Phospholipid Binding, Phosphorylation by Protein Kinase C, and Filament Assembly of the COOH Terminal Heavy Chain Fragments of Nonmuscle Myosin II Isoforms MIIA and MIIB[†]

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ABSTRACT: Previously, we showed that myosin II heavy chains bind to phosphatidylserine (PS) liposomes via their COOH terminal regions and that protein kinase C (PK C) phosphorylates the PS-bound heavy chains [Murakami et al. (1994) *J. Biol. Chem. 269*, 16082−16090]. In this report, we studied the phospholipid binding, the kinetics of phosphorylation by PK C, and the effect of PK C-mediated phosphorylation on assembly using 46−47 kDa fragments from the COOH termini of macrophage ($MIIA^{F46}$) and brain type ($MIIB^{F47}$) heavy chain isoforms. Binding of the fragments to PS or phosphatidylinositol liposomes increased turbidity, but $MIIA^{F46}$ gave higher turbidity than $MIIB^{F47}$. Both fragments were sedimented similarly by ultracentrifugation in PS concentration and mole percent of PS dependent manners. With mixed PS/phosphatidylcholine (PC) liposomes, at least 70 mol % PS was required for heavy chain binding. A similar level of PS was required for phosphorylation of fragments by PK C, indicating that binding of tail regions to PS is a prerequisite for phosphorylation by PK C. PK C phosphorylated $MIIB^{F47}$ with V_{max} values 4−5 times higher than those of $MIIA^{F46}$, but the K_m values for the two substrates were similar. The apparent K_m values for PS liposomes (K_{lipid}) were also similar for phosphorylation of both isoforms. Mixing PS with PC increased the K_{lipid} and reduced the V_{max} values but did not alter the K_m values for the substrates. Assembly of $MIIB^{F47}$, but not $MIIA^{F46}$, was significantly inhibited by the phosphorylation, indicating that nonmuscle myosin assembly can be regulated, in an isoform specific manner, via phosphorylation of heavy chains by PK C.

Myosins are molecular motors expressed in both muscle and nonmuscle cells. Conventional myosins (myosin II) found in cytoplasm are called "nonmuscle" or "cellular" myosin and participate in cell motility in conjunction with filamentous actin. In contrast to the muscle myosins which form stable myofibrills, nonmuscle myosins exhibit a dynamic monomer−filament equilibrium that shifts according to changes in cellular functions and motilities (Jennings et al., 1981; Yumura & Fukui, 1985). In higher vertebrates, at least two genes of nonmuscle myosin II exist, MIIB[1] (brain type) and MIIA (macrophage type) (Saez et al., 1990; Simons et al., 1991; Toothaker et al., 1991). Expression of these isoforms varies depending upon the developmental stage, the type of cells, and changes in cell functions (Kawamoto & Adelstein, 1991; Murakami & Elzinga, 1992; Murakami et al., 1993). Myosin filaments are often found within different parts of cells in an isoform specific distribution (Borrione et al., 1990; Cheng et al., 1992; Maupin et al., 1994), suggesting the presence of regulation mechanism(s) *in vivo* for isoform specific filament formation. Myosin filament formation is regulated by phosphorylation within both the light chains and the heavy chains. Phosphorylation of light chains by myosin light chain kinase increases the actin-activated ATPase activity and promotes filament formation of both smooth muscle and nonmuscle myosins in vertebrates [Adelstein and Eisenberg (1980), Kamm and Stull (1985), and Korn and Hammer (1988) for reviews]. In contrast, phosphorylation of heavy chains is known to regulate myosin functions in lower invertebrates such as *Dictyostelium*, *Acanthamoeba*, and *Physarum*; myosins II from *Dictyostelium* and *Acanthamoeba* have multiple phosphorylation sites at or near the COOH end of their heavy chains (Collins et al., 1982a; Vaillancourt et al., 1988; Lück-Vielmetter et al., 1990), and phosphorylation at these sites inhibits their actin-activated ATPase activities (Kuczmarski & Spudich, 1980; Truong et al., 1992; Collins et al., 1982b; Kuznicki et al., 1983). Phosphorylation of *Dictyostelium* myosin heavy chains tends to inhibit filament formation, or make filaments shorter (Kuczmarski & Spudich, 1980; Kuczmarski et al., 1987), while phosphorylation of *Acanthamoeba* myosin has no obvious effect on its assembly properties (Sinard & Pollard, 1989). Heavy chains of vertebrate myosins II are also known to be phosphorylated both *in vivo* and *in vitro*. Phosphorylation of heavy chains was first reported from *in vivo* studies (Andreasen et al., 1975; Muhlrad & Oplatka, 1977; Hesketh et al., 1978; Trotter, 1982; Fechheimer & Cebra, 1982; Sagara et al., 1983). Since then, there have been many reports regarding the phosphorylation of myosin II heavy chains from nonmuscle (Matsumura et al., 1982; Tanaka et al., 1986; Rieker et al., 1987; Kawamoto et al.,

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[1] Abbreviations: $MIIA^{F46}$, COOH terminal 46 kDa fragment of macrophage type myosin heavy chain; $MIIB^{F47}$, COOH terminal 47 kDa fragment of brain type myosin heavy chain; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; DG, diacylglycerol; PK C, protein kinase C; CK II, casein kinase II; PCR, polymerase chain reaction.

