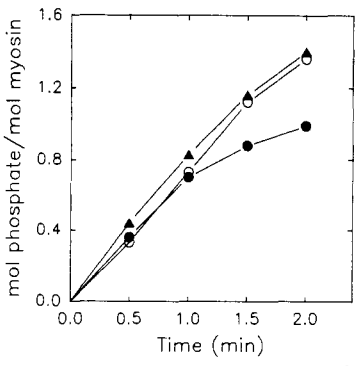

FIGURE 5: Phosphorylation of myosin II at 25 °C by MHCK A. MHCK A was assayed without preincubation (O) or following preincubation in the presence of Mg^{2+} and ATP (●) or Mg^{2+} (▲) as described under Experimental Procedures. Incorporation of ^{32}P into *Dictyostelium* myosin II (0.3 mg/mL) was initiated by the addition of MHCK A to the assays to give a final kinase concentration of 0.6 μg/mL and was measured at the times indicated by spotting 45-μL samples onto phosphocellulose filter papers.

indicated that the initial and final ^{32}P-labeled peptides derived from MH-2 corresponded to the sequences G-E-S-E-K-T-K-T-K and G-E-S-E-K-T-K.

The shorter tryptic peptide derived from both MH-1 and MH-2 contains only the threonine corresponding to Thr-2029. Quantification of the amount of ^{32}P present in the tryptic peptides demonstrates that after a 2-day digest of MH-1 (Figure 3, lane G) 90% of the ^{32}P is in the shorter peptide. Therefore, at least 90% of the ^{32}P initially incorporated into MH-1 must be present in the threonine corresponding to Thr-2029. The remaining ^{32}P may be on either Thr-2029 or Thr-2031. Similarly, at least 60% of the ^{32}P incorporated into MH-2 is present on Thr-2029 (Figure 3, lane D).

To confirm that the threonine corresponding to Thr-2029 is the major site of phosphorylation, a third peptide, termed MH-3, identical with MH-1 except for the replacement of the residues corresponding to Ser-2026 and Thr-2031 with alanines, was synthesized. Phosphorylation of MH-3 with MHCK A showed a lag phase, and yielded K_m and V_{max} values virtually identical with those obtained for MH-1 (Table II).

Correlation between Autophosphorylation and Activity of MHCK A. A time course of the autophosphorylation of MHCK A is shown in Figure 4. By 40 min, phosphate incorporation into MHCK A levels off at 10 mol of phosphate per 130-kDa MHCK A subunit. Preliminary experiments indicate that the rate of autophosphorylation of MHCK A is largely independent of the concentration of the kinase, suggesting that autophosphorylation occurs mainly through an intramolecular mechanism.

In parallel assay carried out under identical conditions (except for the presence of 160 μM MH-1), the activity of MHCK A with the peptide substrate was measured (Figure 4). The presence of MH-1 does not significantly alter the rate of autophosphorylation of MHCK A (see Figure 6). By comparing the curve showing MHCK A activity with that indicating the amount of phosphate incorporated into the kinase, it can be seen that increases in MHCK A activity coincide with the incorporation of three phosphate molecules into each 130-kDa MHCK A subunit. Incorporation of additional phosphate, up to 10 mol/mol of 130-kDa subunit, has no effect on MHCK A activity. It should be noted that the assays in Figure 4 were performed with a higher than usual amount of MHCK A, resulting in a final KCl concentration in the assays of 100 mM. This high concentration of KCl is responsible for the low maximal MHCK A activity (Côté & Bukiejko, 1987).

