Detection of a Calcium-Activated Protein Kinase in Mougeotia by Using Synthetic Peptide Substrates

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ABSTRACT

By using a synthetic peptide, KM-14, a protein kinase was detected and partially purified from Mougeotia sp. The peptide contains the sequence of the regulatory light chain of smooth muscle myosin that is phosphorylated by calcium-calmodulin-dependent myosin light chain kinase (MLCK). The Mougeotia kinase was stimulated 40-fold by calcium with half-maximal stimulation occurring at 1.5 micromolar. The enzyme was fractionated from calmodulin and was depleted of calmodulin based on enzyme activation analysis. The calmodulin-depleted enzyme was fully active and calcium dependent, and was not stimulated further by exogenous calmodulin nor by the calcium effectors phosphatidylinerine and diacylglycerol. The enzyme phosphorylated intact chicken gizzard myosin light chain as well as the KM-14 substrate. KM-13, a peptide analog of KM-14 with a deletion of a glutamine at position 5, was a poor substrate with a Vₘₐₓ/Kₘ ratio 200-fold lower than KM-14. Thus, similarly to vertebrate MLCK, the Mougeotia enzyme is very sensitive to changes in sequence surrounding the phosphorylation site. Calcium-dependent KM-14 kinase activity was also detected in other algae, Mesotaenium caldarium and Spirogyra sp., as well as in pea seedlings. The data suggest that plant and algal tissues possess an enzyme with a substrate specificity similar to MLCK, but unlike MLCK, does not appear to require calmodulin for activity.

The regulation of a number of motility responses in plant and algal tissues appears to be mediated by calcium signal transduction (for reviews see refs. 10 and 24). Among the well-studied biological model systems of calcium regulation of organellar motility is light-dependent chloroplast movement in Mougeotia. Mougeotia has a single, ribbon-shaped chloroplast that is rotated about the cell axis in response to changes in light direction and intensity (9). Upon administering a low-intensity, directional light stimulus, the chloroplast is reoriented so that it faces the stimulus. Based on the action spectrum of the response, it has been proposed that phytochrome is the primary photoreceptor under conditions of low light fluence (9).

Numerous studies have implicated a role for calcium in mediating the effects of light on chloroplast movement in Mougeotia. Transfer of Mougeotia to calcium-deficient media resulted in a decrease in the levels of intracellular calcium stores (35) that parallels a loss in the ability to rotate the chloroplast in response to changes in light direction (34). Transfer of these calcium-deficient cells back to a medium containing a normal calcium concentration results in the recovery of light-dependent chloroplast movement (34). In subsequent studies Dreyer and Weisenseel (6) found that irradiation of Mougeotia with red light stimulates an uptake of exogenous ⁴²Ca ions from the external media. This effect is antagonized by far red light. Based on these results it was suggested that stimulation of phytochrome causes an increase in the concentration of intracellular calcium. Serlin and Roux (30) showed that the application of the calcium ionophore A23187 to Mougeotia cells resulted in a rotation of the chloroplast in the absence of a light stimulus. Further, the ionophore was only effective if applied to the cell surface on the sides of the cell that are perpendicular to the wide face of the chloroplast, and only if calcium was present in the media.

Early inhibitor studies have implicated the involvement of the actomyosin cytoskeleton in chloroplast movement. For example cytochalasin B reversibly inhibits the reorientation of the chloroplast (33). Additionally, ultrastructural studies indicate the presence of filaments that are the approximate size of actin microfilaments, and it appeared as if these structures linked the chloroplast envelope to the cell membrane (34). These structures can be decorated with heavy meromyosin fragments in a manner similar to actin filaments (17).

Based on the observations discussed above, it has been suggested that light activation of phytochrome leads to a localized increase in intracellular calcium that activates the actomyosin cytoskeleton that drives chloroplast movement (9). Although this proposal is attractive, detailed biochemical analyses of the targets of calcium regulation and their modulation of the actomyosin cytoskeleton are lacking. Presently, calmodulin is the only calcium-modulated protein that has been detected and isolated from Mougeotia (12, 36). Due to the ubiquitous distribution of calmodulin in eukaryotes and the numerous activities that appear to be regulated by calmodulin in vitro (28), its finding in Mougeotia is not unexpected. More uncertain, however, is the role that calmodulin, or other calcium-receptor proteins, might play in calcium-dependent regulation in this organism.