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1989; Ludowyke et al., 1989) and muscle tissues and cells (Kawamoto & Adelstein, 1988; Kelley & Adelstein, 1990), both *in vivo* and *in vitro*. In contrast to the invertebrate myosins, however, the effect of heavy chain phosphorylation on vertebrate myosin functions is not clear.

A number of kinases have been shown to phosphorylate heavy chains; casein kinase II (CK II) phosphorylates a single site localized at the tip of the heavy chain within the nonhelical tail piece of both bovine brain myosin *in vitro* (Murakami et al., 1984, 1990) and aortic myosins *in vitro* and *in vivo* (Kelley & Adelstein, 1990). Protein kinase C (PK C) phosphorylates myosin heavy chains from human platelets and rat basophil leukemic cells both *in vivo* and *in vitro* (Kawamoto et al., 1989; Ludowyke et al., 1989). In the human platelet myosin heavy chain, the PK C site was identified as a single serine located near the end of the predicted α-helical region (Conti et al., 1991), approximately 30 amino acid residues upstream from a predicted CK II site. It is evident that the tail end region has a key role in assembly of all muscle and nonmuscle myosins (Tashiro et al., 1985; Cross & Vanderkerckhove, 1986; Maeda et al., 1991; Ikebe et al., 1991; Hodge et al., 1992) and that the sequences around the PK C and CK II sites are conserved among nonmuscle myosins (Saez et al., 1990; Kuro-o et al., 1991; Phillips et al., 1992; Sun & Chantler, 1992; Shohet et al., 1989; Takahashi et al., 1992). In the previous study, we found that myosin II heavy chains bound to phosphatidylserine (PS) via the tail regions and that the extent of heavy chain phosphorylation by PK C was highly correlated with this phospholipid binding (Murakami et al., 1994). Since (1) light chains are also phosphorylatable by PK C (Nishikawa et al., 1984; Kawamoto et al., 1989; Ludowyke et al., 1989), (2) brain myosin is phosphorylated by PK C within its head region (Ikeda et al., 1990), and (3) myosin preparations purified from most tissues and cells contain multiple heavy chain isoforms (Murakami & Elzinga, 1992), native myosins are not suitable for studies on the isoform specific interaction with phospholipid and phosphorylation by PK C of heavy chain isoforms at their tail region. Therefore, as a model system, we expressed two heavy chain isoform fragments in *Escherichia coli*, a 46 kDa fragment corresponding to the COOH terminal 396 amino acid residues of human macrophage myosin (MIIA^{F46}) and a 47 kDa fragment (405 residues) of rabbit brain type myosin heavy chain (MIIB^{F47}) from an equivalent region. Rabbit MIIB was used since its amino acid sequence is almost identical to that of human MIIB (Kuro-o et al., 1991; Phillips et al., 1992), and the cDNA sequence of human MIIB at the tail region has not yet been published. In this report, we have studied in more detail the PS dependence for liposome binding and for phosphorylation by PK C, measured the phosphorylation kinetics for PK C, and analyzed the effect of the PK C-mediated phosphorylation on filament formation by using these two heavy chain isoforms.

MATERIALS AND METHODS

Materials. Multiple PK C preparations were obtained from rat brains in protein concentrations of 0.07 to 0.3 mg/mL by the method of Huang et al. (1986). The preparations purified by this method contained α, β, and γ as major and ε and ζ as minor PK C isoforms, detected by immunoblots. [γ-^{32}P]ATP was purchased from ICN. L-α-phosphatidyl-L-serine (PS) (from bovine brain), L-α-phosphatidylcholine (PC) (type III-B from bovine brain), 1,2-dioleoyl-*sn*-glycerol (diacylglycerol, DG), and ATP were from Sigma. Phospholipids were mixed in chloroform at the desired ratios and dried under a stream of argon gas. Liposomes were prepared by suspension of lipids in 40 mM Tris-HCl (pH 7.5) followed by sonication either as described in Bazzi and Nelsestuen (1987) or for 30 min in ultrasonic water bath (E/MC model 250).

