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Replacement of threonine residues by serine and alanine in a phosphorylatable heavy chain fragment of *Dictyostelium* myosin II

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The target sites of soluble myosin heavy chain kinases partially purified from growth phase or aggregation competent cells of *Dictyostelium discoideum* were identified by the use of normal and mutated fragments of the myosin heavy chain. The kinases from both developmental stages phosphorylated two previously established threonine residues, as well as an additional one. The newly identified site is located within the putative core region of the coiled-coil formed by the myosin tail. A lysine following the phosphorylated threonine residue is the only common feature of the sequences around these sites. The kinases, which specifically phosphorylate threonine residues in wild-type myosin, did accept serine if it was in the right structural context.

Cell motility; Chemotaxis; *Dictyostelium discoideum*; Myosin phosphorylation

1. INTRODUCTION

Phosphorylation of myosin II, the double-headed myosin of *Dictyostelium discoideum*, is regulated upon stimulation of cells with the chemoattractant cAMP [1–3]. Phosphorylation inhibits thick filament formation and reduces the actin-activated Mg^{2+}-ATPase activity of the myosin [4,5]. These effects suggest an involvement of myosin phosphorylation in the control of cell motility during the chemotactic response. In mutants lacking myosin II the average speed of cell movement and precision of the chemotactic response are reduced [6,7]. The most dramatic defect observed in these mutants is the inability of the cells to divide properly [8,9]. This finding shows that myosin II is required for cytokinesis and raises the question whether its phosphorylation is also involved in controlling this important function.

Upon stimulation of cells with cAMP, changes of both myosin heavy and light chain phosphorylation have been observed [2]. On the light chains specifically serine residues are phosphorylated. On the heavy chains threonine as well as serine residues are phosphorylated, the phosphorylation of threonine residues showing a stronger increase after stimulation by the chemoattractant [3]. The biological question behind the studies on myosin phosphorylation in *Dictyostelium* is how phosphorylation affects the functions of myosin in vivo. Because of the multiple phosphorylation sites and the site specific differences in the regulation of phosphorylation, it is necessary to investigate the consequences of phosphorylation at specific sites separately from phosphorylation at other sites. In order to do that, we have identified threonine residues on the myosin heavy chains which are phosphorylated by a certain class of kinases and have replaced these threonine residues by either serine or alanine residues. In the present paper we report on the results of in vitro phosphorylation obtained with the mutated polypeptides.

Soluble kinases that specifically phosphorylate threonine residues of the MHC have been purified from growth phase ($kinase_{grw}$) and aggregation competent ($kinase_{agg}$) cells [5,10]. Previous results obtained with $kinase_{agg}$ indicated that the phosphorylated sites are restricted to a tail region of the myosin close to the C-terminus of the heavy chain [11]. By comparing the lengths of phosphorylated and non-phosphorylated MHC fragments in partial proteolytic digests and by the analysis of phosphopeptide patterns, it was predicted that at least two phosphorylation sites are located in a stretch of amino acids containing four threonine residues, and that the residues are separated by cleavage sites for trypsin and chymotrypsin [11]. $Kinase_{grw}$ was shown by heavy chain peptide sequencing to phosphorylate one of the four residues, T-1833, and in addition a distant site, T-2029 [12]. The region containing all established and putative phosphorylation sites is

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Abbreviations: DTT, dithiothreitol; $kinase_{agg}$, soluble myosin heavy chain kinase from aggregation competent cells of *Dictyostelium discoideum*; $kinase_{grw}$, soluble myosin heavy chain kinase from growth phase cells; MHC, myosin heavy chain; SDS, sodium dodecyl sulfate

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included in a cDNA-encoded, bacterially expressed fragment of the MHC which can be used as a substrate of MHC kinases from *D. discoideum* [13,14]. We have engineered this fragment by site-directed mutagenesis for three reasons: (i) in order to establish that the kinases from both developmental stages have the same site specificity, (ii) to test whether these kinases accept serine instead of threonine when the serine is in the position of a phosphorylation site, and (iii) in order to eliminate all the sites phosphorylated by these kinases.

