Baculovirus Expression of Chicken Nonmuscle Heavy Meromyosin II-B

CHARACTERIZATION OF ALTERNATIVELY SPLICED ISOFORMS*

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We have expressed two truncated isoforms of chicken nonmuscle myosin II-B using the baculovirus expression system. One of the expressed heavy meromyosins (HMMexp) consists of two 150-kDa myosin heavy chains (MHCs), comprising amino acids 1-1231 as well as two pairs of 20-kDa and 17-kDa myosin light chains (MLCs) in a 1:1:1 molar ratio. The second HMMexp was identical except that it contained an insert of 10 amino acids (PESPKPVKHKQ) at the 25-50-kDa domain boundary in the subfragment-1 region of the MHC. These 10 amino acids include a consensus sequence (SPK) for proline-directed kinases. Expressed HMMs were soluble at low ionic strength and bound to rabbit skeletal muscle actin in an ATP-dependent manner. These properties afforded a rapid purification of milligram quantities of expressed protein. Both isoforms were capable of moving actin filaments in an in vitro motility assay and manifested a greater than 20-fold activation of actin-activated MgATPase activity following phosphorylation of the 20-kDa MLC. HMMexp with the 10-amino acid insert was phosphorylated by Cdc2, Cdk5, and mitogen-activated protein kinase. HMMexp with the 10-amino acid insert had a 50% faster translocation rate than the noninserted HMMexp.

Myosin II is a hexamer composed of two heavy chains of approximately 200 kDa and two pairs of light chains of 20 and 17 kDa. It is a member of an expanding family of myosin motor proteins (1) and can be subdivided into a number of different isoforms present in striated muscle, smooth muscle, and nonmuscle cells. Our recent interest has focused on vertebrate nonmuscle myosins (1) and can be subdivided into a number of different isoforms present in striated muscle, smooth muscle, and nonmuscle cells. To date, two separate genes, located on two different human chromosomes (2, 3), have been shown to encode vertebrate nonmuscle myosin heavy chains (MHCs), which we refer to as MHC II-A (22q11.2) and MHC II-B (17p13). Since it is now clear that some of these two isoforms are present in distinct locations in a single cell (4, 5), there is reason to believe that each isoform may have a specific function, in addition to possibly overlapping functions. Moreover, previous studies have shown that these two isoforms are expressed in a tissue-dependent manner with brain and testes being particularly enriched for MHC II-B and spleen and intestines containing mostly MHC II-A, while human platelets and rat basophil leukemic cells contain only MHC II-A (6–8).

MHC subfragment-1 (S-1) can be proteolytically cleaved at two sites, one located about 25 kDa and the second about 75 kDa from the amino-terminus. The 10-amino acid insert differs from the 16-amino acid insert in 6 amino acids, consistent with the idea that these sequences are generated by alternative splicing of pre-mRNA and that the 16-amino acid insert differs from the 10-amino acid insert in that it is encoded by both exons instead of just one (19) (Fig. 1). Sequence of human genomic DNA from this area revealed the presence of two exons, one encoding 10 amino acids and the second encoding 6 amino acids, consistent with the idea that these sequences are generated by alternative splicing of pre-mRNA and that the 16-amino acid insert differs from the 10-amino acid insert in that it is encoded by both exons instead of just one (19) (Fig. 1). The 10-amino acid insert has been shown to be highly expressed in mammalian cerebral cortex and retina, whereas mRNA encoding the 16-amino acid insert has only been detected in human retinoblastoma cell lines.

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1 The abbreviations used are: MHC, myosin heavy chain; myosin S-1, myosin subfragment-1; MLC, myosin light chain; MLG, 20-kDa regulatory light chain; MLC17, 17-kDa essential light chain; HMM, heavy meromyosin; MOPS, 3-(N-morpholino)propanesulfonic acid; MAP kinase, mitogen-activated protein kinase; PMSF, phenylmethylsulfonyl fluoride.