Preparation of cDNA. mRNA was purified from New Zealand white rabbit cerebral cortex according to the method in McNally et al. (1991) and used for preparation of cDNA by reverse transcriptase (Gibco) as described by the supplier. Primers (20-mer, upsteam sequence: 5′-GAGAGGCCAGAGAGAAGGAA-3′; 22−mer, downstream sequence, 5′-GGATGAGGAGAATCTGGAACAC-3′) corresponding to the rabbit SM$_{emb}$/brain myosin heavy chain cDNA sequences 2−21 and 1729−1750 (Kuro-o et al., 1991), respectively, were used for the first polymerase chain reaction (PCR). A second PCR was performed using the primary PCR product as template and two primers that contained restriction sites. The upstream primer was a 49-mer, 5′-AGCTGAATTCAAAATTAAGGAGGATCCATATGAAGGCCCACTTCGAGCG′-3. It contained a *Bam*HI site (underlined) in the noncoding region. The initiation codon ATG (bold) corresponds to bases 289−291 of the SM$_{emb}$ sequence. The downstream primer was a 34-mer, 5′-TCTCTCGGATCCATGTTGATCTTTCGTAAGGAGT-3′. It contained a *Bam*HI site (underlined) in the noncoding region. Base 15 (bold G) corresponds to base 1632 of the SM$_{emb}$. The PCR product had 1343 bases, 1215 of which coded for a sequence of 405 amino acid residues. The PCR fragment was purified, restricted with *Bam*HI, repurified, and ligated into a *Bam*HI site in the plasmid pND1-R. Expression and purification of the fragments from *E. coli* (BL21[DE3]pLysS) were done essentially as described previously for MIIA^{F46} (Murakami et al., 1994). *E. coli* transformed with the plasmid expressed two proteins; one has a calculated M_r of 46 927 and is called MIIB^{F47}, while the other has a calculated M_r of 33 783 and is called MIIB^{F34}. MIIB^{F47} was separated from MIIB^{F34} on a Sephacryl S-300 column (1.5 × 150 cm) equilibrated with 0.25 M NaCl, 10 mM Tris-HCl (pH 7.5), and 6 M urea. The fractions from the column were monitored by A_{280} and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (Laemmli, 1970). The fractions containing MIIB^{F47} were pooled and dialyzed against 50 mM NaCl, 10 mM imidazole/HCl (pH 7.0), 10 mM $MgCl_2$, and 0.1 mM EGTA. The MIIB^{F47} precipitated by centrifugation at 25000*g* for 20 min was solubilized with buffer A [0.6 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1 mM EGTA]. Protein concentrations were determined using bovine serum albumin as a standard (Lowry et al., 1951) unless otherwise indicated.

Binding Assay. Liposomes containing various mole percents of PS and PC were made as 5 mM stock solutions. Heavy chain fragments (10 μg) were mixed with various liposome solutions in the presence of 10 mM Tris-HCl (pH 7.5), 0.175 and 0.2 M NaCl for MIIA and MIIB^{F47}, respectively, 0.1 mM EGTA, and 0.1 mM dithiothreitol (DTT). After incubation for 20 min, $MgCl_2$ was added to a final concentration of 5 mM in a final mixture volume of 0.15 mL and further incubated for 3 h at room temperature. Aliquots of the mixtures (100 μL) were centrifuged in a Beckman TL-100 (using a TLA-100.1 rotor) at 80 000 rpm

