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Phosphorylation of threonine residues on cloned fragments of the *Dictyostelium* myosin heavy chain

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A tail fragment of *Dictyostelium discoideum* myosin has been cloned and expressed as a fusion protein with the N-terminal region of MS-2 polymerase. The cloned fragment was phosphorylated with myosin heavy chain kinase II from aggregation-competent *D.discoideum* cells that specifically phosphorylate threonine residues on the myosin tail. Phosphopeptide maps showed the same site specificity of phosphorylation with the fusion protein as a substrate as with native myosin. An improved assay for the kinase was developed in which the fusion protein is precipitated with a monoclonal antibody that inhibits polymerization of the myosin tails without preventing their phosphorylation. Sites of phosphorylation were tentatively localized to a sequence in the C-terminal region of the heavy chain where four threonine residues are found.

Myosin phosphorylation; Protein kinase; Cell motility; Cloned myosin tail fragment; *(Dictyostelium discoideum)*

1. INTRODUCTION

Phosphorylation of the MHC in *Dictyostelium discoideum* is regulated by the chemoattractant cyclic AMP [1–4]. A MHC kinase, designated as MHC kinase II, was partially purified from aggregation-competent cells and found to be inactivated by Ca^{2+} and calmodulin [5]. This result suggested that the kinase is involved in signal transmission, although the mechanism of this inactivation remained unknown. Phosphopeptide mapping showed that two or more threonine residues are phosphorylated by MHC kinase II [5]. A myosin kinase purified from growth-phase cells also specifically phosphorylated threonine residues on the heavy chain [6]. The activity of this kinase was not significantly altered by Ca^{2+} and calmodulin, and its relationship to MHC kinase II is unclear. Direct comparison of the kinases and their regulatory properties depends on the availability of a more specific substrate than intact myosin, since in the latter serine residues on the light chains and both serine and threonine residues on the heavy chains are phosphorylated [3,4]. We have subcloned the MHC for the use of fusion proteins as better defined substrates for kinases, in order to specify phosphorylation sites and to eliminate two difficulties in quantitating MHC kinase activities. One difficulty is the consumption of ATP by the myosin ATPase activity, another the polymerization of myosin under the low-salt conditions under which the kinases are active [5,6].

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; DTT, dithiothreitol; MHC, myosin heavy chain; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *MHC specific DNA clones*

A λgt11 expression library containing *Eco*RI cut genomic DNA from *D. discoideum* strain AX2 [7] was screened with ^{125}I-labeled mAb 253. Phage λgm159 expressed a protein recognized by the antibody and contained a 1.5 kb insert. The sequence of both strands of the insert was determined using the dideoxynucleotide chain termination method [8] after subcloning into M13mp18 or mp19 and pUC19 [9].

Since the fusion protein produced in λgt11 proved to be instable, the insert of λgm159 was cloned into the *Eco*RI site of

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plasmid pEx33B (Schaller, H., personal communication) and expressed in *E. coli* C600 [10]. The plasmid contains the λPL promoter followed by the coding sequence for the N-terminal 99 amino acids of phage MS-2 polymerase and a synthetic polylinker. The plasmid containing the insert of λgm159 was designated as *pm159*. *Xho*II fragments of the insert were cloned into the *Eco*RI and *Bam*HI sites of pEx33A or B. A subclone containing the 0.9 kb fragment in pEx33B was designated as *pm12*, and a subclone containing the 0.6 kb fragment in pEx33A as *pm4*.

2.2. *Purification of proteins*

For expression of fusion proteins, transformed *E. coli* cells were cultivated overnight at 28°C in LB medium containing 50 μg per ml each of ampicillin and kanamycin, then diluted 5-fold into prewarmed LB medium and incubated for 2 h at 42°C. Bacteria from a 200-ml culture were washed twice in cold 30 mM Tris-HCl buffer, pH 7.5. For partial purification of the proteins, a procedure described for the c-myc protooncogene [11] was modified. Lysozyme-, DNase- and RNase-treated bacteria were lysed by sonication on ice, and the 10000 × g pellet was extracted with 20 ml TEDGA buffer (20 mM Tris-HCl buffer, pH 7.5; 1 mM EGTA; 1 mM DTT; 5% glycerol; 0.02% NaN_3) supplemented with 10 mM benzamidine, 0.5 mM PMSF and 10 mM Chaps (Calbiochem). The pellet was extracted on ice with 20 ml TEDGA buffer and 5 M urea (Biorad, deionized with Amberlite). The extract was centrifuged as above and the supernatant loaded onto a 20 ml DE-52 column (Whatman) equilibrated with TEDGA buffer and 5 M urea. In a linear gradient of NaCl in TEDGA buffer and 5 M urea the fusion protein eluted between 50 mM and 100 mM NaCl. The partially purified fusion protein was dialysed against 20 mM TDSA buffer (Tris-HCl, pH 7.5; 15% sucrose; 1 mM DTT; 0.02% NaN_3) and, after removal of debris, stored with 50% glycerol at −20°C.

