Protein Kinase C Phosphorylates Nonmuscle Myosin-II Heavy Chain from Drosophila but Regulation of Myosin Function by This Enzyme Is Not Required for Viability in Flies†

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ABSTRACT: Conventional myosins (myosin-IIs) generate forces for cell shape change and cell motility. Myosin heavy chain phosphorylation regulates myosin function in simple eukaryotes and may also be important in metazoans. To investigate this regulation in a complex eukaryote, we purified the *Drosophila* myosin-II tail expressed in *Escherichia coli* and showed that it was phosphorylated in vitro by protein kinase C(PKC) at serines 1936 and 1944, which are located in the nonhelical globular tail piece. These sites are close to a conserved serine that is phosphorylated in vertebrate, nonmuscle myosin-IIs. If the two serines are mutagenized to alanine or aspartic acid, phosphorylation no longer occurs. Using a 341 amino acid tail fragment, we show that there is no difference in the salt-dependent assembly of wild-type phosphorylated and mutagenized polypeptides. Thus, the nonmuscle myosin heavy chain in *Drosophila*, which is encoded by the *zipper* gene, appears to be similar to rabbit nonmuscle myosin-IIA. In vivo, we generated transgenic flies that expressed the various myosin heavy chain variants in a *zipper* null or near-null genetic background. Like their wild-type counterparts, such variants are able to completely rescue the lethal phenotype due to severe *zipper* mutations. These results suggest that while the myosin-II heavy chain can be phosphorylated by PKC, regulation by this enzyme is not required for viability in *Drosophila*. Conservation during 530−1000 million years of evolution suggests that regulation by heavy chain phosphorylation may contribute to nonmuscle myosin-II function in some real, but minor, way.

Conventional myosins (myosin-IIs) form part of a large superfamily of motor proteins (*1*−*3*) and generate forces for cell movements, cell shape changes, and other cellular motilities in both muscle and nonmuscle cells. In nonmuscle cells, these movements have been genetically characterized in *Dictyostelium* and *Drosophila* (see references below) through microinjection studies using anti-myosin antibodies (e.g., *4*), through pharmacological studies in cultured cells (e.g., *5*), and through numerous cell biological studies (reviewed in *6*).

Native myosin-II is a hexamer formed from a pair of heavy chains, a pair of essential or alkali light chains, and a pair of regulatory light chains. The carboxyl-terminal rod domains of two myosin-II heavy chains fold into the α helical, coiled-coil tail of native myosin-II. In turn, these tails self-assemble to form bipolar filaments whose morphology is characteristic of the tissue from which the myosin-II comes (*7*−*11*). These bipolar filaments are the fundamental unit of myosin-II contractile activity, because they provide the structural arrangement that allows myosin-II to drive force production among anti-polar actin filaments.

Regulation of myosin-II function involves molecular switches that influence both the motor activity of the myosin-II head and the ability of myosin-II to form filaments (reviewed in *6*, *12*). In metazoan smooth and nonmuscle cells, phosphorylation of the regulatory light chain causes a conformational change in the myosin-II tail that promotes assembly of filaments and also can activate the motor activity of the myosin-II head.

Regulation of myosin-II function in metazoans may also depend on post-translational modifications of the myosin-II tail (reviewed in *6*). In simpler eukaryotes, like *Acanthamoeba* or *Dictyostelium*, in vitro studies suggest that regulation of myosin-II contractility is largely through phosphorylation of serine or threonine residues in the tail region of the myosin-II heavy chain (*13*, *14*, reviewed in *15*, *16*). In *Dictyostelium*, molecular genetic analysis shows that myosin variants that replace the relevant threonines with aspartic acid, which mimics the phosphorylated state of myosin, behave as myosin nulls, demonstrating that modulation of heavy chain phosphorylation is essential in this organism (*15*, *17*).

Phosphorylation of sites on the tail of metazoan myosin-IIs has also been observed, but its biological significance is not so clear. Vertebrate nonmuscle myosin-IIs have been

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studied most extensively, and both protein kinase C[1](PKC) and casein kinase II (CKII) phosphorylate a number of different vertebrate nonmuscle myosin-II heavy chains near their carboxyl termini (see *18*−*23*). More specifically, in rabbits, two or perhaps three distinct genes encode three distinct protein isoforms (MIIA, MIIBα, and MIIBβ) which, pertinent to our studies, differ in sequence at their carboxyl termini, near the tip of the myosin-II tail (the sequences diverge in other regions as well). Murakami and co-workers have investigated the effects of heavy chain phosphorylation of each isoform by both enzymes using in vitro assays designed to evaluate the assembly of fragments of the myosin-II tail (*20*). Independent of phosphorylation, the solubility of the fragments derived from the different isoforms differed as a function of salt concentration. In addition, fragments from the different isoforms responded differently to phosphorylation by PKC and CKII. Phosphorylation by both PKC and CKII favored disassembly of MIIBα at low salt concentration, whereas phosphorylation by neither enzyme had an effect on the assembly of MIIA. In contrast, phosphorylation by PKC favored disassembly of MIIBβ at low salt concentration, but CKII phosphorylation had only minimal effects on the solubility of this isoform. Together, these results suggest the hypothesis that heavy chain phosphorylation is biologically significant and plays a role in the regulation of myosin-II function through effects on myosin-II filament formation. The experiments we describe here are designed to directly evaluate the biological significance of PKC phosphorylation of nonmuscle myosin-II heavy chain.