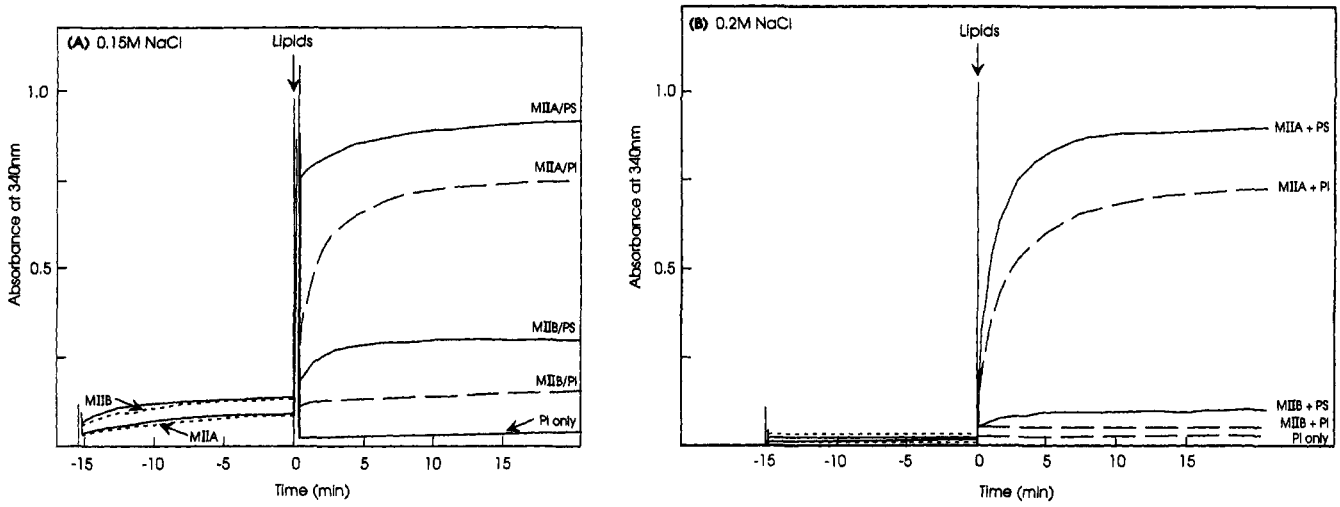

FIGURE 1: Changes in turbidity at 340 nm of MIIA^{F46} and MIIB^{F47} by addition of PS and PI. Thirty micrograms of MIIA^{F46} (0.65 nmol of heavy chain) and MIIB^{F47} (0.64 nmol) were mixed with (A) 0.15 M NaCl or (B) 0.2 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1 mM EGTA. At time 0, liposomes of PS (solid line) or phosphatidylinositol (PI) (dotted line) were added to the mixtures to a final concentration of 0.1 mM in a final volume of 0.5 mL.

for 20 min. The precipitates were suspended in 100 μL of distilled water by sonication. The amounts of heavy chain fragments recovered in the supernatants and in the precipitates were measured by SDS−PAGE followed by Coomassie blue staining; in brief, 45 μL of each sample solution was mixed with 20 μL of SDS sample buffer containing 1.5 μg of MIIB^{F34} as an internal standard, boiled at 100 °C for 10 min, and subjected to SDS−PAGE (12.5% gel). Standard curves were made by simultaneous runs of various amounts of MIIA^{F46} and MIIB^{F47} which also contained the internal standard. The protein bands stained with Coomassie blue were cut out and extracted with 0.75 mL of 25% pyridine overnight as described (Fenner et al., 1975). Areas without any protein from corresponding gels were used to determine background, the value of which was subtracted from each sample. The amounts of the fragments recovered from the SDS−PAGE were normalized by using the recovery rates of the internal standard in individual samples.

Phosphorylation Assay. Heavy chain fragments were preincubated with liposomes, 40 mM Tris-HCl (pH 7.5), PK C, 0.15 M NaCl, 0.1 mM [γ-^{32}P]ATP, 5 μg/mL leupeptin, 0.1 mM DTT, and 5% glycerol for 20 min at room temperature. The phosphorylation reaction was initiated by addition of $CaCl_2$ to 0.2 mM and $MgCl_2$ to 5 mM and was carried out at 30 °C. To test the effect of the PK C-mediated phosphorylation on assembly, fragments (1.0 mg) were phosphorylated by PK C (15 μg) in a final volume of 1.5 mL in the presence of 0.5 mM cold ATP. Before addition of cold ATP, small aliquots from each tube were taken from the mixtures and incubated with [^{32}P]ATP to measure the extent of phosphorylation. When the phosphorylation reaction reached plateau (0.6 mol of phosphate per mole of MIIA^{F46} heavy chain and 0.9 mol per mole of MIIB$^{\alpha F47}$ heavy chain), 20 mM EDTA was added to the mixtures to stop the reaction. The heavy chain fragments were precipitated by addition of EtOH to 66% and collected by centrifugation. The resultant precipitates were washed with 75% EtOH twice and with 100% EtOH once and incubated twice with ether/EtOH (3:1) for 20 min. Finally, the fragments were solubilized with 6 M guanidine/HCl and dialyzed against buffer A containing 25 mM NaF. Nonphosphorylated controls were treated similarly, except no kinase was added.

RESULTS

Phospholipid−Heavy Chain Interaction. We first checked the relationship between liposome concentrations and turbidity increases by mixing MIIA^{F46} with various concentrations of liposomes (0−0.4 mM) made with PS, PS/PC (1:9, 1:4, or 1:3 mol/mol), and PC in the presence of 0.2 M NaCl. Liposomes made of PS alone at about 0.1 mM gave the greatest increase in turbidity (data not shown). Increases in the PS concentrations above 0.2 mM caused a significant increase in the background turbidity and gave a decreased level of specific turbidity due to the liposome−heavy chain binding. In contrast to pure PS, liposomes made from PS mixed with PC (1:9, 1:4, and 1:3 in PS:PC molar ratios) as well as pure PC did not give a specific turbidity increase over the entire range of lipid concentrations, indicating that this inhibition was not simply due to the decrease in PS concentrations.