2 We refer to the noninserted MHC as MHC II-B, the MHC with the 10-amino acid insert as MHC II-B1, and one in loop 2, referred to as MHC II-B2. The insert in loop 1 starts just after amino acid 211, and consists of either 10 or 16 amino acids (see Fig. 1) (14).
Baculovirus Expression of Myosin Heavy Chain II-B and II-B1

A. 10 Amino Acid Inserts

Chicken MHC II-B: RRDHNIP......GEQLER

MHC II-B1: RKDHNIPPEGSKPKVHGEQLER

Human MHC II-B: RRDHNIPPEGSKPKVHGEQLER

B. 16 Amino Acid Inserts

Human: RRDHNIPQESPSKPVKHGEQLER

Xenopus: KKDHTPTEFSPAIKQGSGLYGEQLER

C. Tryptic Cleavage of MHC II-B

RRDHNIPPEGSKPKVHGEQLER

Fig. 1. Amino acid sequences in loop 1 of myosin heavy chain II-B. The 10-amino acid (A) and 16-amino acid (B) inserts present at loop 1 are shown in bold type for three species. C, the major sites of tryptic cleavage of chicken MHC II-B1 in this region are indicated by large arrows. A possible minor tryptic cleavage site is also marked. The phosphorylatable serine in the chicken sequence is underlined.

follow the treatment with butyrate (14).

Expression of MHC II-B1 in nonmuscle cells appears to vary in both a species- and tissue-dependent manner. In Xenopus, an almost identical insert of 16 amino acids in MHC II-B, starting after amino acid 211, is present in all cells examined to date (20). Unlike avian and mammalian cells, MHC II-B (lacking the insert) is not expressed in Xenopus. In contrast to its ubiquitous expression in Xenopus cells, expression of MHC II-B1 (with the 10-amino acid insert) in avian and mammalian cells is almost always confined to neuronal tissue and neuronal cell lines, where it is accompanied by expression of the noninserted isoform. Of note, the constitutively expressed Xenopus MHC II-B1 isoform has been shown to be phosphorylated by cyclin p34<sup>cdc2</sup> (Cdc2) kinase both in vitro and in situ within the inserted region (21).

To date, there has been no careful characterization of the differences between myosin containing MHC II-B1 and MHC II-B. This has largely been due to the inability to obtain sufficient quantities of pure myosin II-B1 from avian and mammalian cells. In this paper, we focus on the differences between nonmuscle MHC II-B and MHC II-B1, the isoform containing the 10-amino acid insert. We used the baculovirus system to express a heavy meromyosin (HMM)-like product of these two alternatively spliced isoforms along with both the 20-kDa and 17-kDa myosin light chains. We then characterized each isoform with respect to its ATPase activity and ability to propel actin filaments in an in vitro motility assay. The MHC II-B1, but not the MHC II-B isoform, contains a putative phosphorylation site for proline-directed kinases, which we demonstrate can be phosphorylated by cyclin-dependent and mitogen-activated protein (MAP) kinases. The site phosphorylated is within the 10-amino acid insert. We also show that MHC purified from bovine brains contains this site and can also be phosphorylated.

EXPERIMENTAL PROCEDURES

Construction of Transfer Vectors—Three different transfer vectors were employed for transfection of Sf9 cells. CDNA encoding the chicken MHC II-B HMM-like isoform was obtained by utilizing clone S-1, previously derived from a chicken brain library (13), which is a Not fragment that contains 74 nucleotides of untranslated mRNA at the 5' end and terminates at nucleotide 3,693. Single-stranded oligonucleotide adaptors with the following sequences: 5'-CT AGC GAT CAG CTG TAG CAC TAT TC-3'; 3'-G CTA GTC GCA ATC GTC ATG ATA AGC CCG-5'; 5'-GCC GAA TAG TGC TAA CGC TGA TCG-3'; and 3'-CTT ATC ACG ATT GCG ACT AGC GAT C-5', were annealed and ligated to the NotI sites. In addition to providing NheI sites for ligation into pBlueBac, the double-stranded oligonucleotides also provided multiple stop sites.