In some systems, for example in vertebrate smooth muscle and nonmuscle cells (29), the phosphorylation of R-MLC² by

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1 Abbreviations: R-MLC, regulatory myosin light chain from chicken gizzard; Chaps, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate; KM-14, synthetic peptide based on the MLCK phosphorylation site of the regulatory light chain of chicken gizzard; KM-13, synthetic peptide that is identical to KM-14 with the exception of a deletion of glutamine at position 5; MLCK, myosin light chain kinase; PS/DAG, phosphatidylinerine and diacylglycerol; TPCK, 1-chloro-3-[4-tosylalanyl]-4-phenyl-2-butanone; YA-10, synthetic peptide substrate of cAMP-dependent protein kinase.

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calcium and MLCK is associated with the onset of contractile activity. In the present study, we have used a synthetic peptide substrate with the phosphorylation sequence of smooth muscle regulatory myosin light chains for the detection and partial purification of a calcium-dependent, calmodulin-independent protein kinase from Mougeotia.

MATERIALS AND METHODS

Algal and Plant Growth

Mougeotia sp., Spirogyra sp., and Mesotaenium caldariorum cultures were obtained from the culture collection at the University of Texas, Austin. Large scale liquid cultures were grown in continuous light with aeration in Woods Hole media (21) except that Hapes was substituted for Tris in the media formula. Filamentous algae were harvested by vacuum filtration on a sintered glass funnel and unicellular algae were collected by centrifugation. All cells were frozen in liquid nitrogen and were stored at -80°C. Pea plants were grown for 2 weeks in a greenhouse prior to harvesting for extraction and analysis.

Materials

Calmodulin was purified from bovine brain (5) as previously described. Regulatory myosin light chains were purified from chicken gizzards according to Roberts et al. (26). Calmodulin-dependent myosin light chain kinase was purified from chicken gizzards by the method of Adelstein and Klei (1). Synthetic peptides were prepared on a Biosearch SAMII automated solid phase peptide synthesizer by the Analytical Service Facility at the University of Tennessee, Knoxville. Peptides were purified by molecular exclusion chromatography on a Biogel P-2 resin or by reverse phase HPLC on a Vydac C-18 column. Peptide concentrations were determined by amino acid composition analysis on a Waters picotag instrument by using precolumn derivatization with phenylisothiocyanate. γ-32P-ATP was prepared by using a commercial gamma prep synthesis system (Promega Corp.). Mixed histone (Histone II-A), dephosphorylated casein, phosphoserine, and phosphoethyline were purchased from Sigma Chemical Co. Phospholipidase and 1,2-dioleyl glycerol were purchased from Avanti Lipids. All other reagents were of reagent quality or higher.

Analytical Methods

Protein kinase assays were performed by using the general procedure described by Roberts et al. (26). Briefly, incubations were done at 30°C in a 50 μL volume containing 25 mM Mops NaOH (pH 7.0), 7.5 mM 2-mercaptoethanol, 10 mM magnesium acetate, either 0.5 mM CaCl2 or 1 mM EGTA, 1 mM γ-32P-ATP (90 μCi per μmol ATP), protein or peptide substrates, and enzyme. The incorporation of 32P into peptide was determined by the phosphocellulose filter paper method as previously described (26).

For calcium dependence curves, the reaction mixture contained 50 mM Hepes NaOH (pH 7.2), 1 mM γ-32P-ATP, and various amounts of MgCl2, CaCl2, EDTA, peptide substrates, and enzyme. A computer program (23) and the stability constants of Martell and Smith (20) were used to determine the amounts of MgCl2, CaCl2, and EDTA required to yield a Mg2+ concentration of 4 mM and a free Ca2+ concentration that varied from 10^-4 to 10^-9 M. All buffers and substrates were treated with Chelex resin to remove metal ions. The metal ion content of standard solutions were determined by atomic absorption spectroscopy by using an Instrumentation Laboratories model 551 atomic absorption spectrometer.