In a previous report, we showed that native myosin II increased in turbidity when mixed with PS liposomes and that the levels of turbidity increase were heavy chain isoform specific (Murakami et al., 1994). MIIA^{F46} and MIIB^{F47} also gave isoform specific turbidity increases when mixed with liposomes made from acidic phospholipids, PS or PI. At 0.15 M NaCl (Figure 1A), both PS liposomes and PI liposomes increased the turbidity of the MIIA solution, and the magnitudes were similar. The PS and PI liposomes also increased the turbidity of MIIB^{F47}, but the extents of these increases were much smaller than those seen with MIIA^{F46}. The turbidity levels of MIIA^{F46}/PS and MIIA^{F46}/PI mixtures were similar at 0.15 and 0.2 M NaCl, although the rate of turbidity increase was slower in 0.2 M NaCl (Figure 1B). However, the turbidity of MIIB^{F47} mixed with either PS or PI liposomes was significantly lower at 0.2 than at 0.15 M NaCl. Even though the turbidity increase was significantly lower, the elution positions of MIIB^{F47} from the Sepharose 4B column were shifted toward the void volume after incubation with either PS or PI liposomes (data not shown). Sedimentation analysis also confirmed that both MIIB^{F47} and MIIA^{F46} bound to PS liposomes (see below). These results confirm our previous finding obtained by using native myosins II. (1) The increase in turbidity due to heavy

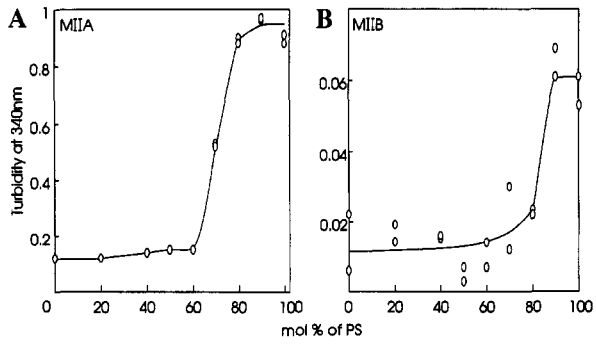

FIGURE 2: PS requirement for turbidity increases using liposomes containing PS in various molar ratios. Liposomes containing various mole percents of PS were made as 1 mM stock solutions. The liposome solutions contained pure PS, pure PC, or PS/PC in molar ratios of 2:8, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1. Twenty-five micrograms of MIIA^{F46} (0.54 nmol) (A) or MIIB^{F47} (0.53 nmol) (B) was incubated with 0.1 mM liposomes in the presence of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM EGTA, and 0.2 M NaCl in a final volume of 0.5 mL at room temperature for 1.5 h. The turbidities due to the liposome background were subtracted from those of the heavy chain/liposome mixtures.

chain−phospholipid binding depends upon the PS concentrations, liposome composition, and salt concentrations, and (2) the effects are isoform specific. In the following studies, we used PS liposomes because PS is essential for phosphorylation of heavy chains by PK C.

We then measured the minimum requirement of mole percent of PS for turbidity increases in a mixed liposome system composed of various mole ratios of PS to PC. For this, liposomes containing 0−100 mol % PS were used in the presence of 0.2 M NaCl, to avoid filament formation but still permit detection of turbidity changes. As shown in Figure 2, with liposomes containing less than 60 mol % PS, the turbidities of MIIA^{F46} solutions did not increase, but the turbidity was half-maximal at 70 mol % PS and maximal at 80 mol % PS. MIIB^{F47} gave significantly lower turbidity increases for the entire range of mole percents of PS, confirming the result shown in Figure 1. Also, turbidity increases with MIIB^{F47} required a higher mole percent of PS; at 80 mol % PS, the turbidity increase was negligible, while at 90 mol % PS, it was maximal.

Sedimentation Analysis for Heavy Chain−Phospholipid Binding. Although turbidity measurement is a sensitive and convenient method to detect protein−liposome interaction, it does not allow determination of the amounts of heavy chains bound to various phospholipid liposomes. To quantify the binding, MIIA^{F46} and MIIB^{F47} were incubated with liposomes in the presence of 0.175 and 0.2 M NaCl, respectively. These salt concentrations were chosen as the lowest concentrations that would prevent the fragments from aggregating (MIIB^{F47} required higher salt concentrations than MIIA^{F46} to inhibit filament formation; see Figure 8). In the turbidity analysis, we eliminated Mg^{2+} since millimolar levels of divalent cations bind PS-containing liposomes and, as a result, increase turbidity. For sedimentation analysis, we added 5 mM $MgCl_2$ since we found that millimolar levels of Mg^{2+} enhanced the amounts of heavy chains sedimented with liposomes, and 5 mM $MgCl_2$ was used for the PK C phosphorylation assay. In the absence of PS liposomes, less than 20% of the MIIA^{F46} was sedimented (Figure 3), and 5 mM $MgCl_2$ did not increase the amount of protein sedimented. In the presence of PS liposomes, the amount of

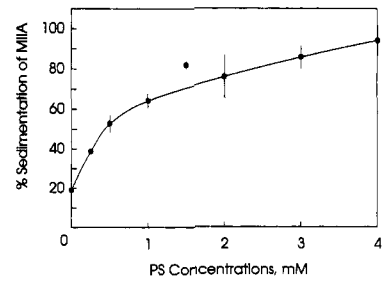

FIGURE 3: PS concentration dependent binding of MIIA^{F46}. Various concentrations of PS liposomes were incubated with heavy chain fragments (10 μg, 0.22 nmol) in a final volume of 0.15 mL, and the protein recovered in the pellets was measured as described in Materials and Methods. Numbers are an average of two independent measurements.