For construction of the transfer vector containing the 30-nucleotide insert between T633 and G634, the MHC II-B cDNA encoding the HMM fragment was removed from pBlueBac using BamHI and subcloned into pBlueScript. Clone S4, which is derived from the same chicken brain library and contains the 30-nucleotide insert flanked by NsiI sites (13), was restricted and the resulting 539-base pair fragment was ligated into the same site in pBlueScript containing the truncated done MHC II-B (13). The cDNA encoding the MHC II-B1 HMM-like fragment was then ligated into pBlueBac II at the NheI site.

cDNAs encoding bovine nonmuscle MLC<sub>17</sub> (22)(a gift of Dr. David Hathaway, Bristol Myers Squibb Co., Princeton, NJ) and chicken nonmuscle MLC<sub>20</sub> (23)(a gift of Dr. C. Chandra Kumar, Schering-Plough Corp., Bloomfield, NJ) were cloned into the PACUV51 transfer vector system using polymerase chain reaction-derived oligonucleotides. CDNA encoding MLC<sub>17</sub> was cloned into the BglII site, placing it under control of the p10 promoter, and cDNA encoding MLC<sub>20</sub> was cloned into the BamHI site, under control of the polyhedrin promoter. Orientation and sequence of both light chains were verified using the dideoxy chain termination method (24).

Transfection—Transfection of 3×10<sup>6</sup> Sf9 insect cells with either the MHC II-B or MHC II-B1 truncated constructs was carried out using 3 μg of plasmid DNA, 1 μg of linear AcMNPV DNA, 1 ml of Grace’s medium, and 20 μl of a cationic liposome solution as per the manufacturer’s instructions (Invitrogen, San Diego, CA). The vector containing both the MLC<sub>17</sub> and MLC<sub>20</sub> was transfected using 2 μg of plasmid DNA and 0.5 μg of BaluGold DNA using the Ca<sub>9</sub>(PO<sub>3</sub>)<sub>6</sub> method as per the manufacturer’s instructions (PharMingen, San Diego, CA). Plaque assay purification and viral amplification was carried out according to the manufacturer’s instructions. Some plaque assays, amplifications, and Sf9 infections were carried out by Cell Trends (Middletown, MD).

Introduction and Preparation of Myosin—1×10<sup>6</sup> Sf9 cells were coinfectected at a multiplicity of infection of 5 with respect to both the MHC virus (II-B or II-B1) and with the virus containing both MLCs. Infected cells were harvested by sedimentation after 72 h of growth, and the pellet was washed twice with phosphate-buffered saline, quick-frozen in liquid nitrogen, and stored at −80 °C.

The partially thawed pellet was extracted (5–5 ml of packed cells) with 0.6 M NaCl, 25 mg Tris HCl (pH 7.5), 50 μM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 5 μg EDTA, 10 μg ATP, 0.1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg dithiothreitol, 5 μg leupeptin, 3 mM NaN<sub>3</sub>, and 1% Nonidet P-40 following homogenization in a ground glass homogenizer. Extraction at 4°C with stirring continued for 1 h followed by sedimentation at 47,000 g for 10 min. This step effectively separates soluble from insoluble MHCs. The supernatant is sedimented at 300,000 g for 1 h and the resulting supernatant made 5 mM ATP, 10 mM MgCl<sub>2</sub>, and subjected to (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation. The 40–60% fraction was solubilized in 10 ml of 0.5 M NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EDTA, 3 μg leupeptin, 10 μg MgCl<sub>2</sub>, and subjected to (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractionation. The 40–60% fraction was solubilized in 10 ml of 0.5 M NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EDTA, 3 μg leupeptin, 0.1 mM PMSF, and 5 mM dithiothreitol (Buffer A) and dialyzed in 50 volumes of Buffer A overnight with one change.

Actin Selection—Final purification of the HMMSs was made by use of the ability of actin to bind myosin in the absence, but not the presence, of ATP. The 40–60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction (10 ml) was made 5 μM with respect to F-actin and 5 μM with respect to phalloidin and sedimented at 543,000 g for 15 min in a Beckman TLX Ultracentrifuge. The pellet was suspended in 2–3 ml of Buffer A containing 5 μg phallolidin and the supernatant homogenized in a Teflon glass homogenizer. Following sedimentation, the resulting pellet was solubilized in 0.5–1.0 ml of Buffer A, 5 μg phallolidin, 1 μl ATP, and 5 μg MgCl<sub>2</sub>. Resedimentation at 543,000 g for 15 min results in a supernatant that contains the purified HMMS<sub>exp</sub>. Practically all of the HMMS<sub>exp</sub> appears to be soluble and cycles appropriately with actin in this procedure. Typically, there was no contamination with reticulogenin (59% cells). Prior to characterization, the purified HMMS was dialyzed in a 500-fold excess of 25 mM KCl, 10 mM MOPS (pH 7.2), 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 5 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 2 mM leupeptin, 0.1 mM PMSF, 5 μg dithiothreitol, and 5 μg leupeptin.