2. MATERIALS AND METHODS

2.1. *Purification of proteins*

D. discoideum myosin II was prepared from aggregation competent AX2 cells as described by Maruta et al. [5]. MHC kinase from growth phase cells was prepared essentially according to Côté and Bukiejko [10]. However, instead of a Bio Gel A-1.5 m column a Sephacryl S-300 column (2.5 × 115 cm) from Pharmacia was used and the final aminohexyl-Sepharose 4B column was omitted. The MHC kinase from aggregation competent cells was partially purified following procedure II of Maruta et al. [5], omitting the final Blue Sepharose and Sephadex G-100 steps. Wild-type and mutated 74 kDa fusion proteins containing 11 kDa of an MS-2 polymerase fragment and a 58 kDa portion of MHC were purified from transformed *E.coli* C600 cells as described [14].

2.2. *Phosphorylation of myosin and fusion proteins; identification of the phosphorylated amino acids*

For phosphorylation 20 μg of myosin or 50 μg of the fusion proteins were supplemented with 1 mM EGTA, 5 mM $MgCl_2$ and 0.2 mM [γ-^{32}P]ATP (100 kBq per assay) in 50 μl of Tris-HCl, pH 7.5, containing 30% sucrose, 1 mM DTT, and 0.02% NaN_3. After incubation for 15 min at 30°C with either kinase$_{grw}$ or kinase$_{agg}$ the samples were boiled in SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis in 10% gels. Protein bands were excised from the gels and digested with trypsin and chymotrypsin [15]. The peptides were hydrolyzed and subjected to two-dimensional electrophoresis as described by Hunter and Sefton [16]. Phosphorylated amino acids were identified by autoradiography and staining of coelectrophoresed, authentic amino acids with ninhydrin.

2.3. *Site-directed mutagenesis*

A 1.5 kb *Eco*RI fragment of cDNA encoding amino acids 1534 to 2032 of *D. discoideum* MHC was cloned into the *Eco*RI site of plasmids pMa/c5-8 provided to us by H.-J. Fritz [17]. Site-directed mutagenesis was performed using the gapped duplex DNA method of Stanssens et al. [17] with oligonucleotides synthesized on an Applied Biosystems DNA synthesizer. Partial sequencing using the dideoxynucleotide chain termination method [18] confirmed the mutations. For expression of the fusion proteins the mutated 1.5 kb fragments