There are several advantages to the use of the fly as a model system. Sequence similarity among metazoan myosin-II tails near the site of phosphorylation suggests that *Drosophila* might be an appropriate model for the analysis of the biological significance of myosin-II heavy chain phosphorylation in metazoans (*24*, *25*). Further, the use of *Drosophila* might shed light on the regulation of myosin-II by phosphorylation in vertebrates. In addition, there is only one gene for nonmuscle myosin-II heavy chain (*26*, *27*); the genomic sequence of the fly myosin-II heavy chain is completely known, therefore, we can rule out differential splicing that could give rise to isoform diversity at the carboxyl-terminal end of the protein (*24*, *26*), and null or near-null mutations in the nonmuscle myosin-II heavy chain are available (*28*). Thus, appropriate transgenic methods, coupled with classical genetic strategies, can be used to introduce mutagenized myosin-II transgenes into genetic backgrounds that otherwise lack myosin-II function. This strategy allows us to test the importance of individual residues or specific regions of myosin-II for the in vivo assessment of phosphorylation effects on myosin-II function.

Here, we expressed fragments of the *Drosophila* myosin-II tail that span close to its entire length in *Escherichia coli* and showed that it is phosphorylated in vitro by PKC (α, β, and γ) at two sites (serine 1936 and serine 1944), which are located in the nonhelical globular tail (which starts at lysine 1925) near its carboxyl terminus. These sites are close to, but not identical to, a conserved serine that is phosphorylated in vertebrate, nonmuscle myosin-IIs. We also show that after the two serine sites are mutagenized to either alanines or aspartic acids, phosphorylation by PKC no longer occurs. Using a 341 amino acid tail fragment in assembly studies, we show that there is no observable difference in the salt-dependent assembly of wild-type, phosphorylated, and mutagenized polypeptides. Thus, the single nonmuscle myosin heavy chain in *Drosophila* appears to be most like the rabbit nonmuscle myosin-IIA and its counterparts in other mammalian organisms. Further, in vivo genetic studies on transgenic flies that carry myosin-II variants with alanine or aspartate mutations at the sites we show are phosphorylated in *Drosophila* nonmuscle myosin-II heavy chain, or that correspond to the site phosphorylated in vertebrate nonmuscle myosin II heavy chain, are able to completely rescue the lethal phenotype due to severe (near null) *zipper* mutations. These results suggest that while myosin-II heavy chain can be phosphorylated by PKC, regulation of myosin function by this enzyme is not required for viability in *Drosophila*.

[1] Abbreviations: PKC, protein kinase C; MHC, myosin heavy chain.

EXPERIMENTAL PROTOCOLS

Materials. Rat brain protein kinase C (PKC, a mixture of α, β, and γ isoforms) was a gift from Dr. Patrick Casey (Department of Molecular Cancer Biology, Duke University) or was purchased from Promega (Madison, WI). In each case, the enzyme was purified as described elsewhere (*29*). Rabbit skeletal muscle myosin-II was a gift from Dr. Meg Titus (Department of Cell Biology, Duke University). Coomassie Plus protein assay reagent was obtained from Pierce Biochemicals (Rockford, IL). ^{32}P-γ-ATP was obtained from Amersham Life Science (Arlington Heights, IL). L-α-Phosphatidyl-L-serine (PS) was obtained from Sigma (St. Louis, MO). Liposomes for the phosphorylation assays were prepared from PS, according to the procedure of Murakami et al. (*21*). TLC silica gel plates were purchased from Sigma, and TLC cellulose plates were purchased from Merck (Whitehouse Station, NJ). Other reagent-grade chemicals were from Sigma.

Preparation of the Expression Construct for a Myosin-II Tail Fragment. A *Nco*I-*Eco*RI cDNA fragment that encodes a 341 amino acid peptide from a *Drosophila melanogaster* nonmuscle MHC tail was subcloned from a previously available construct, pCasPer-hs-mhc-1, which contains a full-length cDNA for MHC (see Figure 1, accession number M35102, refs *24*, *28*, *30*), in the expression vector pMW 172 (*32*). Standard cloning methods were used here and throughout these studies (*33*). The final construct adds two amino acids (a methionine and a glycine) to the *N*-terminal end of the 341 amino acid myosin-II tail fragment. This construct was named pMW-C-LMM-341 (Figure 1A).

Expression and Purification of the MHC Tail Fragment, C-LMM-341 from E. coli. The pMW-C-LMM-341 construct was transformed into a bacterial expression strain, BL21-(DE3) from Novagen (Madison, WI). Protein was expressed and purified as described elsewhere (*34, 35*)

Preparation of Rabbit Skeletal Muscle Light Meromyosin. Light meromyosin was prepared from rabbit skeletal muscle by the method of Margossian and Lowey (*34*). Purity of LMM was assayed by SDS−PAGE (data not shown).

Electron Microscopy. Myosin-II tail preparations, at 0.15 mg/mL in high salt buffer, were diluted with 3 volumes of

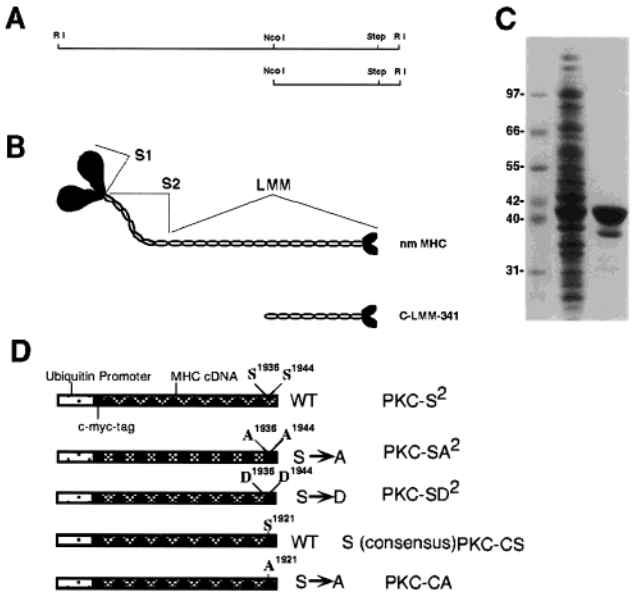

FIGURE 1: Subcloning, expression, and purification of a tail fragment of nonmuscle myosin-II. (A) A schematic diagram of the cDNA that encodes the entire nonmuscle myosin II heavy chain shows selected restriction sites and the translation stop codon. The *Nco*I-*Eco*RI fragment that encodes a tailpiece of 341 amino acids (C-LMM-341) was used for subcloning into an expression vector, pMW172. (B) A diagram of the nonmuscle myosin II heavy chain and C-LMM-341 shows the length and location of C-LMM-341 relative to MHC. (C) A Coomassie-stained SDS−PAGE gel shows molecular weight standards (molecular mass shown in kDa, to the left), high-speed supernatant of the whole bacteria lysate, and the C-LMM-341 fragment after purification. The minor contaminant is likely a proteolytic breakdown, because the longer the protein is stored at 4 °C, the more the contaminant. (D) A schematic shows the mutagenized heavy chain constructs with both PKC phosphorylation site serines mutagenized to either alanine or aspartic acid.