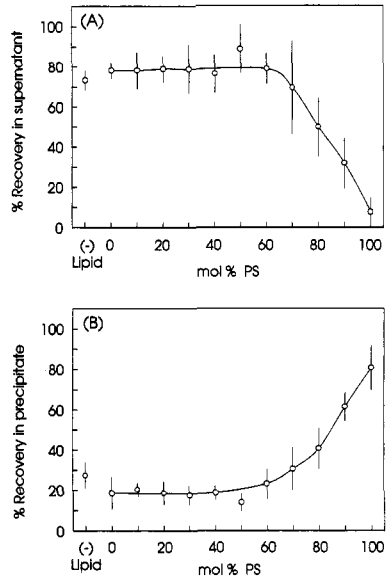

FIGURE 4: Mole percents of PS required for heavy chain/liposome binding. Heavy chains (10 μg) were mixed with 4 mM liposomes containing PC and PS, 0 to 100 mol%. MIIA^{F46} recovered in supernatants (A) and pellets (B) was measured as described in Materials and Methods. Data represent an average of five separate measurements.

protein sedimented varied with the liposome concentrations. At 4 mM PS, almost all the MIIA^{F46} (Figure 3) and MIIB^{F47} (not shown) sedimented. Therefore, in the following experiments, the minimum mole percent of PS in PS/PC liposomes required for sedimentation of heavy chains was determined with 4 mM liposomes. As shown in Figure 4A, 70−80% of MIIA^{F46} was recovered in the supernatant at a PS ratio below 70 mol %. With liposomes containing PS of at least 80 mol %, the amounts of the fragments recovered in the sediments increased (Figure 4B). MIIB^{F47} incubated with liposomes (at 0.2 M NaCl) showed practically the same pattern as MIIA^{F46}, and approximately 90% of MIIB^{F47} was recovered in the precipitates when pure PS liposomes were used (data not shown). The results shown in Figures 2 and 4, obtained by two different analytical methods, both demonstrate that binding of heavy chain fragments to liposomes requires at least 70−80 mol % of PS.

Liposome Composition and PK C-Mediated Phosphorylation. Using the same preparations of mixed liposomes used for the binding analysis (Figure 2), we next determined the minimum requirement of mole percent of PS for phosphorylation of MIIA^{F46} and MIIB^{F47} by PK C. PK C did not appreciably phosphorylate MIIA^{F46} with liposomes contain-

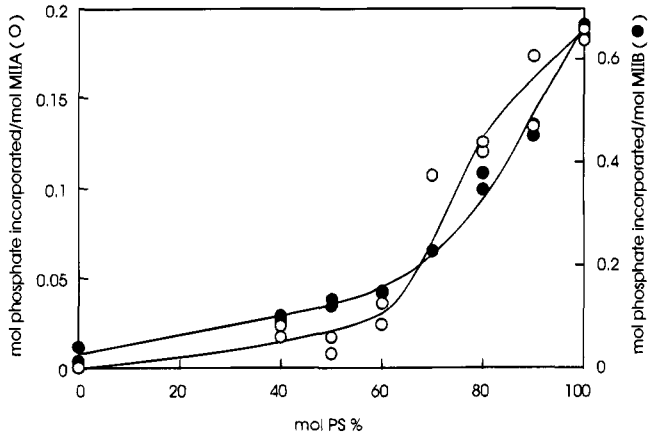

FIGURE 5: Determination of the mole percent of PS required for phosphorylation of MIIA^{F46} and MIIB^{F47} by PK C. Heavy chain fragments (10 μg) were preincubated with PK C (0.3 μg) for 10 min in the presence of 0.1 mM liposomes containing various mole percents of PS in a final incubation volume of 0.1 mL, as described in Materials and Methods. The reaction was terminated by addition of 3 N H_2SO_4/4% silicotungstate, and the protein-bound radioactivities were counted as described previously (Murakami et al., 1991). The specific phosphate incorporation into the fragment was calculated by subtraction of the radioactivities incorporated into the kinase fractions from the total amount of [^{32}P]phosphate incorporated into proteins incubated with the kinase: ○, MIIA^{F46}; ●, MIIB^{F47}.