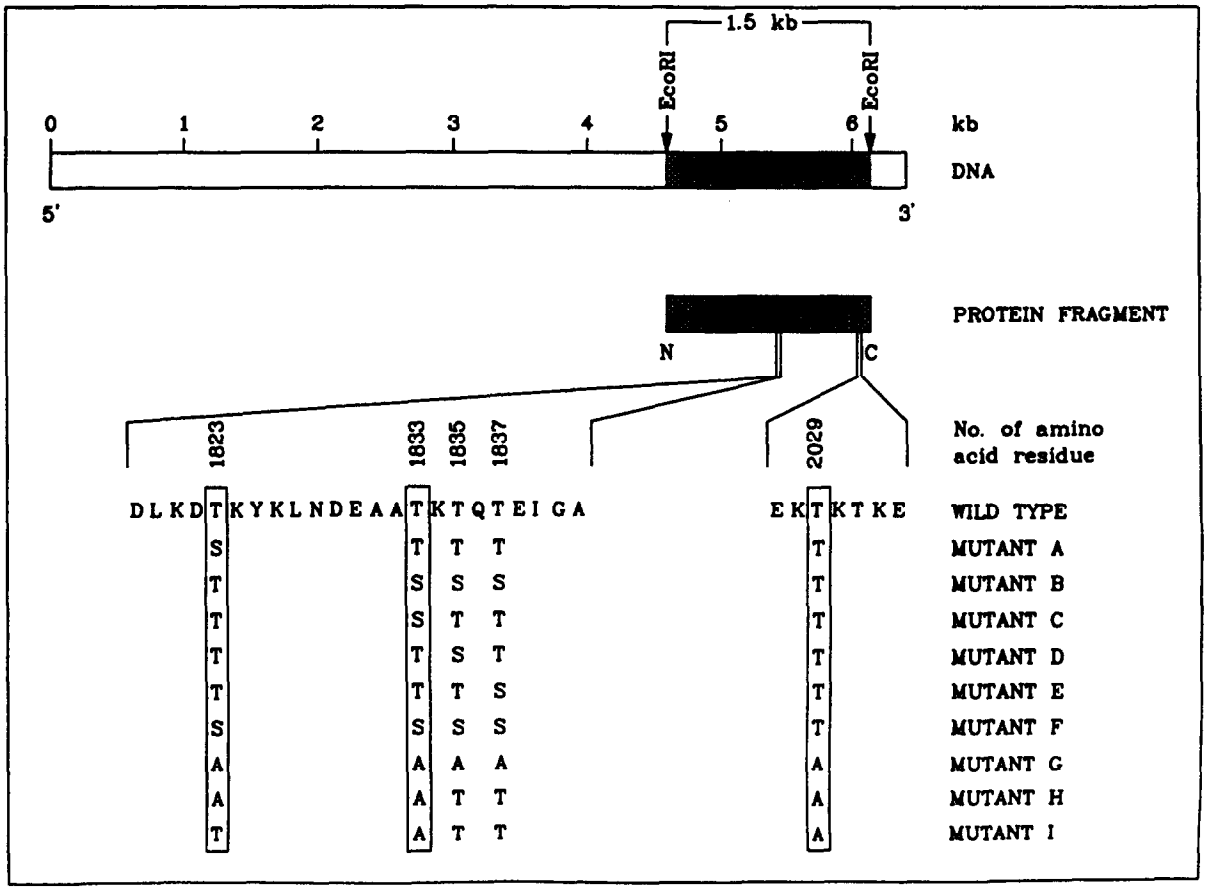

Fig. 1. (Top) Map of cDNA for the *D. discoideum* MHC showing the position of a 1.5 kb *Eco*RI fragment that encodes the protein fragment used as a substrate for MHC kinases. (Bottom) Location of mutated threonine residues on the fragment, and diagram of constructs assayed as kinase substrates. The mutated amino acids are numbered according to the MHC sequence of Warrick et al. [19]. The three residues identified as phosphorylation sites are boxed.