buffer that contained no salt (10 mM imidazole−Cl, pH 7.0) to a final salt concentration of 150 mM NaCl and prepared for negatively stained electron microscopy as described elsewhere (*35*).

Sedimentation Assay. Nonphosphorylated, phosphorylated, and mutated C-LMM-341 were diluted into buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA), containing a range of NaCl concentrations, to a final protein concentration of 160 μg/mL, in a volume of 100 μL. As a control, rabbit skeletal myosin-II LMM was treated in parallel. The mixtures were kept on ice for 10 min and centrifuged (100000*g*, 30 min). Ten microliters of supernatant was taken from each mixture in order to measure protein concentrations. The protein concentration was measured in 96-well plate that was read at 595 nM, using Coomassie Plus protein assay. The percentage of proteins that remained in the supernatant was determined and the data were plotted by Cricket Graph (Cricket software, Malvern, PA).

Phosphorylation Assay. Phosphorylation of nonmuscle myosin-II heavy chain was analyzed by a modification of the methods of Murakami et al. (*20*).

Peptide Mapping and Phosphoamino Acid Analysis. The myosin-II band containing 50 μg of protein that was phosphorylated by PKC was excised from the gel and then peptide mapped using the protocols described elsewhere (*18, 36*).

Purification of the Peptides and Peptide Sequencing. One aliquot of the lyophilized, trypsin-digested, PKC-phosphorylated myosin-II tail was dissolved in 0.1% trifluoroacetic acid in water and injected onto a C18 reverse-phase HPLC column (Vydac, The Nest Group, South Borough, MA). Peptides were eluted with a linear gradient of 10−60% (v/v) acetonitrile, 0.1% trifluoroacetic acid over 60 min, at a flow rate of 1 mL/min. A single radioactive peak that eluted at an acetonitrile concentration of approximately 20% was lyophilized, injected back onto the same C18 column, and eluted with an acetonitrile gradient of 10−30%. Two radioactive peaks were obtained and used for amino acid sequencing. The samples were analyzed by mass spectrometry and Edman degradation at the Harvard University Microchemistry Facility (Cambridge, MA).

Mutagenesis of the PKC Phosphorylation Sites and P Element-Mediated Germline Transformation. PKC phosphorylation sites were mutagenized by standard PCR strategies (*15*), using primers that contained codons for either alanine or aspartic acid at the respective serine sites. The PCR product was subcloned into appropriate vectors, and the DNA flanking the restriction sites was sequenced to verify cloning junctions. The mutated proteins were expressed and purified from bacteria (see above). The MHC cDNA containing various mutations was also cloned into a P-element-based vector, pWUM, for the production of transgenic flies and subsequent in vivo genetic analysis (*45*). pWUM carries the P-element insertion sequences (p), a *white*$^+$ marker (W, that dominantly marks transgenic flies in a w^- genetic background), the ubiquitin promoter (U), and full length MHC (M) coding sequence (Figure 1D). Figure 1 provides an overview of the relevant *zipper* cDNA clones and the nonmuscle myosin-II heavy chain that they encode. The various transgenic constructs were then introduced into the fly genome by P-element-mediated transformation using standard methods (*37*).

Fly Stocks and Rescue Crosses. The names of the genes and chromosomes are as described (Flybase, http://flybase.bio.indiana.edu:82, and *38*). Various transgenic myosin-II constructs (encoding wild-type myosin-II or with the introduced PKC site mutations) were followed in flies during genetic analysis by their tight linkage to the w^+ marker in the pWUM transgene vector. These flies were crossed to flies of appropriate genetic backgrounds that included *zipper* mutations on visibly marked chromosomes in trans to second chromosome balancers, which suppress meiotic recombination and ensure absolute linkage between visible marker mutations and the *zip* alleles. Alleles of *zipper* included a weak allele ($zip^{1.6}$) and two severe (null or near-null) alleles (zip^2 and zip^{1D16}). The ability of PKC site mutations to rescue MHC mutant flies was tested by evaluating the viability and morphology of genetically marked, trans-heterozygous *zipper* flies in the presence or absence of the rescue construct. The use of trans-heterozygous combinations of mutant alleles is common practice in such rescue experiments because it avoids complications in the interpretation of experimental results caused by the accumulation of second-site lethal mutations on the balanced chromosome stocks. In the absence of the rescue construct, we confirmed that the trans-heterozygous combinations of *zip* alleles (specifically *Sco sax cn bw* $zip^{1.6}$/*bw sp* zip^2 or *bw sp* zip^2/*cn bw sp* $zip^{1D1.6}$) were lethal. For each cross, the number of rescued flies was compared to that expected on the basis of total number of