D. discoideum myosin was purified from aggregation-competent AX2 cells as described [5]. MHC kinase II was partially purified from aggregation-competent AX2 cells essentially by procedure II of Maruta et al. [5], omitting the final Blue Sepharose and Sephadex G-100 steps.

2.3. *Immunoblotting, precipitation and phosphorylation*

IgG of mAb 96 or mAb 253 [12] was purified from hybridoma culture supernatants. For immunoblotting of fusion proteins, bacteria were lysed in SDS-sample buffer, and proteins separated in 7.5% or 10% SDS-polyacrylamide gels were transferred to BA85 nitrocellulose filters (Schleicher and Schüll). The blots were labeled with mAb 253 or mAb 96 that were iodinated using the chloramine T method.

For immunoprecipitation, 40 μg IgG in TDSA buffer and 100 μg of partially purified fusion protein or 20 μg of purified myosin were incubated overnight at 4°C in TDSA containing 150 mM NaCl. After the addition of 40 μl swollen protein A-Sepharose beads (Pharmacia) in 160 μl TDSA-NaCl, incubation at 4°C was continued with gentle shaking for 30 min. The beads were pelleted by short centrifugation and washed twice with 500 μl TDSA in the cold. For phosphorylation with partially purified MHC kinase II, the beads were suspended in TDSA supplemented with 1 mM EGTA, 5 mM $MgCl_2$ and 10 μM ^{32}P-ATP (10^8 cpm per nmol). After 30 min of incubation at 35°C, the beads were washed twice with TDSA, boiled in SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis.

2.4. *Phosphopeptide mapping and identification of phosphothreonine*

The 74 kDa fusion protein or purified *D. discoideum* myosin was precipitated using mAb 96 and ^{32}P-phosphorylated with MHC kinase II. After SDS-polyacrylamide gel electrophoresis, bands of proteins were cut from the dried gel and extracted under digestion for 30 h at 35°C with 5 μg of trypsin and 5 μg of chymotrypsin in 0.5 ml of 10 mM NH_4HCO_3 (pH 8.0) [13]. Peptides were separated by thin-layer electrophoresis and chromatography on cellulose sheets [5]. For identification of phosphoamino acids, the extract was hydrolysed for 2 h at 110°C with 6 M HCl under vacuum. After addition of unlabeled authentic phosphoamino acids the mixture was separated by two-dimensional electrophoresis [14], autoradiographed and stained with ninhydrin.

3. RESULTS

3.1. *Characterization and partial purification of cloned myosin heavy-chain fragments*

The sequence of a genomic *Eco*RI fragment proved to be identical with that of nucleotides 4598 to 6097 of the complete MHC sequence which consists of one continuous exon [15]. The 1.5 kb fragment encodes amino acids 1534 to 2032 of the MHC, lacking at its 3′-end the coding region for the 84 C-terminal amino acids. The fusion protein that contained 11 kDa of MS-2 polymerase in addition to the 58 kDa of the MHC fragment showed an apparent molecular mass of 74 kDa in 7.5% SDS-polyacrylamide gels. It was labeled with mAb 253 and mAb 96. The latter antibody binds close to the threonine residues phosphorylated by MHC kinase II [16].

Subcloning of the 1.5 kb *Eco*RI fragment gave rise to a 0.9 kb 5′-fragment that was expressed as a 46 kDa fusion protein, and to a 0.6 kb 3′-fragment that yielded a 32 kDa fusion protein. The 46 kDa protein bound only mAb 253, the 32 kDa protein only mAb 96.

3.2. *Phosphorylation of fusion proteins*

The partially purified 74 kDa fusion protein was incubated with mAb 96 or mAb 253 and precipitated with protein A-Sepharose. Both antibodies prevent the polymerization of myosin [12]. Phosphorylation of the immunoprecipitates drastically reduced background due to phosphorylation of other proteins in the partially purified preparations. After incubation of the im-