The calmodulin content of various samples was determined by enzyme activator analysis as previously described (26). Samples to be analyzed were heated at 80°C for 5 min. This treatment destroys endogenous protein kinase activity but does not affect calmodulin activity. The heat-treated samples were assayed for their ability to activate calmodulin-depleted chicken gizzard myosin light chain kinase in a calcium-dependent manner. Protein analyses were done by the method of Bradford (4). The dependence of the enzyme upon exogenous calmodulin (26) and phosphatidylethanolamine and diacylglycerol (15) was determined by using previously described procedures.

To analyze for phosphoamino acid content, KM-14 peptide (40 μg) was labeled at 30°C for 45 min in a reaction mixture (150 μL) consisting of purified KM-14 kinase (15 μL of an enzyme preparation with an activity of 20 nmol 32P incorporated/min/mL), 0.4 mM γ-32P-ATP (364 μCi per μmol), 0.5 mM CaCl2, 7.5 mM 2-mercaptoethanol, 10 mM magnesium acetate, and 25 mM Mops NaOH (pH 7.0). The mixture was applied to a Waters C18 Sep-Pak cartridge and was washed with 18 mM ammonium acetate, 10% (v/v) acetonitrile (pH 6.0) to remove the unincorporated ATP. The peptide was eluted with 12 mM ammonium acetate, 40% (v/v) acetonitrile (pH 6.0). Acid hydrolysates of the peptide were done in 6 N HCl in vacuo at 105°C for 2 h (14). The hydrolyzed samples were dried and analyzed for phosphothreonine and phosphoserine by electrophoresis (900 V, 2 h) on cellulose TLC plates in 7.8% acetic acid, 2.5% formic acid (pH 2.0). Included as internal standards were 0.4 μg of phosphoserine and phosphothreonine. Unlabeled amino acids were visualized with ninhydrin and radiolabeled amino acids were detected by autoradiography.

Extraction and Purification of the Calcium-Dependent Protein Kinase

Buffer compositions are: buffer A, 50 mM Tris HCl (pH 7.5), 1 mM NaCl, 5 mM 2-mercaptoethanol, 50 μg/mL PMSF, 50 μg/mL TPCK; buffer B, 50 mM Tris HCl (pH 7.5), 3 mM MgCl2, 1 mM EGTA, 5 μg/mL leupeptin, 3 μg/mL pepstatin, 90 μg/mL TPCK, 90 μg/mL PMSF, 1 μg/mL soybean trypsin inhibitor, 10 mM sodium isosaccharate, 2% (w/v) Chaps, 0.5% (w/v) polyvinylpolypyrrolidone; buffer C, 20 mM Mops NaOH (pH 7.0), 0.1 mM NaCl, 1 mM MgCl2, 0.5 mM EGTA, 5 mM 2-mercaptoethanol, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, 50 μg/mL TPCK, 50 μg/mL PMSF, 1 μg/mL soybean trypsin inhibitor; buffer D, 20 mM Mops NaOH (pH 7.0), 0.1 mM NaCl, 1 mM sodium isosaccharate, 0.5 mM EGTA, 5 mM 2-mercaptoethanol, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, 50 μg/mL TPCK, 50 μg/mL PMSF, 0.075% (w/v) Chaps; buffer E, 20 mM Mops NaOH (pH 7.0). 0.5 mM EGTA,
The concentrated homogenate (200 mg of cells) was sonicated (Artek, model 301 probe sonifier) for 10 s with a large probe on a setting of 5, with a duty cycle of 40%. The mixture was sonicated again (10 s) after a 60 s rest. The extract was centrifuged at 17,000 rpm in a Sorvall SS-34 rotor at 4°C for 10 min. The supernatant was removed and saved. The pellet was resuspended in a fresh portion of buffer B and was sonicated and centrifuged as described above. The supernatants were combined and applied to a column of DEAE cellulose DE-52 (2.5 mL resin per mL sample). The column was equilibrated with buffer D. The column was washed extensively with buffer D until no protein was detected in the effluent. The protein kinase then was eluted with 0.1 to 0.6 M linear NaCl gradient in buffer D. The fractions with enzyme activity were pooled and were concentrated by ultrafiltration in an Amicon stirred cell with a YM-30 membrane. The concentrated enzyme was loaded onto a 2.5 × 50 cm Biogel A-1.5 column and was eluted with buffer E at a flow rate of 15 mL/h. Active fractions were concentrated by dialysis overnight against 10 mM Mops NaOH (pH 7.0), 0.25 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.005% (w/v) Chaps, 0.1 µg/mL pepstatin, 0.15 µg/mL leupeptin, 25 µg/mL TPCK, 25 µg/mL PMSF, 75 mM NaCl 50% (w/v) sucrose. Hydroxylapatite (Biogel HT) was added directly to the sample (1 mL resin per mg protein) and was mixed on ice for 15 min. The resin and enzyme were loaded into a column and were washed with buffer E without sodium isascorbate. The column was eluted with a linear gradient of 0 to 0.4 M KPO 4 in buffer E. Active fractions were pooled and were concentrated by dialysis against 10 mM Mops NaOH (pH 7.0), 0.25 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.005% (w/v) Chaps, 25 µg/mL TPCK, 25 µg/mL PMSF, 50% (w/v) sucrose. The enzyme was stored at −80°C. Throughout the extraction and purification procedures, the protein kinase activity was followed by testing for the phosphorylation of the synthetic peptide substrate KM-14.