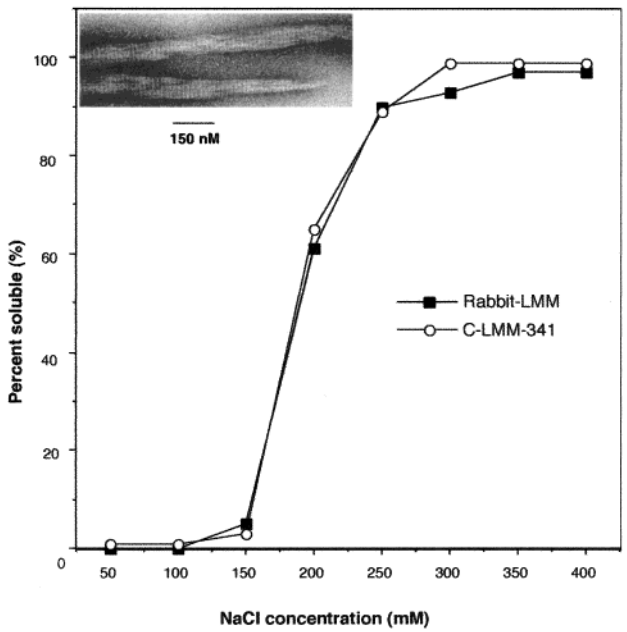

FIGURE 2: The purified nonmuscle myosin-II fragment, C-LMM-341, assembles in a salt-dependent manner. Sedimentation assays (open circles) and electron microscopy of negatively stained specimens (inset). Light meromyosin from the rabbit skeletal myosin-II heavy chain serves as a control (closed squares). The protein was adjusted to a concentration of 2 mg/mL and was diluted into various salt concentrations, with a final volume of 100 μL. The mixtures were incubated on ice for 10 min and then centrifuged at 100000 g for 30 min. The protein concentrations in the supernatant were measured, and the percentage of the protein in the supernatant was plotted as a function of salt concentration using Cricket Graph.

the progeny in the nontestor classes and is expressed as % Mendelian expectations.

Nomenclature and zip Coding Sequence. The *zipper* gene is differentially spliced to yield four potential transcripts (we do not know if all four are made or are stable). The cDNA used in these experiments is designated 1b2a by the nomenclature of Mansfield et al. (*24*), which means that it includes a short version of exon 1 that results in translation start in exon 2 and does not include exon 7, which inserts a 40 amino acid sequence into the myosin-II head region. The corrected sequence of this *zip* transcript is given at accession M35012, and that of the *zip* gene is at U35816, and alterations are introduced in the coding sequence of *zip* based on exon sequencing by Halsell et al. (*31*).

RESULTS

Purification and Characterization of a Phosphorylatable, Self-Assembling Fragment of the Nonmuscle Myosin-II Tail. The myosin tail fragment C-LMM-341 (amino acid 1631−1972) from *D. melanogaster* nonmuscle myosin-II heavy chain was expressed in *E. coli* and purified as described in the Materials and Methods (Figure 1B). The purified C-LMM-341 fragment behaved like the light meromyosin fragment from rabbit skeletal muscle myosin-II in a sedimentation assay (Figure 2). The fragment was nearly 100% sedimentable at 50 mM NaCl, more soluble when the salt concentration was increased, and nearly completely soluble above 300 mM NaCl. To verify that the sedimentable species was myosin assembled into paracrystals, negatively stained specimens were examined by electron microscopy. C-LMM-341 formed paracrystals (inset in Figure 2) that were very similar to the ones formed by rabbit light meromyosin (e.g., *35*).

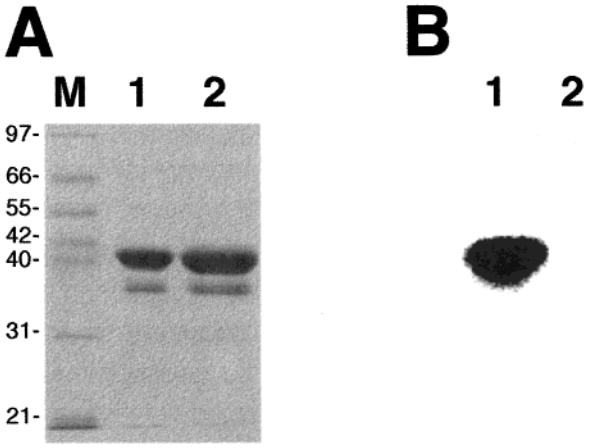

FIGURE 3: C-LMM-341 is phosphorylated by PKC. C-LMM-341 was incubated with or without PKC for 30 min at 30 °C, in the presence of ^{32}P-γ-ATP, and then treated as described in the Experimental Protocols. (A) Coomassie-stained SDS−PAGE gel. Lane M, molecular weight markers with molecular masses in kDa shown to the left. Lane 1, C-LMM-341 phosphorylated by PKC. Lane 2, C-LMM-341 "mock" phosphorylated in incubation buffer, but no PKC. (B) Autoradiograph of the gel shown in A shows that ^{32}P labels C-LMM-341 in the presence (lane 1) but not in the absence of PKC (lane 2).

D. melanogaster Nonmuscle Myosin-II Heavy Chain is Phosphorylated by Protein Kinase C at Two Serines. To examine if *Drosophila* nonmuscle myosin-II can be phosphorylated by PKC, we used the entire myosin-II tail (or "rod") and two fragments of the tail that together span the equivalent of light meromyosin (LMM) as substrates. In the *Drosophila zipper* myosin-II heavy chain, the hinge region that marks the beginning of LMM spans residues 1240−1256. Specifically, the three fragments were (1) the myosin-II rod (the tail distal to the proline at residue 841), (2) a fragment of the rod (N-LMM-687) that starts at residue 1037 and ends at residue 1723, and (3) a C-terminal fragment of the LMM (C-LMM-341) fragment that starts at residue 1631 and extends to the end of the tail. Because myosin-II head fragments are insoluble when made in *E. coli*, we did not directly test whether PKC could phosphorylate residues in the *zipper* myosin-II head. However, it is highly unlikely that there are residues that are phosphorylated by PKC in the *zipper* myosin-II head because serine and threonine residues, known to be phosphorylated in other myosin heads (*39*), are substituted with charged amino acids in the *zipper* myosin head. We found that the C-LMM-341 contained all sites that were phosphorylated by PKC. The phosphorylated protein was analyzed by SDS−PAGE (Figure 3A) and autoradiography (Figure 3B). C-LMM-341 was phosphorylated by PKC in the presence of calcium, ^{32}P-γ-ATP, and liposomes that were formed from phosphotidylserine. The maximum incorporation of phosphate into the myosin-II tail was reached after 1 h incubation at 30 °C, to a molar ratio of 1.8 (Figure 4). This ratio was stable for at least 24 h. The incorporation rate was not affected by salt concentration from 100 to 300 mM NaCl, conditions that favor myosin-II assembly and disassembly, respectively (data not shown). To determine the number of phosphorylated peptides, and the amino acids that were phosphorylated, we performed