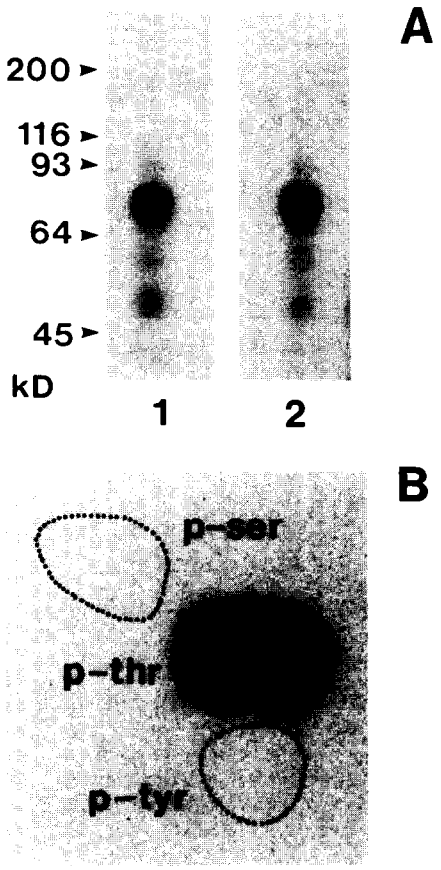

Fig.1. Phosphorylation of the 74 kDa fusion protein after immunoprecipitation (A), and identification of threonine as the phosphorylated amino acid (B). (A) The partially purified fusion protein was immunoprecipitated with mAb 96 (lane 1) or mAb 253 (lane 2), phosphorylated with MHC kinase II and [γ-^{32}P]ATP, and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Positions of molecular mass markers are indicated on the left. After incubation of either the kinase preparation or the substrate alone with [γ-^{32}P]ATP, no labeled band was seen (not shown). (B) The 74 kDa fusion protein was hydrolysed and phosphoamino acids were separated. Positions of authentic phosphoamino acids are indicated on the autoradiogram by dotted lines.

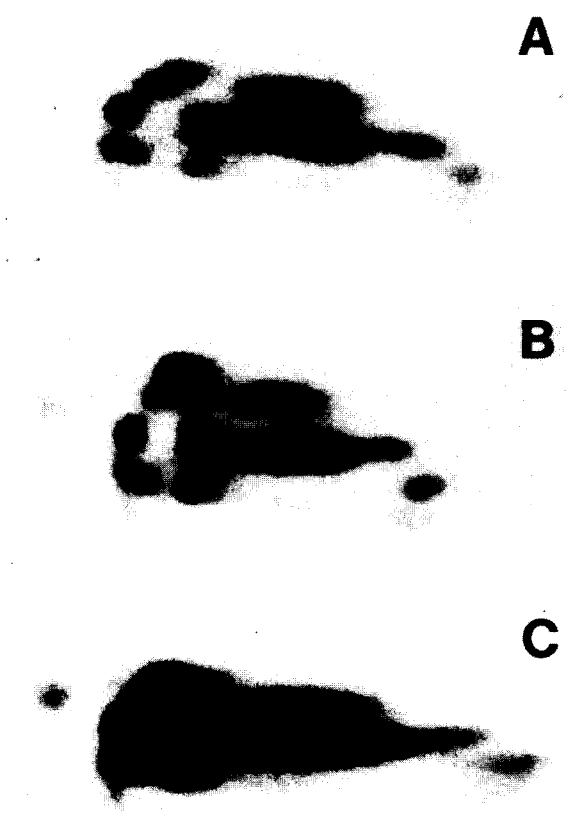

Fig.2. Two-dimensional phosphopeptide patterns obtained from intact myosin heavy chains (A), the 74 kDa fusion protein (B), or a mixture of both (C). After incubation of myosin with MHC kinase II and [γ-^{32}P]ATP, the heavy chains were digested with trypsin and chymotrypsin. The direction of electrophoresis of the phosphopeptides was from left (+) to right (−). The direction of chromatography was from bottom to top.

munoprecipitates with [γ-^{32}P]ATP and MHC kinase II, the ^{32}P label was primarily found in the 74 kDa band of the fusion protein (fig.1A). Minor phosphorylated polypeptides were most likely degradation products of the 74 kDa protein. These existed already in the bacteria and were copurified with the fusion protein.

Only phosphothreonine residues of the fusion protein were labeled with MHC kinase II (fig.1B), as was the case with normal myosin [5]. Phosphopeptide mapping of myosin and fusion protein resulted in superimposable phosphopeptide patterns (fig.2). Thus all threonine residues phosphorylated by MHC kinase II appear to be located in the cloned 58 kDa fragment of the myosin tail.