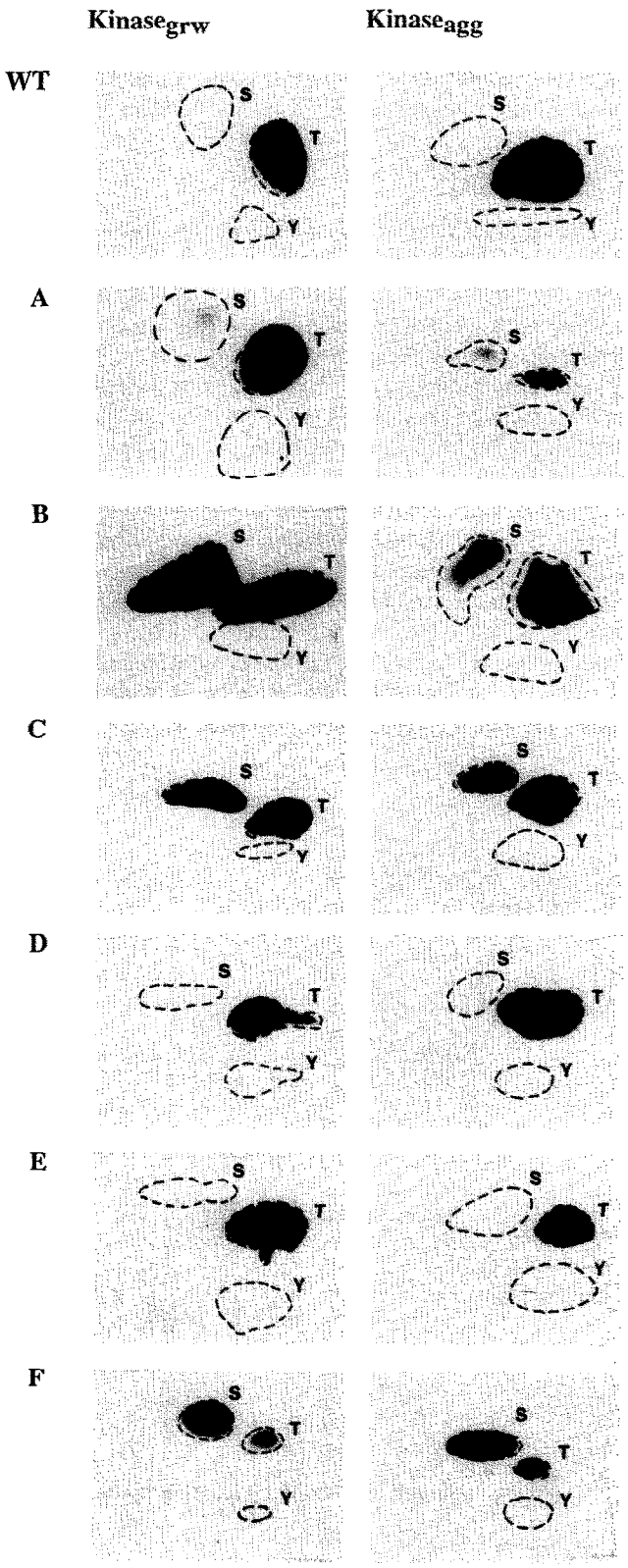

Fig. 2. Identification of the phosphoamino acids of wild-type (WT) and mutated MHC fragments A–F as diagrammed in Fig. 1. The fragments were either phosphorylated with kinase$_{grw}$ (left column) or kinase$_{agg}$ (right column). Broken lines indicate positions of authentic carrier phosphoamino acids.

were cloned into the *Eco*RI site of pEx33B as described for the wild-type fusion protein [14].

3. RESULTS

MHC kinase$_{grw}$ was partially purified from growth phase cells of *D. discoideum* essentially as described by Côté and Bukiejko [10] and MHC kinase$_{agg}$ from aggregation competent cells, following a procedure similar to that of Maruta et al. [5]. In accord with the published results, the kinases of both preparations phosphorylated specifically threonine residues on the heavy chains of intact *D. discoideum* myosin.

As substrates for the kinases, the normal and mutated MHC fragments shown in Fig. 1 were used. In a first set of mutageneses four threonine residues between positions 1823 and 1837 were replaced by serine (Fig. 1, mutants A–F). After phosphorylation of the mutated MHC fragments by kinase$_{grw}$ or kinase$_{agg}$ the phosphoamino acids were analyzed (Fig. 2). When only the single residue T-1823 was changed, a small but significant amount of phosphoserine was found (Fig. 2, mutant A). If the cluster of residues 1833, 1835, and

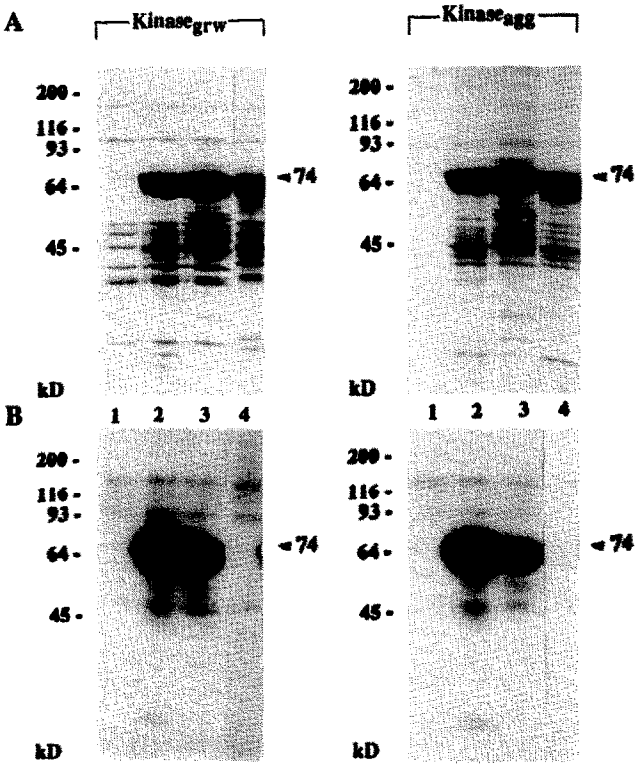

Fig. 3. Coomassie-Blue polyacrylamide gels (A) and autoradiograms (B) showing normal and mutated MHC fragments phosphorylated with either kinase$_{grw}$ (left panel) or kinase$_{agg}$ (right panel). Lanes 1: Controls, containing kinase preparations without added substrate protein. Lanes 2: With wild-type MHC fragment added. Lanes 3: With the mutated fragment H of Fig. 1 added. Lanes 4: With fragment G added in which five threonine residues are replaced by alanine.