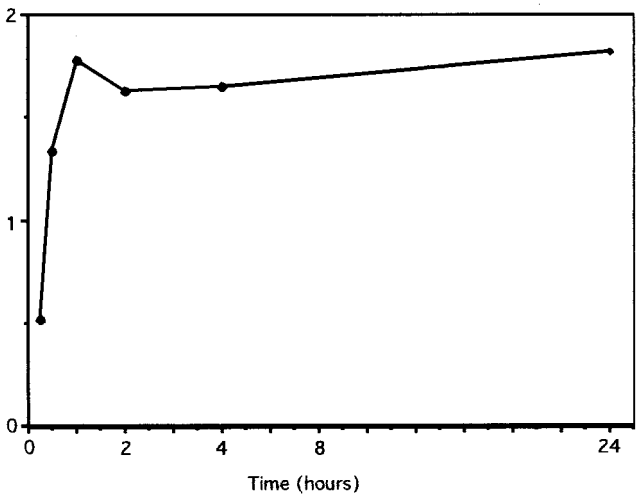

FIGURE 4: PKC rapidly phosphorylates C-LMM-341 to approximately 2 mol of PO_4 per mol of protein. Phosphate incorporation was determined as described in the Experimental Protocols. The molar ratio of phosphate to C-LMM-341 was calculated and plotted as a function of time. The peak incorporation was reached after 1 h at 30 °C and remained stable over a 24 h course.

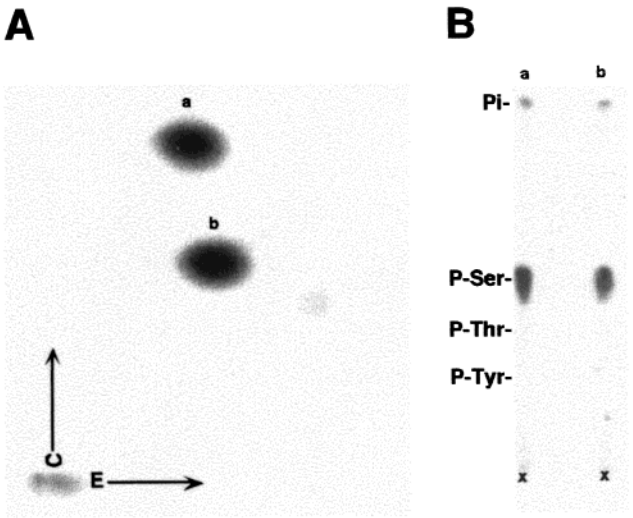

FIGURE 5: Peptide mapping and phosphoamino acid analysis demonstrates that PKC phosphorylates C-LMM-341 at two different serine residues. (A) PKC-phosphorylated C-LMM-341 myosin tail fragment was subjected to complete trypsin digestion and then analyzed by electrophoresis and ascending chromatography on silica gel plates, as described in the Experimental Protocols. Phosphate is incorporated into two distinct peptides as detected by autoradiography (a third, minor peptide seen on the gel resulted from incomplete trypsin digestion as demonstrated through analysis of a time course of tryptic digestion (data not shown)). (B) Phosphoamino acid analysis reveals phosphorylation of serine residues. The tryptic peptides that incorporated PO_4 were subjected to amino acid analysis as described in the Experimental Protocols. Only phosphorylated serine residues were detected by autoradiography.

peptide mapping and phosphoamino acid analysis. As shown in Figure 5A, two peptides were phosphorylated when C-LMM-341 was digested to completion with trypsin. Phosphoamino acid analysis indicated that only phosphoserine was present in each peptide (Figure 5B). To map the locations of the phosphorylated serines, we purified two peptides by reverse-phase HPLC. Mass spectrometry analysis indicated that there was only one phosphoserine in each peptide. The exact positions of the phosphoserines were determined by Edman amino acid sequencing of the two

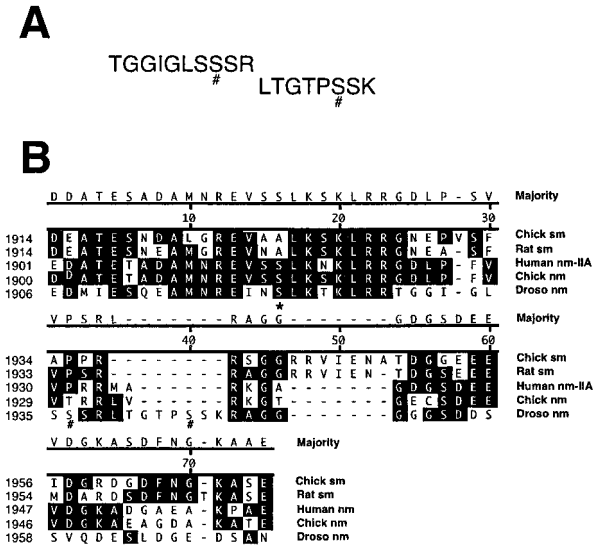

FIGURE 6: Determination of the peptide sequences and the phosphorylation sites. (A) Phosphopeptides were purified and sequenced as described in the Experimental Protocols. There is a single phosphorylated serine in each peptide (marked by a # sign). (B) Sequences of the fly nonmuscle myosin-II heavy chain near the two serines that are phosphorylated by PKC are compared to sequences from various vertebrate myosin heavy chains. The PKC phosphorylation sites mapped in *Drosophila* nonmuscle myosin heavy chain do not match with the sites found in other myosin heavy chains.