4. DISCUSSION

The results reported here are combined in fig.3 with previous data [12,16,17] indicating that the threonine residues phosphorylated by MHC kinase II are located in a 38 kDa fragment from the MHC

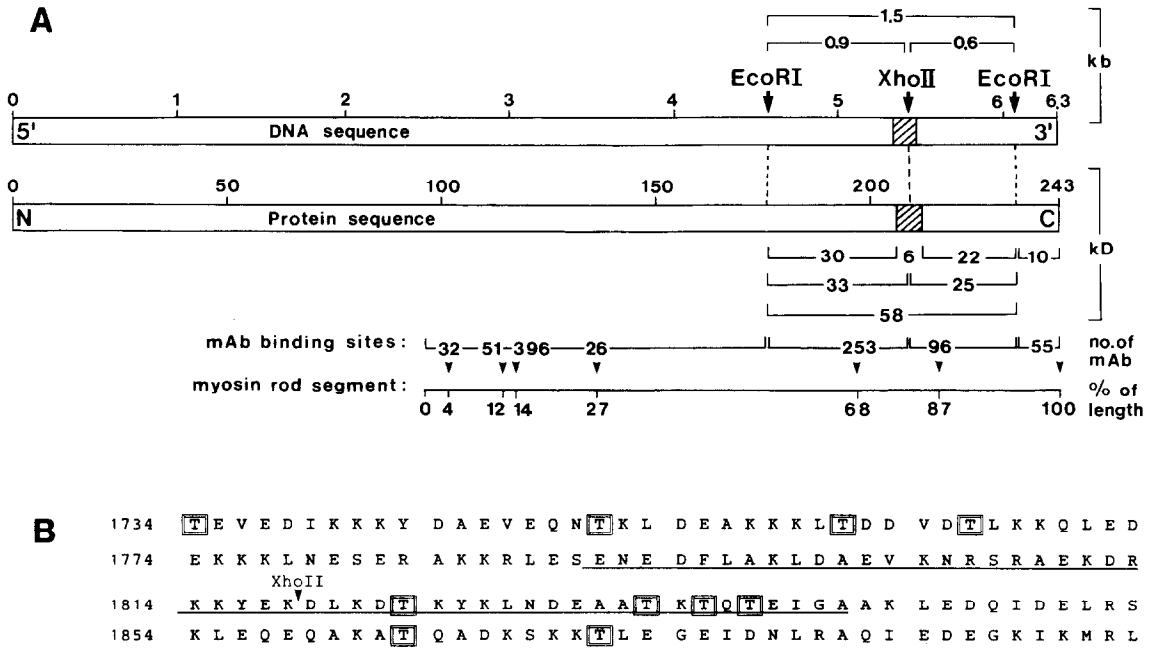

Fig.3. Map of the *D. discoideum* myosin heavy chain and of the cloned fragments (A) and sequence of a portion of the MHC that contains putative phosphorylation sites (B). This sequence has been derived from the DNA sequence of the insert of λgm159 that codes for the 58 kDa polypeptide shown in A. (A) *Eco*RI and *Xho*II sites used for cloning and subcloning are indicated on top, and boundaries of the encoded polypeptides below. The hatched area indicates a region containing phosphorylation sites, as predicted from the apparent molecular masses of partial chymotryptic fragments of myosin phosphorylated with MHC kinase II [16]. The two lines on bottom indicate the positions of mAb binding sites on the myosin tail as revealed by electron microscopy [12,17]. All antibodies were tested for binding to the cloned fragments. Only mAbs 253 and 96 bound to the 58 kDa fragment; mAb 253 to the 33 kDa subfragment and mAb 96 to the 25 kDa one. (B) Amino acid residues are numbered according to the complete coding sequence of the MHC gene [15]. Threonine residues are boxed, the approximate extension of the region hatched in A is underlined. The position of the *Xho*II site used for subcloning of the DNA is indicated.

C-terminal region. This fragment was recognized by mAb 96 and 55, but not by mAb 253. A smaller C-terminal fragment of 32 kDa lacked both the mAb 96 binding site and the phosphorylated threonine residues. These data suggested that the phosphorylated sites are located within the hatched area of fig.3A. This region is underlined in the sequence of the 58 kDa fragment shown in fig.3B. Among the 24 threonine residues encompassed by the cloned fragment, residues 1823, 1833, 1835 and 1837 are located in that region, and four others in the flanking sequences up to a distance of 30 amino acids on both sides. In the central group of four residues, which are the most likely sites phosphorylated by MHC kinase II, a single threonine residue is separated from a cluster of three by an amino acid stretch that contains cleavage sites for trypsin and chymotrypsin. This fits to the phosphopeptide pattern which indicates at least two phosphorylatable threonine residues which are separated by cleavage sites for both these enzymes [5].

The results presented show that intact *D. discoideum* myosin can be replaced as a substrate for MHC kinase II by a fusion protein containing an internal MHC fragment which represents about 20% of the total size of the heavy chain. The fusion protein is easier to prepare and is a better standardized substrate for the kinase than myosin which might retain phosphate when it is purified from *D. discoideum* cells [5,6]. Use of the fusion protein will facilitate distinction of MHC kinase II from myosin kinases that phosphorylate other residues on heavy or light chains, and thus will help to elucidate the mechanisms by which these kinases are regulated.

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