ing less than 60 mol % PS (Figure 5), which was very similar to the patterns for the heavy chain−liposome binding analysis (Figures 2 and 4); with liposomes of 60 mol % PS, phosphorylation of MIIA^{F46} was less than 20% of that obtained with pure PS. However, the phosphorylation efficiency increased linearly at PS levels greater than 60 mol %. Similarly, phosphorylation of MIIB^{F47} by PK C was low with liposomes containing less than 60 mol % PS. With liposomes containing more than 70 mol % PS, the phosphorylation increased linearly with the increase in mole percent of PS (Figure 5). It should be noted that, for phosphorylation of these heavy chain fragments, micromolar levels of Ca^{2+} activated PK C fully and that increases in the Ca^{2+} concentration to millimolar levels did not alter the phosphorylation efficiencies (data not shown). Our results from Figures 2, 4, and 5 suggest that phosphorylation of heavy chain fragments by PK C is highly dependent upon binding of the heavy chains to phospholipids.

Kinetic Studies of the PK C-Mediated Phosphorylation. PK C incorporated up to 1 mol of phosphate per mole of both heavy chain isoforms, but MIIB^{F47} was phosphorylated at a faster rate than MIIA^{F46}. To understand the basis for this difference, kinetic parameters of the phosphorylation reaction were determined. First, the phosphorylation was performed in the presence of a constant concentration of liposomes and various concentrations of heavy chain fragments. The rate of phosphorylation of MIIB^{F47} was faster than that of MIIA^{F46} with all types of liposomes (Figure 6A,B), and both the rate and extent of phosphorylation were the highest with pure PS liposomes for the entire range of substrate concentrations. The K_m and V_{max} values were calculated from double reciprocal plots (Figure 6A,B, insert), and the results are summarized in Table 1. With PS liposomes, the V_{max} value for MIIA^{F46} phosphorylation was around 80 nmol min^{-1} (mg of PK C)$^{-1}$ while those of MIIB^{F47} were 400−500. The K_m values, in contrast, were rather similar for MIIA^{F46} and MIIB^{F47}, 0.3−0.4 mg/mL (7−9 μM) and 0.4−0.6 mg/mL (9−13 μM), respectively. Mixing PS with PC significantly lowered the phosphorylation levels from those with PS liposomes for the entire range of substrate concentrations, for both MIIA and MIIB (Figure 6A,B). Double reciprocal plots of the data revealed that the V_{max} values for phosphorylation of MIIA^{F46} and MIIB^{F47} decreased significantly when PS was mixed with PC. Mixing PS with PC, however, did not alter the K_m values noticeably (Table 1). We also determined the kinetic parameters of phosphorylation of heavy chain fragments with liposomes containing DG in addition to PS and PC (Figure 6, Table 1). Confirming our previous results using native myosins (Murakami et al., 1994), we found that phosphorylation of MIIA^{F46} and MIIB^{F47} in the presence of PS/DG liposomes gave K_m and V_{max} values very similar to those with PS liposomes (not shown). Similarly, with PS/PC/DG liposomes (1:3.84:0.16), the V_{max} and K_m values for MIIA^{F46} and MIIB^{F47} phosphorylation were essentially the same as those with PS/PC (1:4).

Table 1: Kinetic Parameters for the PK C-Mediated Phosphorylation of MIIA^{F46} and MIIB^{F47} Determined by Using Various Concentrations of Substrates in the Presence of Various Types of Liposomes[a]

liposome	K_m [mg of substrate/mL]	V_{max} [nmol min^{-1} (mg of kinase)$^{-1}$]
MIIA^{F46}		
PS	0.38 ± 0.06	82 ± 5
PS/PC (1:4)	0.38 ± 0.02	11 ± 1
PS/PC (3:2)	0.43 ± 0.01	25 ± 1
PS/PC/DG (1:3.84:0.16)	0.37 ± 0.06	13 ± 3
MIIB^{F47}		
PS	0.59 ± 0.03	456 ± 66
PS/PC (1:4)	0.53 ± 0.12	56 ± 16
PS/PC (3:2)	0.47 ± 0.11	158 ± 31
PS/PC/DG (1:3.84:0.16)	0.58 ± 0.09	67 ± 17

[a] The heavy chain fragments MIIA^{F46} and MIIB^{F47} were incubated with PK C (0.1−0.26 μg) at 30 °C for 10 min in a final volume of 0.1 mL in the presence of 0.15 M NaCl and a total of 0.5 mM liposomes containing various mole percents of PS. The other conditions were as described in the legend to Figure 6. The apparent K_m values for fragments and V_{max} values were calculated from double reciprocal plots of the data. Numbers listed are the average of three independent measurements.