peptides, which showed that serines 1936 and 1944, which map to the nonhelical globular tail, were phosphorylated (Figure 6A). As shown in Figure 6B, the PKC phosphorylation sites in *Drosophila* nonmuscle myosin-II heavy chain are near, but do not match precisely, the single residue that is phosphorylated by PKC in vertebrate smooth and nonmuscle myosins-II (the equivalent site would be 1921, see Figure 6B, and *24*, Figure 1). Moreover, the SSR/K sequence context in which each of the two phosphoserine residues reside is consistent with the common PKC substrate, S/T−X_{2-0}−R/K_{1-3} (S or T is phosphorylated and X is any amino acid) (*40*). Furthermore, this SSR/K sequence matches the PKC phosphorylation sites, serine 1 or 2, in the vertebrate nonmuscle myosin-II regulatory light chain. The identification of the two phosphoserine sites provided us with targets for the mutagenesis that were analyzed biochemically in vitro and genetically in vivo.

Mutagenesis of the PKC Phosphorylation Sites. As a first step in understanding if phosphorylation of MHC has a role in regulating nonmuscle myosin-II functions in *Drosophila*, we mutagenized the two PKC phosphorylation sites to either alanines or aspartic acids by PCR-based mutagenesis using the expression vector pMW172- C-LMM-341 as a template. These mutagenized tail fragments were purified with the same procedures used for the purification of their wild-type counterpart (Figure 7A). We showed that this myosin-II fragment, which extends from amino acid 1631 to the normal stop codon and contains the S1936A and S1944A or S1936D and S1944D mutations, could no longer be phosphorylated by PKC. This confirms that the serine 1922, chosen by sequence alignments with other myosin-IIs as a likely substrate candidate for PKC phosphorylation, is not phosphorylated in fly MHC by PKC (Figure 7B).

Phosphorylation by Protein Kinase C Does Not Inhibit Myosin-II Filament Formation in Vitro. To investigate the

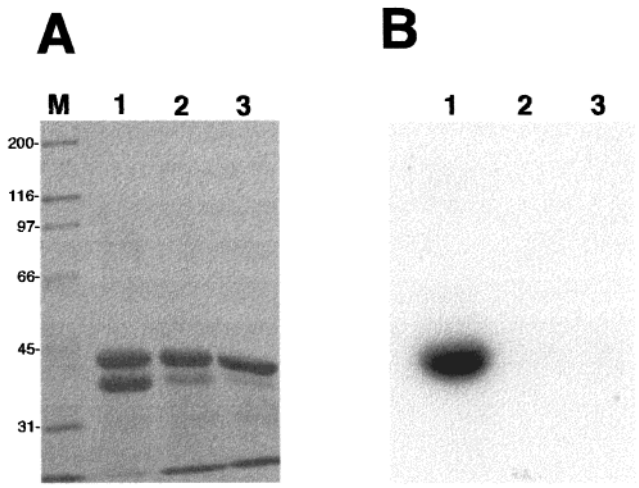

FIGURE 7: Site-directed mutagenesis of the serine residues that are phosphorylated by PKC yields variants of C-LMM-341 that can no longer be phosphorylated by PKC. (A) A Coomassie-stained SDS−PAGE gel shows the purified PKC mutant proteins. Lane M, molecular weight markers with mass in kDa shown to the left. Lane 1, wild-type C-LMM-341 (the lower band is due to sample degradation that occurs during storage). Lane 2, ser1936ala, ser1944ala double mutant. Lane 3, ser1936asp, ser1944asp double mutant. (B) An autoradiograph of the gel shown in A shows that only the wild type, and not the mutagenized C-LMM-341 polypeptides, can be phosphorylated by PKC.

mechanism by which phosphorylation regulates myosin-II function, we performed sedimentation assays that allowed us to assess the effect of phosphorylation on myosin-II filament formation, and measured protein left in the supernatant (see Materials and Methods). The average of four experiments was graphed (Figure 8). Surprisingly, phosphorylation of *Drosophila* nonmuscle myosin-II heavy chain by PKC has no significant effect on the myosin-II assembly. We saw neither over assembly when the serines were mutated to alanine nor less assembly when they were changed to aspartate residues.

Phosphorylation of Two Serines in the Nonhelical Globular Tail Is Not Required for Viability. We made cDNA constructs that carry PKC site mutations, p*WUM-PKC-SA*^{2} and p*WUM-PKC-SD*^{2}, and generated transgenic flies that carry these mutations by P-element-mediated germline transformation. Our results from these in vivo genetic studies show that PKC phosphorylation of MHC is not required for myosin-II function. Flies that are trans-heterozygous for *zipper* are viable in the presence of either p*WUM-PKC-SA*^{2} or p*WUM-PKC-SD*^{2}. Moreover, rescue of the testor class is exactly or nearly equivalent to the Mendalian expectation. Tables 1 and 2 show that the number of progeny that were *zip* trans-heterozygotes and carried the rescue transgene was comparable to the number of progeny of *zip*/+ (the SM6a balancer chromosome carries a wild-type *zipper* allele). In contrast, all flies that were trans-heterozygous for these severe *zipper* alleles, but did not carry the transgene, died as expected. An alternate way to express this rescue is as survival rate, defined as the number of flies in the rescue class divided by the total number of progeny from the cross. In each case, 20% of the flies were expected to be in the rescued class or classes (see Tables 1 and 2). The survival rates for the expected rescued flies are 20% for p*WUM-PKC-SA*^{2} (Table 1) and 18.5% for p*WUM-PKC-SD*^{2} (Table 2), respectively, which is comparable to that obtained by the wild-type construct (data not shown). We used combinations of various *zipper* alleles that comprise severe alleles (*zip*^{2}, *zip*^{1D16}) and a weaker allele (*zip*$^{1.6}$), and the results are nearly identical. In all cases, we had rescued male and female flies that are fertile. For completeness, we also tested whether serine 1921, which sits at the consensus phosphorylation site for the vertebrate nonmuscle myosin heavy chains, is essential for myosin function. Recall that we have no evidence that this residue is ever phosphorylated in the *Drosophila* nonmuscle myosin II heavy chain. As with the variants at serines 1936 and 1944, we found that flies carrying the PKC-CA transgene are completely rescued (data not shown).