RESULTS

To test for a calcium-dependent protein kinase in Mougeotia we used a synthetic peptide, KM-14 (the amino acid sequence is Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ala-Met), as a substrate. The sequence of this substrate is based on the site on the regulatory light chain of chicken gizzard myosin that is necessary for recognition and phosphorylation by calmodulin-dependent MLCK (14, 27). Phosphorylation occurs on serine 9 of the peptide (14). As shown in Table I, in the absence of added substrate, phosphorylation of endogenous proteins was not detected under the conditions of the filter paper assay (see "Materials and Methods" for details of this assay). However, in the presence of KM-14 substantial activity was observed, and the activity was 45-fold higher in the presence of calcium than in the presence of EGTA. Gel electrophoresis of the assay contents showed phosphorylation of a low mol wt peptide that migrates ahead of endogenous Mougeotia proteins, and has an electrophoretic mobility identical to KM-14 (Fig. 1). For the sake of discussion this enzyme activity will be referred to as KM-14 protein kinase in the present paper. Phosphorylation of endogenous proteins are not apparent in the autoradiogram, presumably due to the low amounts of Mougeotia protein used in the assay. By using higher concentrations of protein and higher specific activity of γ-32P-ATP, phosphorylation of Mougeotia proteins can be detected in these fractions by autoradiography (data not shown). The overall activity, as well as the calcium dependence, of the KM-14 protein kinase in the extract varied from preparation to preparation. Such an observation has been made with other calcium-dependent protein kinases (cf. 16, 37) and could be the result of partial proteolysis that results in the removal of a regulatory region, and the generation of a constitutive activity. In addition, the enzyme was highly unstable in the extract and lost activity rapidly upon storage. To obtain a more stable preparation of the enzyme and to analyze for calcium and calmodulin dependency, the enzyme was partially purified.

Calmodulin is generally more acidic than the enzymes that it modulates, and anion exchange chromatography in the presence of calcium chelators is often used to fractionate calmodulin from calmodulin-stimulated enzymes (28). The results of DEAE-cellulose chromatography of the KM-14

| Table I. Calcium and Calmodulin Dependence of KM-14 Protein Kinase |
|-----------------|-----------------|-----------------|
|                 | Conditions      | Specific Activity# |
| Fraction        | Ca2+           | KM-14            | −KM-14          |
| Extract         | Ca2+           | 0.64             | 0               |
|                 | EGTA            | 0.014            | 0               |
| HA purified enzyme | Ca2+           | 96.1             | 2.02            |
|                 | EGTA            | 2.4              |                 |
|                 | Ca2+/CaM        | 98.2             |                 |