1837 was mutated, a large amount of phosphoserine was obtained in addition to phosphothreonine (Fig. 2, mutant B), indicating at least one phosphorylation site within this cluster (Fig. 1). This site was identified by replacing the three threonine residues in the cluster separately. Phosphoserine was produced only when T-1833 was mutated (Fig. 2, mutants C-E).

Production of phosphothreonine after mutation of all four threonine residues into serine (Fig. 2, mutant F) indicated the presence of a third target site for both $kinase_{grw}$ and $kinase_{agg}$ which is located in another region of the cloned MHC fragment. Since it appeared likely that the additional site is T-2029, which was shown to be phosphorylated in intact myosin by $kinase_{grw}$ [12], this threonine residue was replaced in addition to the four other ones by alanine (Fig. 1, mutant G). The resulting fragment was not detectably phosphorylated with either kinase (Fig. 3, lanes 4). Similarly, no phosphorylation was obtained with a MHC fragment in which only at the positions 1823, 1833 and 2029 the threonine residues were replaced by alanine (Fig. 1, mutant H). Mutation of T-1833 and T-2029 to alanine was employed in order to confirm the newly identified phosphorylation site at position 1823 (Fig. 1, mutant I). Substantial phosphorylation observed after elimination of these two sites (Fig. 3, lanes 3) established that in wild-type myosin three threonine residues are phosphorylatable by the kinases used.

4. DISCUSSION

The soluble MHC kinases used in this study showed the same specificity, although previous reports indicating different regulatory properties of the MHC kinases from growth phase and aggregation competent cells inferred that these enzymes might not be the same [5]. Because $kinase_{grw}$ and $kinase_{agg}$ phosphorylated the same threonine residues on the heavy chains, the results obtained with both kinase preparations will be discussed together.

In vivo and in vitro phosphorylation studies have shown that *D. discoideum* cells contain kinases that phosphorylate serine residues on light and heavy chains [3]. The kinases used in the present study phosphorylate only threonine residues of the heavy chains [5,10]. The finding that replacement of phosphorylatable threonine residues by serine does not abolish phosphorylation (Fig. 2), shows that these kinases do not require