Phosphorylation of Dictyostelium Myosin II. In contrast to the results obtained with the synthetic peptides, no lag phase in phosphate incorporation was observed when MHCK A was assayed with *Dictyostelium* myosin II under the standard assay conditions (low salt, 25 °C) (Figure 5). Samples of MHCK A that were preincubated with Mg^{2+} or with Mg^{2+} and ATP, or not preincubated, all yielded similar time courses. Comparable results were obtained with myosin II concentrations ranging from 0.1 to 1.5 mg/mL. While it is difficult to obtain accurate kinetic constants with myosin II as a substrate (it is filamentous under the assay conditions), we estimate that the K_m of MHCK A for *Dictyostelium* myosin II filaments is close to 1 μM (0.55 mg/mL). At this myosin II concentration, the specific activities of both the unphosphorylated and autophosphorylated forms of MHCK A, measured over the first minute of the assay, are near 1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

These results are surprising, since they imply that autophosphorylation of MHCK A is required only for activity with the peptide substrates. However, it is also possible that the interaction with *Dictyostelium* myosin II alters the autophosphorylation of MHCK A. Indeed, as shown in Figure 6A,C, the initial rate of autophosphorylation of MHCK A increases significantly in the presence of *Dictyostelium* myosin II. Experiments with three separate preparations of *Dictyostelium* myosin II and MHCK A have consistently yielded a 3–4-fold increase in the rate of MHCK A autophosphorylation when myosin II is added. In contrast, MH-1 was found to have little effect on the rate of MHCK A autophosphorylation (Figure 6B,D).

The increased autophosphorylation of MHCK A in the presence of *Dictyostelium* myosin II does not result from contamination of the myosin II preparations with MHCK A, since no phosphorylation of the myosin II occurs in the absence of added MHCK A. The myosin II preparations used in these experiments were also examined to ensure that they were free of degradation products with molecular weights around 130000. Furthermore, when samples of myosin II were

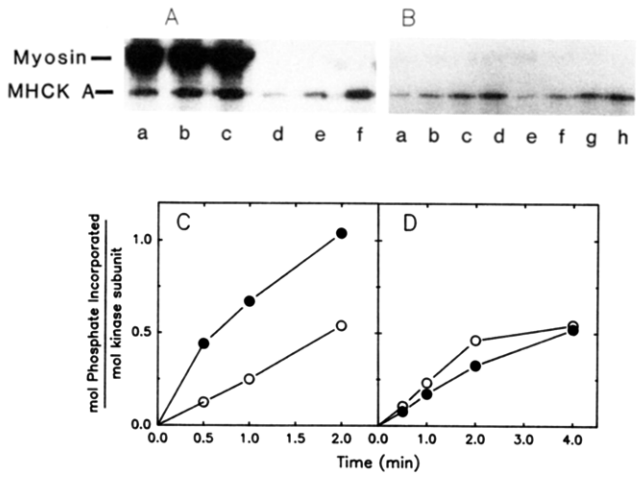

FIGURE 6: Effect of myosin II and peptide MH-1 on the rate of phosphorylation of MHCK A. (A) Incorporation of ^{32}P into MHCK A (4 μg/mL) was performed under the conditions described under Experimental Procedures in the presence (lanes a–c) or absence (lanes d–f) of 0.1 mg/mL *Dictyostelium* myosin II (in 10 mM TES, 2 mM $MgCl_2$, and 1 mM dithiothreitol). Phosphate incorporation into the kinase (and into myosin) was initiated by the addition of 30 μM ATP (2.3×10^4 cpm/pmol). At 0.5 (a, d), 1 (b, e), and 2 min (c, f), 20-μL aliquots were removed, boiled in SDS sample buffer, and electrophoresed on an SDS–polyacrylamide gel. An autoradiogram of the gel is shown. (C) Bands representing MHCK A were cut from the gel shown in (A) and counted in scintillation fluid, and the amount of ^{32}P incorporated at each time point in the presence (●) and absence (○) of myosin II is plotted. (B) Phosphorylation of MHCK A was performed as described in (A) in the presence (lanes a–d) or absence (lanes e–g) of 0.32 mM peptide MH-1. Samples of 20 μL were taken 0.5 (a, e), 1 (b, f), 2 (c, g), or 4 min (d, h) after addition of ATP, electrophoresed on an SDS–polyacrylamide gel, and autoradiographed. (D) Bands representing MHCK A were cut from the gel shown in (B) and counted in scintillation fluid, and the amount of ^{32}P incorporated at each time point in the presence (●) and absence (○) of peptide is plotted.

phosphorylated using MHCK A previously autophosphorylated with cold ATP, no ^{32}P-labeled band in the region of 130 kDa was observed.