# Specific activity is expressed as the nmol of 32P-phosphate incorporated per min per mg protein. The values show the differences between the activity in the presence or absence of 80 μM KM-14. The values represent the average of two determinations. The samples shown represent the soluble extract and hydroxylapatite (HA) purified enzyme (see "Materials and Methods" for details). The activities in the presence of 0.5 mM CaCl2, 1 mM EGTA, and 100 mM bovine brain calmodulin/0.5 mM CaCl2 are shown.
Mougeotia were 1616 ROBERTS N the position 1. lane 2, 80 μM KM-14 and 1 mM EGTA; lane 3, 0.5 mM CaCl₂ with no peptide; lane 4, 1 mM EGTA with no peptide. Each lane contains 2.8 μg of Mougeotia protein. Lanes 1 and 2 contain 0.4 nmol of KM-14. A, Coomassie blue stained gel; B, autoradiogram. Gel electrophoresis was done on 17.5% (w/v) gels by the method of Laemmli (18). The mol wt × 10⁻³ of standard proteins are indicated. The arrow marks the position where the KM-14 peptide migrates.

Figure 1. SDS-PAGE of phosphorylated KM-14. Extracts of Mougeotia were prepared and were tested in standard protein kinase assays in the presence of: lane 1, 80 μM KM-14 and 0.5 mM CaCl₂; lane 2, 80 μM KM-14 and 1 mM EGTA; lane 3, 0.5 mM CaCl₂ with no peptide; lane 4, 1 mM EGTA with no peptide. Each lane contains 2.8 μg of Mougeotia protein. Lanes 1 and 2 contain 0.4 nmol of KM-14. A, Coomassie blue stained gel; B, autoradiogram. Gel electrophoresis was done on 17.5% (w/v) gels by the method of Laemmli (18). The mol wt × 10⁻³ of standard proteins are indicated. The arrow marks the position where the KM-14 peptide migrates.

Figure 2. DEAE-cellulose chromatography of KM-14 protein kinase. A Mougeotia extract was chromatographed on DEAE-cellulose DE-52 as described in "Materials and Methods." Ten μL aliquots of the fractions were tested for Mougeotia KM-14 protein kinase activity (filled circles). A second set of 10 μL aliquots of the fractions were heat-treated to destroy endogenous protein kinase activity and were then tested for calmodulin activity by the ability to activate calmodulin-depleted MLCK (unfilled circles) in a calcium-dependent manner. In both cases the nmol ³²P incorporated into KM-14 per min are plotted. The dashed line represents conductivity.

Figure 3. Calcium dependence of purified KM-14 kinase. The activation of the purified KM-14 protein kinase by increasing amounts of calcium was determined by using the calcium/EDTA buffer system described in "Materials and Methods." The points shown are the average of three determinations and the error bars show the standard error of the mean.

Table II. Partial Purification of the KM-14 Protein Kinase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Total Activity</th>
<th>Yield %</th>
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<td>7.4</td>
<td>536</td>
<td>100</td>
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<td>18.4</td>
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<td>96.1</td>
<td>42.0</td>
<td>18.8</td>
<td>3.5</td>
</tr>
</tbody>
</table>
calcium ion of 1.5 μM in the absence of added calcium effectors. The addition of exogenous calmodulin had no effect on the overall activity of the enzyme (Table I). As discussed above, calmodulin was fractionated away from the enzyme during purification, and was not detected in the final enzyme preparation. To assay for protein kinase C-like activity, the effects of PS/DAG were tested. The inclusion of PS/DAG (20 μg/mL PS and 0.4 μg/mL DAG) had no influence on the maximal level of calcium activation nor on the concentration of calcium required for half-maximal activation (data not shown). Thus, the KM-14 kinase exhibits a micromolar dependence on calcium in the presence of physiological concentrations of Mg²⁺, and in the absence of two known calcium effectors, calmodulin and PS/DAG.

The KM-14 peptide possesses two possible sites of phosphorylation, threonine-8 and serine-9. Previous work (14) has shown that 97% of the phosphate incorporated into the peptide by MLCK is located on the serine residue. In the case of KM-14 labeled by the Mougeotia KM-14 kinase, only phosphoserine is detected (Fig. 4).