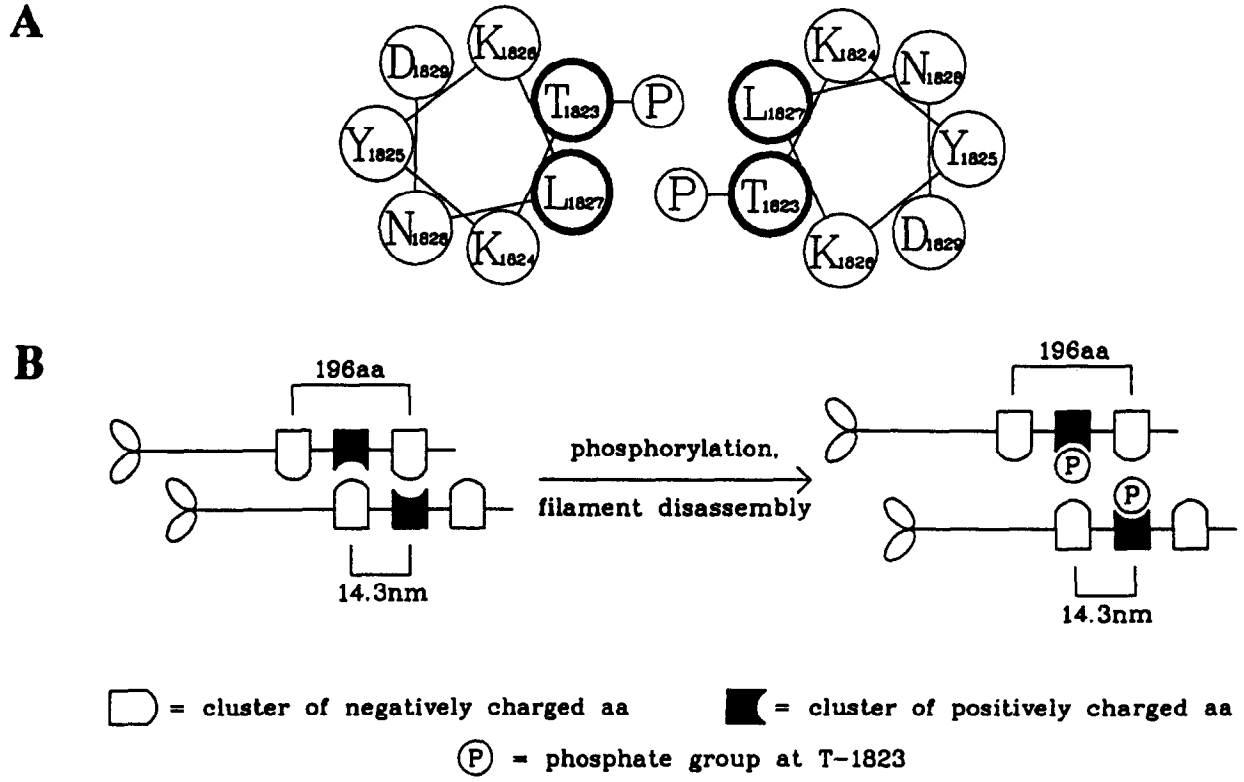

Fig. 4. Diagram of the location of threonine-1823 within the α-helix (A) and possible effect of its phosphorylation on myosin polymerization (B). (A) Seven amino acid residues representing two turns of the α-helices of the two heavy chains that comprise the myosin tail are illustrated in a top view. Residues indicated by dark circles make up the hydrophobic core of the coiled coil [19]. T-1823 in its phosphorylated state might decrease the hydrophobic interaction between the two subunits. (B) Model of the assembly of two myosin molecules by charge-charge interaction and its suppression by phosphorylation. T-1823 is situated within a positive charged stretch of the MHC sequence which is flanked by two negatively charged stretches. Charge-charge interaction between the positive and negative regions would produce a stagger interval of 14.3 nm, as suggested by H.M. Warrick (personel communication), and phosphorylation of T-1823 might affect the intermolecular bonds.

threonine as a target site. We conclude that the site specificity of phosphorylation is either determined by the sequence context or by restrictions due to the conformation of the region into which the phosphorylatable residues are embedded.

By systematically mutating putative phosphorylation sites into serine residues we have found a third phosphorylatable residue, T-1823, at the myosin heavy chain. The phosphorylatable residues T-1823, T-1833, and T-2029 have a lysine at their C-terminal side as the only obvious sequence context in common. Since there are other T-K pairs in the cloned MHC fragment used as a kinase substrate where no phosphorylation was detected, the lysines might be necessary but are not sufficient for phosphorylation to occur.

The tail of the MHC contains a 7-residue repeat, characteristic of α-helical coiled-coil proteins in which the first and fourth amino acids are highly hydrophobic [19]. This pattern allows the formation of an internal hydrophobic core of the coil. Whereas T-1833 and T-2029 lie adjacent to but not within the predicted hydrophobic core [12], T-1823 is located at a core position (Fig. 4A). Therefore, the hydrophilicity produced by its phosphorylation may cause a strong disturbance of the coiled-coil structure. Moreover, T-1823 lies within a stretch of amino acids where 11 out of 23 residues are positively charged. This is the highest accumulation of positive charges within the complete MHC tail sequence. This region of positively charged amino acids falls into the middle of two sequences of negatively charged amino acids which are 196 residues apart (Warrick, H.M., personal communication). The 14.3 nm stagger of a myosin filament is known to correspond to one-half of 196 amino acids [19]. A possible mechanism of filament formation would be the charge-charge interaction of the positive stretch of amino acids with the negative stretch at another myosin molecule (Fig. 4B). Decrease of the net charge in the positive region by phosphorylation might contribute to the inhibition of filament formation as has been observed as a consequence of MHC phosphorylation [4].

The results shown provide a basis for studies on the functional consequences of phosphorylation at defined threonine residues of the myosin tail. The three phosphorylation sites in question are shown to be phosphorylated independently of each other and they can be eliminated by site-directed mutagenesis separately or in concert. Since it is unknown whether the MHC kinases discussed are the products of only one gene or of several genes, elimination of the phosphorylation sites might have a stronger effect on myosin functions in vivo than inactivation of one gene encoding a kinase. *D. discoideum* strains that lack certain phosphorylation sites can be produced by transformation of MHC null mutants [20] with vectors carrying mutated MHC coding regions. By recording cell motility and chemotaxis using image processing systems [7,21] it should be possible to quantitate effects of altered phosphorylation on cell behavior.

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