These results suggest that in the presence of *Dictyostelium* myosin II, autophosphorylation, and hence activation, of MHCK A might occur too rapidly for a lag phase in the time course of myosin II phosphorylation to be observed. To decrease the rate of MHCK A autophosphorylation, phosphate incorporation into *Dictyostelium* myosin II was measured at 4 °C. Under these conditions, a definite lag phase was observed when unphosphorylated MHCK A was assayed, but no lag phase was apparent when autophosphorylated MHCK A was assayed (Figure 7). The activity over the first 15 s of the assay was 4-fold greater for the autophosphorylated than for the unphosphorylated MHCK A.

Discussion

The major sites phosphorylated by MHCK A on the *Dictyostelium* myosin II heavy chain, threonines-1833 and -2029, are both located within the carboxyl-terminal one-quarter of the myosin II tail (Vaillancourt et al., 1988). Phosphorylation of these sites inhibits the actin-activated ATPase activity of myosin II and its ability to assemble into filaments (Côté & Bukiejko, 1987; Côté & McCrea, 1987) so that factors which regulate the activity of MHCK A may play an important role in determining whether *Dictyostelium* myosin II participates in contractile events in vivo. Initial studies, using intact *Dictyostelium* myosin II as the phosphate-accepting substrate, showed that the activity of MHCK A was unaffected by cAMP, cGMP, and Ca^{2+} and calmodulin and indicated that the kinase required only Mg^{2+} and ATP

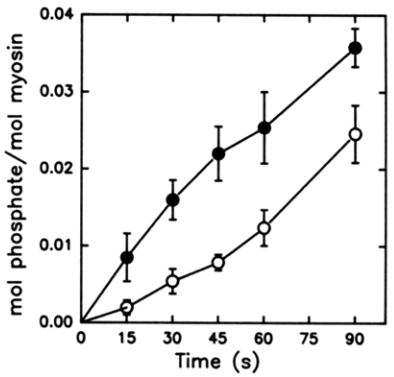

FIGURE 7: Phosphorylation of myosin II at 4 °C by MHCK A preincubated in the presence and absence of Mg^{2+}ATP. MHCK A was preincubated in the presence (●) and absence (○) of Mg^{2+}ATP as described under Experimental Procedures and then cooled to 4 °C. Incorporation of ^{32}P into *Dictyostelium* myosin II (0.75 mg/mL) at 4 °C was initiated by the addition of MHCK A to the assays to give a final kinase concentration of 2 μg/mL. Samples were taken at the times indicated, the assay was stopped as described under Experimental Procedures, and phosphate incorporation was determined by the phosphocellulose filter papers assay. Results shown are the mean and standard deviation for four separate experiments.

for activity (Côté & Bukiejko, 1987).

While *Dictyostelium* myosin II is a good substrate for MHCK A, several factors combine to make the characterization of MHCK A activity with *Dictyostelium* myosin II problematic. Myosin II assembles at low ionic strength into insoluble filaments, has a high molecular weight, and can be assayed at concentrations no greater than a few micromolar. In addition, the ability of myosin II to take up different monomeric or filamentous conformations depending on its state of phosphorylation and the presence or absence of effector molecules (i.e., ATP, Mg^{2+}, KCl) (Kuczmarski et al., 1987; Pasternak et al., 1989) introduces the possibility of substrate-directed effects on the rate of phosphorylation. So far, attempts to find other protein substrates for MHCK A have not been successful. We show here, however, the soluble synthetic peptides based on the sequence around Thr-2029 in the *Dictyostelium* myosin II heavy chain can be used as substrates for MHCK A.