Purification of Bovine Brain Myosin—Blood vessels and meninges were carefully removed from fresh bovine brains and the brains washed with ice-cold physiological saline solution. The tissue was homogenized in a buffer containing 20 mM MOPS (pH 7.2), 2 mM EDTA, 2 mM MgCl<sub>2</sub>, 3 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 2 mM dithiothreitol, 0.1 mM PMSF, 5 μg leupeptin. The mixture was sedimented (10,000 g for 60 min) and myosin was
extracted from the pellet in the presence of 5 mM ATP, 30 mM MOPS (pH 7.2), 1 mM NaCl, 2 mM EGTA, 4 mM EDTA, 3 mM Na2MoO4, 2 mM dithiothreitol, 0.1% PMSF, and 5 μg/ml leupeptin. Following sedimentation (80,000 × g for 60 min), the supernatant was subjected to ammonium sulfate fractionation, and the 35–60% fraction was solubilized and chromatographed on a Sepharose CL-4B column. Fractions containing actin and myosin were pooled, concentrated, and used for phosphorylation.

Phosphorylation—The following kinases were used to phosphorylate myosin and HMM: Cdc2 kinase, MAP kinase, Cdk5 kinase, protein kinase C, cAMP-dependent kinase, and calmodulin-dependent kinase II (CaM kinase II). Human HeLa cell Cdc2 kinase was a gift of Fumio Murakami, University of Wisconsin, Madison, WI. Recombinant CaM kinase II was obtained from Upstate Biotechnology, Ithaca, NY. Recombinant human Cdc2 kinase was purchased from Upstate Biotechnology, Ithaca, NY, and baculovirus expressed human Cdc2 kinase was purchased from New England Biolabs (Beverly, MA). Purified chicken MHC exp or bovine brain myosin purified from bovine brain cerebral cortex (0.2–1 mg/ml), was incubated at 30°C for 1 h in 50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, 0.2 mM EGTA, 1 mM dithiothreitol, 0.5 μM protein kinase A inhibitor, 0.1 mM ATP (10 μCi of [32P]ATP, 30 Ci/mmol, DuPont NEN), 1–4 μg kinase in a total volume of 0.03–0.05 ml. The synthetic peptide, DHNIPPESPKPVK, duplicating the expected tryptic peptide in MHC, was incubated with myosin and HMM in the presence of 5 μM unlabelled F-actin to improve the quality of movement (30). After phosphorylation and addition of 10 mM EDTA, the MHC exp actin buffer (20 mM KCl, 20 mM MOPS, 5 mM MgCl2, 0.1 mM EGTA, 1 mM dithiothreitol, and 20 mM rhodamine phallolidin actin), the movement was initiated by the addition of assay buffer containing 80 mM KCl, 20 mM MOPS (pH 7.2), 5 mM MgCl2, 1 mM ATP, 0.1 mM EGTA, 0.2 mM CaCl2, 5 mM dithiothreitol, 10 mM calmodulin, 5 × 10−6 M myosin light chain kinase in the presence of 5 μM unlabelled F-actin to improve the quality of movement (30). After incubation and addition of 10 mM EDTA, the MHC exp actin buffer (20 mM KCl, 20 mM MOPS, 5 mM MgCl2, 0.1 mM EGTA, 1 mM dithiothreitol, and 20 mM rhodamine phallolidin actin), the movement was initiated by the addition of assay buffer containing 80 mM KCl, 20 mM MOPS (pH 7.2), 5 mM MgCl2, 1 mM ATP, 0.1 mM EGTA, 200 mM smooth muscle tropomyosin, 50 mM dithiothreitol, 2.5 mM Mg2+ gluco- cose, 0.1 mM Mg2+ glucose oxidase, 2 μM catalase, 0.7% methylcellulose, 30 mM HEPES, and the data were recorded on 2-min fields on SVHS tapes using image averaging and minimal illumination levels. Actin filament movement was quantified according to Homsher et al. (31). Data points were taken at 10-s intervals, which resulted in centroid displacements of 4–5 pixels. Data were taken from individual preparations only if 80–100% of the actin filaments in a field were moving simultaneously.