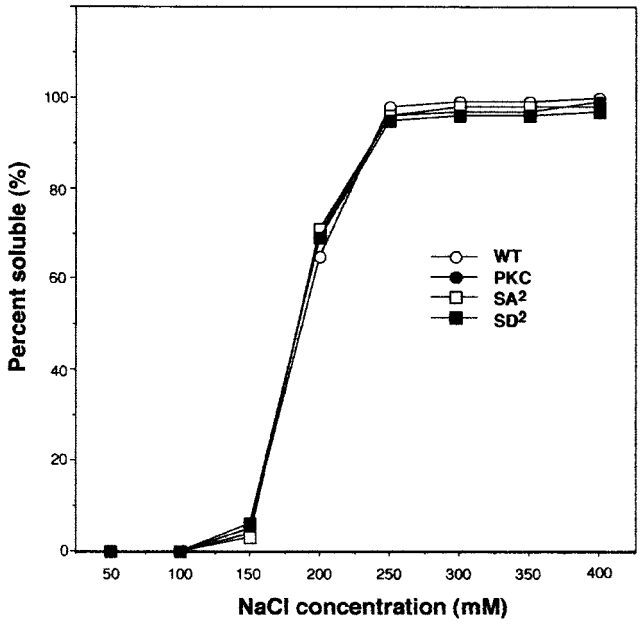

FIGURE 8: PKC phosphorylation does not affect assembly of C-LMM-341 into paracrystals. The effects of PKC phosphorylation on assembly of C-LMM-341 were examined by determining the solubility of the in vitro PKC phosphorylated C-LMM-341, nonphosphorylated C-LMM-341, and the PKC site mutant variants of C-LMM-341. Percent solubility is plotted as a function of salt concentration, as in Figure 2. The four protein preparations have similar solubility under various salt concentrations at pH 7.5. Open circles, C-LMM-341 (WT). Filled circles, PKC phosphorylated C-LMM-341 (PKC). Open squares, serines to alanines double mutant (SA^2). Filled squares, serines to aspartates double mutant (SD^2).

Table 1: Serine to Alanine Mutations of the PKC Phosphorylation Sites Rescue the Lethal Phenotype of Trans-heterozygous *zipper* Flies[a,b]

genotype	*y w*; *bw sp zip*^{2}	*y w*; *SM6a*
PKC-SA^2; *cn bw sp zip*^{1D16}	34[c]	32
Y; *cn bw sp zip*^{1D16}	0	35
PKC-SA^2; *SM6a*	29	
Y; *SM6a*	36	

[a] *pWUM-PKC-SA*^{2} is on an *X* chromosome, *SM6a* is a second chromosome balancer, *zip*^{1D16} is a severe *zipper* allele whose molecular defect has not been determined, and *zip*^{2} is another severe *zipper* allele that encodes a truncated myosin heavy chain that is not stable. [b] Number of rescued flies/number of total progeny: 34/166 = 20%. [c] Rescued flies.

Table 2: Serine to Aspartic Acid Mutations of the PKC Phosphorylation Sites Rescue the Lethal Phenotype of Trans-heterozygous *zipper* Flies[a]

genotype	*yw*; *bw sp zip*^{2};+	*y w*; *SM6a*;+
y w; *cn bw sp zip*^{1D16}; PKC-SD^2	23[b]	24
y w; *cn bw sp zip*^{1D16}; *c*	0	22
y w; *SM6a*; *PKC-SD*^{2}	23	
y w; *SM6a*; *c*	25	
Y; *cn bw sp zip*^{1D16}; *PKC-SD*^{2}	20[b]	24
Y; *cn bw sp zip*^{1D16}; *c*	0	28
Y; *SM6a*; *PKC-SD*^{2}	21	
Y; *SM6a*; *c*	22	

[a] Number of rescued flies/number of total progeny: 43/232=18.5%. [b] Rescued flies. [c] Wild-type chromosome, *pWUM-PKC-SD*^{2} is on third chromosome, *SM6a* is a second chromosome balancer, *zip*^{1D16} is a severe *zipper* allele whose molecular defect has not been determined, and *zip*^{2} is another severe *zipper* allele that encodes a truncated myosin heavy chain that is not stable.