In addition to phosphorylating KM-14, the kinase phosphorylates the intact regulatory myosin light chain from gizzard. The kinetic parameters for each substrate were determined in the presence of 1 mM ATP under the standard assay conditions. The apparent Kₘ and Vₘₐₓ values obtained for the intact light chain and the KM-14 peptide were very similar (Table III). Other exogenous substrates tested were mixed histones, casein, and phosphorylase b. At substrate concentrations ranging from 0.5 to 10 mg/mL, the enzyme activity was less than 5% of the activity obtained with KM-14.

Previous work with smooth muscle myosin light chain kinase shows that it has a high degree of substrate specificity for the regulatory myosin light chains from smooth muscle myosin (31). To address whether the KM-14 kinase from Mougeotia has a similar specificity, two other peptide substrates were tested as substrates (amino acid sequences are shown in Table III). YA-10 is a substrate used to assay the cAMP-dependent protein kinase. cAMP-dependent protein kinase readily phosphorylates this substrate on serine-8; however, this substrate is phosphorylated poorly by MLCK.

As shown in Table III, the KM-14 kinase does not phosphorylate this peptide, even at substrate concentrations as high as 2 mM. The other peptide tested, KM-13, is an analog of KM-14 that lacks the glutamine residue at position 5. Interestingly, this peptide is a much poorer substrate for the enzyme, and has Vₘₐₓ/Kₘ ratio 200-fold less than KM-14 and myosin light chain. Thus, among the substrates tested the kinase shows a high selectivity for the KM-14 sequence, and alterations in the amino terminal sequence of the peptide reduces its suitability as a substrate. This is consistent with previous observations with smooth muscle MLCK (14).

A survey of selected algal and plant tissues as made in order to determine the distribution of the KM-14 kinase. In addition to Mougeotia two other algae, Spirogyra sp. and Mesotaenium caldarium, and a higher plant, Pisum sativum, were tested. Mesotaenium is an unicellular alga that also exhibits a light-dependent chloroplast rotation response (9, 12). Spirogyra was selected since it is another member of the order Zygnematales and is closely related to Mougeotia but does not exhibit the chloroplast rotation response. As shown in Table IV, calcium-stimulated KM-14 activity is not unique to Mougeotia and is readily detected in all organisms tested.

DISCUSSION

By using a 14 amino acid peptide that contains the site phosphorylated by smooth muscle myosin light chain kinase, we have demonstrated the presence of a calcium-dependent protein kinase in three algal species and a higher plant species. Due to extensive evidence implicating light-dependent calcium regulation in chloroplast movement in Mougeotia, and the preliminary evidence showing calcium-enhanced phosphorylation of endogenous proteins (12) and rabbit myosin light chains (2) by Mougeotia extracts, the KM-14 kinase from Mougeotia was selected for further study. The enzyme

![Image](image_url)
exhibits a micromolar dependence on free calcium ion, and thus could be an intracellular target of calcium fluxes. The protein kinase activity is not stimulated to any significant extent by the addition of exogenous calmodulin nor phosphatidylserine and diacylglycerol, and thus appears to be a calcium-dependent, calmodulin- and lipid-independent enzyme.

Among the exogenous peptides and proteins tested, the KM-14 peptide and the regulatory myosin light chain from gizzard were the best substrates for the calcium-dependent protein kinase. The apparent $K_m$ and $V_{max}$ values for the two substrates were similar. The $K_m$ observed for KM-14 (40 $\mu$M) is similar to the range of $K_m$ values (20–27 $\mu$M) reported for the same peptide with chicken gizzard myosin light chain kinase (14, 27).

Based on the observation that the calcium-dependent protein kinase phosphorylates myosin light chains in vitro, it is attractive to suggest that this enzyme is a myosin light chain kinase activity. However, it is important to note that vertebrate smooth muscle myosin light chains can be phosphorylated by multiple protein kinases including the multifunctional calmodulin-dependent protein kinase II (31) and protein kinase C (11), as well as MLCK. In the case of protein kinase C, the site of phosphorylation is at the amino terminus of the light chain and is distinct from the KM-14 site phosphorylated by the other two protein kinases. Protein kinase C phosphorylation of this second site reduces the actomyosin-ATPase activity (11) and is thought to be responsible for the attenuation of the activation of actomyosin by MLCK phosphorylation. The demonstration that the calcium-dependent protein kinase from Mougeotia phosphorylates KM-14 as well as myosin light chains suggests that the site of phosphorylation is the same as that of MLCK and calmodulin-dependent protein kinase II.