Miscellaneous Procedures—Rat brain calmodulin (32), chicken gizzard myosin light chain kinase (33), rabbit skeletal muscle actin (34), smooth muscle HMM (35), and rabbit skeletal muscle tropomyosin (36) were purified as described previously. Protein concentrations were determined using the Bio-Rad Protein Assay. Smooth muscle HMM was used as a standard.

RESULTS

Purification of HMM exp—As detailed under “Experimental Procedures,” Sf9 cells were coinfected with two viral constructs, one containing cDNA encoding an MHC-like fragment (amino acids 1–1231) of chicken nonmuscle MHC II-B and a second virus containing cDNA encoding both MLC150 and MLC17.

In separate experiments, Sf9 cells were coinfected with viruses containing cDNA encoding an MHC-like fragment of MHC II-B, which contains 10 extra amino acids (PESPKPVKHQ) starting after amino acid 211 along with the same MLCs. Our initial experience had shown that expression of the MHC150 fragment (150 kDa) alone or the MHC150 and the MLC20 lead to an insoluble, aggregated product, which sedimented at velocities of 47,000 × g (data not shown). In contrast, coexpression of the MHC150 along with both MLCs resulted in the expressed polypeptides being soluble at both high (0.6 M) and low (0.2 M) NaCl concentrations following sedimentation at 300,000 × g for 1 h.

Purification of HMM without the insert (HMM1) and with the insert (HMM2) is shown in Fig. 2. The only difference between these two isoforms is the presence of the 10 amino acids in loop 1 starting after residue 211 in the MHC. Lanes 1 and 4 show the pattern of polypeptide staining following the initial extraction of Sf9 cells. Examination of both the low speed and high speed (300,000 × g) pellets revealed that most of the MHC exp was soluble under these conditions (data not shown). Lanes 2 and 5 show the polypeptide pattern following fractionation of the extract supernatant with 40–60% ammonium sulfate. Lanes 3 and 6 show the purified HMM exp following release from F-actin by MgATP. Virtually all of the HMM exp bound to actin in the absence of ATP and was released into the supernatant in the presence of ATP (data not shown). Thus, most of the expressed HMM heavy chains combined with light chains was soluble and bound to actin in an ATP-dependent manner. Although both the extract and 40–60% ammonium sulfate fraction showed overexpression of MLC150 compared to MHC150, scanning of the purified HMM exp gave a molar ratio of 1:1.1 for the MHC150 and two MLCs. Using the procedure outlined above and detailed under “Experimental Procedures,” we are able to purify between 0.4 and 2 mg of purified HMM from 108 infected cells (650 ml of 1.5 × 109 cells/ml).

Characterization of the Two Expressed HMMS—Table I compares the Vmax of the actin-activated MgATPase activity, the Km of HMM exp for actin, and the velocity of actin filament propulsion for both isoforms. The data show that the presence...
of the 10-amino acid insert has only a modest effect on these parameters. Fig. 3 depicts the difference in the in vitro motility assays for six different preparations of the two isoforms that were prepared at the same time. Although preparations 2, 4, and 5 appear to show some differences in the rate of actin movement between the two isoforms, this difference does not always appear to be significant. The increase in the average mean velocity for HMMi compared to HMMn is about 20% (Table I). Likewise, HMMi shows a similar small increase in the actin-activated MgATPase activity compared to HMMn.

Phosphorylation of HMM exp—The presence of a consensus sequence for proline-directed kinases (SPK) (37) suggested that the single serine residue in the 10-amino acid insert of the expressed chicken brain MHC II-B1 might be phosphorylated by Cdc2 kinase, MAP kinase, and/or the brain specific Cdk5 kinase. Fig. 4 shows a Coomassie Blue-stained gel and corresponding autoradiogram of HMMn and HMMi following phosphorylation by Cdk5 kinase. As can be seen from the autoradiogram, only the isoform containing the inserted sequence is phosphorylated on the heavy chain. In addition, the MLC20 of both isoforms is also phosphorylated, although to a lesser extent.