When the synthetic peptides substrates are used clear evidence is obtained for an increase in MHCK A activity over the initial few minutes of the assay (Figure 1, Table II). This increase in activity seems to result from the progressive autophosphorylation of MHCK A during the assays. Preincubation of MHCK A under conditions allowing autophosphorylation (in the presence of Mg^{2+}ATP) also increases kinase activity, while no effect on MHCK A activity is observed by preincubation under conditions that do not allow autophosphorylation (Figure 2). Autophosphorylation increases the V_{max} of MHCK A about 50-fold when measured with peptide MH-1 as substrate (Table II).

It is much more difficult to show a lag phase in the rate of phosphate incorporation using *Dictyostelium* myosin II as the substrate. However, when experiments are performed at low temperatures to decrease the rate of autophosphorylation of MHCK A, a lag phase lasting about 1 min can be observed. This lag phase is eliminated if MHCK A is autophosphorylated prior to its addition to the assay (Figure 7). The rapid activation of MHCK A in assays containing *Dictyostelium* myosin II may be a consequence of the increased rate of autophosphorylation of MHCK A that occurs in the presence of myosin II (Figure 6A,C). It is also possible that the order in which sites are autophosphorylated on MHCK A differs in the presence and absence of myosin II. Not all of the

phosphate incorporated into MHCK A has an effect on kinase activity (Figure 4), and those sites leading to activation of the kinase may be preferentially phosphorylated in the presence of myosin II. Peptide maps of MHCK A phosphorylated in the presence and absence of myosin II should provide further information on this question, and on the number and identity of the phosphorylation sites involved in the activation of MHCK A.

Interestingly, another kinase involved in the regulation of cell motility, the *Acanthamoeba* myosin I heavy-chain kinase, has recently been shown to be activated by the incorporation of high amounts of phosphate (7.5 mol of phosphate/mol of enzyme) possibly by an autophosphorylation reaction (Brzeska et al., 1990). The phosphorylation of the *Acanthamoeba* myosin I heavy-chain kinase is stimulated 20-fold by the presence of phosphatidylserine. It will be important to identify factors, in addition to *Dictyostelium* myosin II, that act to regulate the rate of autophosphorylation of MHCK A. Although purified MHCK A rapidly autophosphorylates upon addition of Mg^{2+}ATP, the kinase is isolated from *Dictyostelium* in an unphosphorylated state. This suggest that MHCK A autophosphorylation may be inhibited in vivo, or rapidly reversed by *Dictyostelium* protein phosphatases.

The peptide substrates examined in this paper contain two threonine residues (corresponding to Thr-2029 and -2031). Both of these threonines are flanked by lysines, yet MHCK A specifically phosphorylates the threonine corresponding to Thr-2029, the same residue phosphorylated in myosin II (Figure 4). The group of three basic residues starting seven amino acids to the amino-terminal side of Thr-2029 seems to play an important role in the recognition of substrates by MHCK A, since peptide MH-1 has a 10-fold lower K_m and 4-fold higher V_{max} than MH-2. However, Thr-2029 is preferentially phosphorylated even in MH-2, indicating that other amino acids, which will require the preparation of further synthetic peptides to identify, must be involved in directing MHCK A to the correct threonine residue.

The experiments in this paper also demonstrate that MHCK A can phosphorylate substrates that do not have an α-helical coiled-coil structure. This does not exclude the possibility that the binding site for MHCK A on the *Dictyostelium* myosin II tail involves residue from both chains of the α-helical coiled-coil structure (Vaillaincourt et al., 1988), since the K_m of MHCK A for *Dictyostelium* myosin II is nearly 100-fold lower than its K_m for the best synthetic peptide tested. Studies on the *Dictyostelium* myosin II heavy-chain kinase isolated by Ravid and Spudich (1989) indicate that this kinase efficiently phosphorylates only fragments of the *Dictyostelium* myosin II tail that are in a filamentous state (O'Halloran et al., 1990). In contrast, MHCK A readily incorporates phosphate into a soluble tail fragment of *Dictyostelium* myosin II (Côté & McCrea, 1988). Such differences in substrate specificity may reflect different functions for the two myosin II heavy-chain kinases in *Dictyostelium*.

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