DISCUSSION

Our experiments demonstrate that in vitro, *Drosophila* nonmuscle myosin-II can be phosphorylated by protein kinase C (PKC) at two sites, serines 1936 and 1944, in the nonhelical globular tail piece that constitutes the end of the myosin-II tail. These sites are near, but not identical to, a single serine 1921 (in the helical tail adjacent to the junction with the nonhelical tail piece that starts at lysine 1925) that is conserved in flies and is known to be phosphorylated by PKC in vertebrates (*19*). This site is not phosphorylated in fly nonmuscle myosin-II heavy chain, even when serines 1936 and 1944 are replaced by either alanines or aspartic acids. Our results are somewhat surprising. PKCs do not generally show strict substrate specificity, and the general requirements for positive charges upstream or downstream of the phosphorylated residue are fulfilled for both of the serines that we show are phosphorylated in fly nonmuscle myosin heavy chain (Figure 6). Nevertheless, analysis of synthetic peptides based on the sequences that surround residues phosphorylated by PKC in vertebrate nonmuscle myosin-II, glycogen synthetase, the ribosomal protein S6, and the epidermal growth factor receptor shows that an arginine residue at −3 residues from the phosphorylated serine or threonine contributes strongly to the efficiency with which PKC phosphorylates its substrates (*25, 41*). In the fly, a comparable arginine is at the −3 position for the consensus site that is not phosphorylated, but it is not in place for serines 1936 and 1944, which are phosphorylated. It is formally possible that one or more of the three PKCs found in the fly (see Flybase at http://flybase.bio.indiana.edu:82/), show substrate specificity different from that of the vertebrate enzymes used in our in vitro experiments, and as a consequence, these PKCs phosphorylate the fly myosin-II heavy chain at residue 1921. Unfortunately, there is no good source for sufficient quantities of purified fly PKC protein of any one of the fly homologues, much less all of them, so this cannot be tested directly at this time. In addition, while we showed previously that the fly myosin heavy chain is phosphorylated in vivo (Ketchum and Kiehart, unpublished observations and *42*), we observed a low phosphate-to-protein ratio. Our interpretation is that only a small fraction of the myosin is phosphorylated at any one time. This is reasonable for a nearly ubiquitously expressed chemomechanical force producing protein like myosin, for which there are substantial cellular pools, and for which only a small fraction is actually required to drive specific cellular movements in localized regions of the cell at any given time. We have not attempted to identify conditions that optimize phosphorylation of the myosin heavy chain in more than a cursory fashion, and as a consequence, we have not pursued experiments designed to map the site or sites that are phosphorylated in vivo. Thus we have been unable to identify the enzyme or enzymes that are responsible for this phosphorylation. Overall, we believe that this possibility (that the endogenous, fly enzyme or enzymes phosphorylate serine 1921 in vivo whereas the heterologous enzymes phosphorylate serines 1936 and 1944 in vitro) seems highly unlikely for the following reasons. The vertebrate enzymes are known to phosphorylate the serine in vertebrate nonmuscle myosin-II heavy chains (e.g., serine 1916 in human nonmuscle myosin-IIA, which is equivalent to serine 1921 in fly). Therefore, the vertebrate enzymes would have to switch specificity on the fly myosin substrate and phosphorylate serines 1936 and 1944, while the fly enzyme would phosphorylate the "consensus" serine at residue 1921. To our knowledge, there is no evidence that different residues on a single polypeptide substrate are phosphorylated by different PKC isoforms. We cannot explain why the fly myosin substrate is phosphorylated at serines 1936 and 1944 instead of at serine 1921, but are convinced that it is.

We show that a small fragment of the myosin-II tail can assemble into paracrystals and, as with vertebrate nonmuscle myosin-IIA, assembly of this fragment is not affected by the state of phosphorylation. Expression of various myosin-II tail fragments is a widely used strategy and an accepted model in the study of aspects of myosin-II assembly. These tail fragments readily assemble into paracrystals and provide the best model system available because of the difficulty in the recovery of sufficient quantities of purified, recombinant, whole, native myosins for biochemical studies. We found that the most C-terminal 341 amino acids of the *Drosophila* MHC were able to assemble and contained all phosphorylation sites by PKC. We show that this tail fragment, like its vertebrate counterparts, assembles in a salt concentration-dependent manner even though it consists of only 341 amino acids. In a simple, nucleotide-free buffer, it is essentially insoluble at NaCl concentrations below 150 mM and completely soluble at concentrations above 250−300 mM.

To evaluate the biological significance of PKC phosphorylation, we used site-directed mutagenesis to alter the serine residues that are phosphorylated to either alanine, which cannot be phosphorylated, or aspartic acid, which mimics the constitutively phosphorylated state. Next, we characterized the effect of altered sequences (each carrying a double mutation) on myosin function both in vitro and in vivo. In vitro, the altered sequences have no effect on the assembly of the C-LMM-341 fragment of myosin into paracrystals. Thus, fly nonmuscle myosin-II heavy chain functionally most resembles rabbit nonmuscle myosin IIA. In vivo, we introduced transgenes that carry the mutations into essentially wild-type flies by P-element-mediated transformation. The transgenes encoding the mutant heavy chains were genetically crossed into flies that otherwise lacked the ability to express myosin heavy chain and would, therefore, be expected to die as embryos. We found that both of the double mutant transgenes (SA^2 or SD^2) completely rescued the

viability of the *zipper* flies that would otherwise die. For completeness, we also showed that a mutant transgene that replaced the consensus serine 1921 with alanine (PKC−CA) also completely rescued the viability of the *zipper* flies. Our results demonstrate that MHC phosphorylation by PKC is not required for viability in *Drosophila*.

Our study is the first to address the in vivo function of MHC phosphorylation in metazoan organisms. Our results suggest that the biological significance of MHC phosphorylation by PKC in vertebrates and in *Drosophila* may not be as important as in lower eukaryotes, where regulation by phosphorylation is absolutely essential (*15*). Our data do not address the importance of regulation by heavy chain phosphorylation as measured over evolutionary time. Indeed, the observation that vertebrate PKC can phosphorylate sites on the fly myosin heavy chain substrate suggests that phosphorylation by PKC, or an enzyme like it, has been maintained in arthropods and vertebrates since the two had a common ancestor. The fossil record and molecular clocks suggest that such a common ancestor would have lived some 530 million years (the time of the Cambrian explosion) to 1000 million years ago (see *43* for a discussion of the dates). This suggests that while the advantages of having heavy chain phosphorylation by PKC for regulation of myosin function may not be apparent under laboratory conditions, subtle but significant increases in fecundity result in the maintenance of this regulatory pathway over evolutionary time (*44*). Our overall conclusion is that heavy chain phosphorylation contributes to regulation in a real, but minor, way.

Our data suggest that it is highly likely that at least one abundantly expressed isoform in vertebrates (the MIIA isoform whose assembly does not appear to be regulated by PKC phosphorylation, *20*) also requires heavy chain phosphorylation in a similar real, but minor, way. It may well be that phosphorylation of the other nonmuscle myosin heavy chain isoforms (MIIBα and MIIBβ), for which affects on assembly have been observed in vitro, may be more important for the viability of the organism that expresses them.

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