In order to identify the site(s) phosphorylated by Cdk5 kinase, both the phosphorylated MHC150 and MLC20 bands were excised from the gel, digested with trypsin, and subjected to gel isoelectric focusing. Fig. 5 shows that the major phosphopeptide generated by trypsin comigrates with a synthetic phosphopeptide with the amino acid sequence: DHNIPPESPKVPK. This peptide represents the amino acid sequence of the predicted tryptic peptide from the inserted MHC sequence (see "Experimental Procedures"). Data shown are the mean velocity with error bars indicating the standard deviation.
the bottom of the gel is most likely due to partial cleavage of the RKD sequence at the amino-terminal end of the expected tryptic peptide to yield KDHNIPPESPKPVK, in addition to the RKD sequence at the amino-terminal end of the expected tryptic peptide.

We studied the ability of two other proline-dependent kinases to catalyze phosphorylation of MHCII-B. Both MAP kinase and CaM kinase II, nor protein kinase C could catalyze phosphorylation of HMMi. In addition to proline-directed kinases, we also obtained the results of an in vitro phosphorylation assay using bovine brain myosin as well as HMMi, as substrate. Panel A is a Coomassie Blue-stained SDS-10% polyacrylamide gel, and panel B is the corresponding autoradiogram. The figure shows that purified bovine brain MHC II-B1 is a substrate for Cdc2 kinase, and phosphorylation of the MLC from the gel and digested with trypsin. The tryptic peptides were analyzed by an isoelectric focusing gel, and the major phosphopeptide was identified as containing Ser-214 based on its comigration with standards (see Fig. 5).

**TABLE II**

| Experiment | MHC phosphorylated | MHC unphosphorylated | Stoichiometry |
|------------|-------------------|----------------------|--------------|
| 1 | HMM2 | 0.32 | 0.27 | 0.3 |
| 2 | HMM2 | 0.47 | 0.34 | 0.3 |

**DISCUSSION**

The pre-mRNA encoding MHC II-B, but not MHC II-A, is subject to alternative splicing to produce a number of different isoforms that are only expressed in avian and mammalian neuronal cells. The function of the isoforms produced by this splicing is not known and they appear to be confined to cells that are part of the central nervous system. In chicken brain, the B1 isoform, detected by quantitative polymerase chain reaction, is already present by embryonic day 4 (the first day analyzed) and it reaches a peak on embryonic day 10 (14). Using S-1 nuclease analysis, Takahashi et al. (13) were only able to detect MHCII-B1(14), which had previously been shown to contain both MHC II-B and MHCII-B1(13), could be phosphorylated. Fig. 6 shows that phosphorylation of the heavy chain of this isoform (data not shown). The phosphorylation of the heavy chain of HMMi phosphorylated by Cdk5 kinase from the samples electrophoresed in Fig. 4. Lane 3 shows the focusing of a synthetic phosphopeptide, DHNIPPESPKPVK, that was phosphorylated using the same kinase. This peptide corresponds to the sequence of amino acids 207–219 in HMMi (see Fig. 1). Lane 4 shows the phosphorylation of the peptides of the MLC from the same sample. The identification of the two light chain phosphopeptides as Ser-1 and Ser-128, which had previously been shown to contain both MHC II-B and MHCII-B1(13), could be phosphorylated. The identification of the MHC peptide as containing Ser-214 based on its comigration with standards (see Fig. 5).
able to detect small amounts of mRNA encoding MHC II-B1 in the adult chicken brain. On the other hand, human cerebral cortex and retina have been shown to be highly enriched for mRNA encoding MHC II-B1 and have been shown to express the 10-amino acid inserted peptide (14).