Smooth muscle MLCK can be distinguished from the multifunctional calmodulin-dependent kinase II by differences in substrate specificity (for a review of the catalytic properties of the two enzymes see ref. 31). MLCK has a very narrow specificity, and it is clear that the content and orientation of the arginine and lysine residues in the KM-14 sequence are critical for optimal catalytic activity (14). In contrast, the multifunctional calmodulin-dependent protein kinase has a less stringent requirement for substrate recognition (Arg-x-x-

Ser, with x representing any amino acid). As a result, smooth muscle MLCK is specific for the regulatory myosin light chain, whereas the multifunctional calmodulin-dependent protein kinase II can phosphorylate multiple substrates in vitro, including myosin light chains. Thus, different calcium-dependent protein kinases can have overlapping and yet distinct substrate specificities.

From the perspective of substrate specificity, it is of interest to note that the KM-13 peptide, which has a deletion of a glutamine residue, is a poor substrate for the Mougeotia protein kinase. Thus, it appears that disruption of the orientation of the amino acids at the amino terminus of the phosphorylation site can have a drastic effect on the suitability of the substrate. This is consistent with the narrow substrate specificity observed with MLCK. The results also suggest that a high degree of conservation of the recognition sequences for some calcium-dependent protein kinases. However, as discussed below, whether the plant and algal calcium-dependent KM-14 kinase is a bona fide MLCK that is involved in the regulation of contractile activity will require a detailed examination of the regulatory properties of plant actin and myosin.

Although the substrate specificity of the Mougeotia KM-14 protein kinase is similar to MLCK, the calcium and effector dependency studies suggest that it differs from other MLCK enzymes in its calcium regulatory properties. MLCK enzymes from vertebrate muscle and nonmuscle cells (31) are calcium and calmodulin-dependent. However, the KM-14 kinase described in this report appears to be a calcium-dependent, calmodulin-independent enzyme. In previous work (3, 7, 8), other protein kinase activities with similar calcium regulatory properties have been described. By using a mixed histone preparation as a substrate, Gundersen and Nelson (7) showed a calcium-dependent protein kinase in Paramecium that exhibits a micromolar calcium-dependence, is calmodulin-independent, and binds calcium directly. Similarly, using a lysine-rich histone fraction, Harmon et al. (8) demonstrated a calcium-dependent, calmodulin-independent protein kinase in soybean tissue culture cells. Recently, by using an immunocytological approach, it was shown that antibodies prepared against the soybean protein kinase colocalize with actin filaments (25). This finding, combined with the data reported in the present manuscript, raises the possibility that calcium-dependent calmodulin-independent protein kinases are associated with the actomyosin cytoskeleton and phosphorylate myosin sequences that may be associated with the control of actomyosin contractile activity.

For questions to be addressed: are plant and algal myosins phosphorylated by this protein kinase, and what is the regulatory significance of this phosphorylation event? These questions can only be addressed by a detailed analysis of plant myosin structure and function. Myosin-like ATPase activity (i.e. Mg$^{2+}$ inhibited and F-actin activated) (13, 19, 32) and immunocross-reactivity (22) have been reported in several plant and algal species. Ma and Yen (19) have recently purified and characterized the myosin from pea tendrils. An examination of the polypeptide profiles of the purified myosin showed three polypeptides with apparent mol wt of 165,000, 17,000, and 15,000. This is consistent with the structural
motif of one heavy chain and two light chains (regulatory and essential) that is characteristic of myosin (29). The structural homology between plant and animal myosins, including the presence of putative phosphorylation sites on the regulatory myosin light chain, has not yet been determined. Further study of the structure, function, and regulation of plant myosins is necessary to assess whether regulation of plant actomyosin occurs by calcium-dependent phosphorylation.

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