Kelley et al. (18) found that smooth muscle myosin containing an insert of seven amino acids (QGPSFSY) in the exact same place in loop 1 as that described for the nonmuscle MHC II-B1, translocates actin filaments 2.5 times faster than does smooth muscle myosin, which does not contain the inserted amino acids. Uyeda et al. (38) produced chimeric Dictyostelium myosins in which the amino acid sequence in the loop 2 region was exchanged for the homologous sequence from other types of myosins. These substitutions were found to modulate the actin-activated MgATPase activity of the chimeric myosins in a manner roughly proportional to the rate of the myosin from which the loop was derived, but did not have a similar effect on the rate of in vitro motility. Based on these two biochemical studies and the location of the two inserts in the crystal structure of myosin, Spudich suggested that the sequence in loop 1, which is near the ATP binding pocket, might have a profound effect on the translocation of actin filaments by myosin in the in vitro motility assay, whereas the sequence in loop 2 near an actin binding site may affect the actin-activated MgATPase activity (11).

We found no major effect on either of these two activities when comparing side by side preparations of baculovirus expressed truncated MHC II-B isoforms that either contained or did not contain the inserted sequence in loop 1. Although both Fig. 3 and Table I show higher values for both the in vitro motility assay and the actin-activated MgATPase activity, these increases are, at best, modest. This suggests that the presence of this insert in neuronal cell myosin may have other functional consequences rather than to alter these two parameters of myosin activity or that this is a subtle modulatory mechanism.

The presence of a consensus sequence for proline-directed kinases in the inserted residues raised the possibility that this MHC might serve as a substrate for a number of kinases, including Cdc2, Cdk5, and MAP kinase. Previous work with Xenopus MHC II-B, which contains a similar, although 6 amino acids longer, inserted region at loop 1, has shown that the insert can be phosphorylated by Cdc2 kinase, but not MAP kinase (21). In this paper, we show that a number of proline-directed kinases can phosphorylate the 10-amino acid insert in the chicken nonmuscle MHC. This inability of Xenopus MHC II-B to be phosphorylated by MAP kinase may reflect the difference in sequence (TESPK versus PESPK) between the species (see Fig. 1) and might also be related to the 6 extra amino acids present in the Xenopus insert. We also found that Cdk5 kinase could phosphorylate the MLC20 at the same site phosphorylated by protein kinase C, in agreement with the observation of Satterwhite et al. (39) using Cdc2 kinase. The extent of MLC phosphorylation was considerably less than that of the MHC with Cdk5, an enzyme that appears to be only active in neuronal tissue (37).

Despite multiple additions of kinase, only 30–40% of the MHC was phosphorylated. This did not appear to be due to prior phosphorylation of the MHC since 32P0-labeling of the S9 cells just prior to harvesting showed no evidence for labeling of MHC, following SDS-polyacrylamide gel electrophoresis of a lysed cell extract (data not shown). Failure to obtain more than 40% phosphorylation of Ser-214 in vitro may mean that we have not yet identified the relevant proline-directed kinase, the right conditions for phosphorylation or both. In any case, the partial phosphorylation of the MHC that we observed had no significant effect on the actin-activated MgATPase activity.

What then could be the role of the 10-amino acid insert? Previous work has shown that splicing of the mRNA to introduce the insert is responsive to certain signal transduction pathways. For example, mRNA encoding MHC II-B can be induced to splice in the 30 nucleotides encoding MHC II-B1 by treating rat PC-12 cells with nerve growth factor, but not epithelial growth factor. The cells then cease to divide and initiate neurite outgrowth (14). Since MHC II-B is the major isoform (perhaps the only isoform since the amount of MHC II-A in brain is small and may be expressed only in non neuronal cells) present in neuronal cells, it is conceivable that splicing in the insert acts as a localization mechanism, permitting the myosin II-B1 to be bound in a particular part of the cell. Phosphorylation of the myosin in the inserted sequence might then act to regulate this association. Studies to explore this possibility are presently under way.

Previous investigators have used the baculovirus expression system to express other HMMs, including smooth muscle HMM (40–42) and cardiac HMM (43). Of particular interest have been studies using site-directed mutagenesis to understand the mechanism of smooth muscle myosin regulation (40). To our knowledge, this is the first report on expression of a vertebrate nonmuscle myosin. The ability to express milligram quantities of an enzymatically active myosin fragment and to introduce discrete mutations is proving to be a powerful technique in understanding all forms of myosins. Our study has shown that the system is also a valuable technique for studying different functions of closely related isoforms of myosin that would prove extremely difficult to obtain in pure form.

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