Since binding of heavy chains to phospholipids and phosphorylation of heavy chains by PK C decreased significantly when PS was mixed with the neutral phospholipid PC (Figures 2, 4, and 5), we examined the changes in apparent binding affinities of liposomes to heavy chains (K_{lipid}) by measuring the phosphorylation efficiencies of the heavy chains by PK C. Liposomes are not exactly appropriate for this purpose because they do not exist as homogeneous solutions, but rather as suspensions. Micelles could be better for this purpose. However, our previous work suggested that myosins and heavy chain fragments do not bind to micelles, and more importantly, PK C did not phosphorylate heavy chains in the presence of micelles (Murakami et al., 1994). Therefore, the data obtained from the following kinetic analysis, especially for K_{lipid} values, are presented only for the purpose of comparison among samples assayed under similar conditions, not for determination of the precise kinetic constants. When PS alone liposomes were used, phosphorylation of MIIA^{F46} and MIIB^{F47} by PK C reached a plateau at about 0.1 mM PS (Figure 7A,B). Table 2 summarizes the kinetic parameters calculated from double reciprocal plots of the data (Figure 7 insert). MIIB^{F47} gave slightly higher affinities to PS

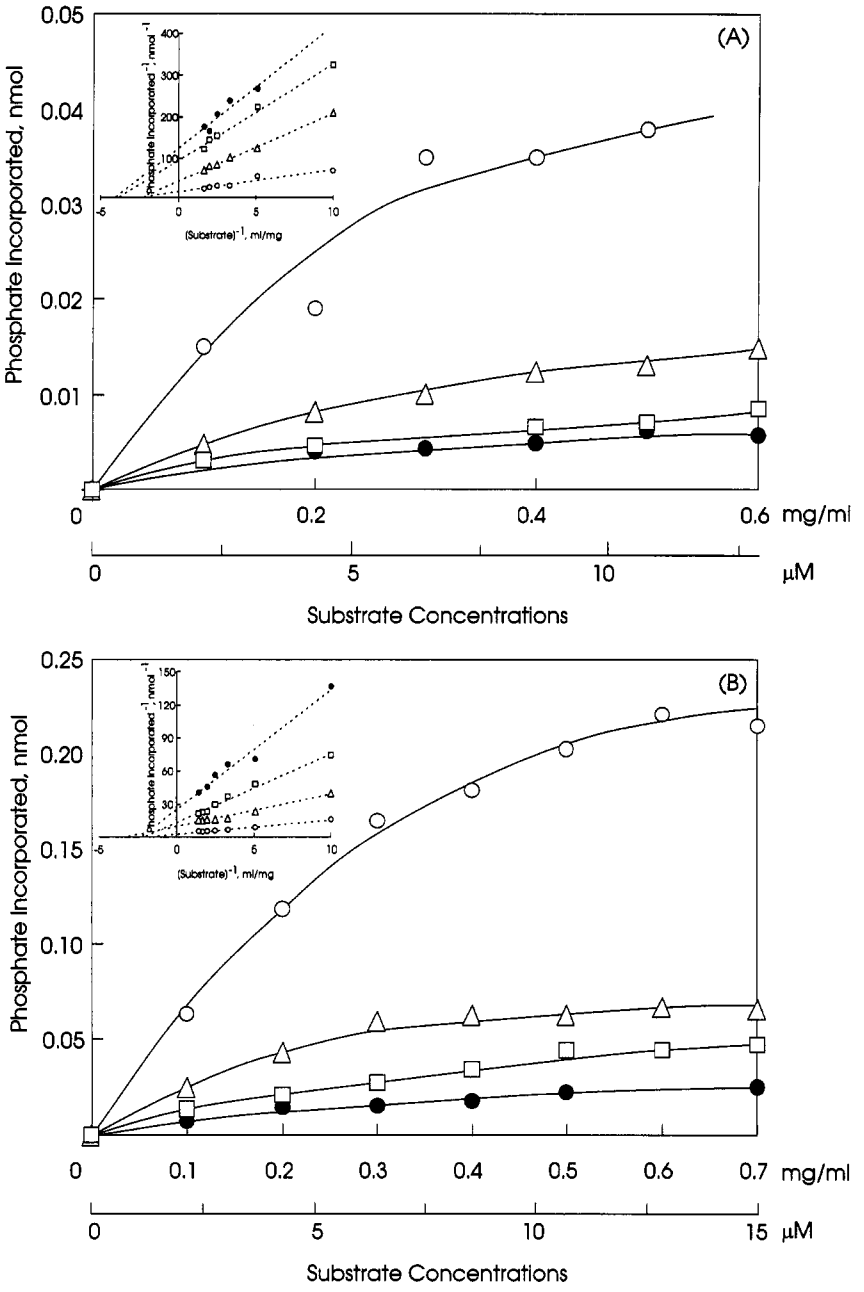

FIGURE 6: Phosphorylation of various concentrations of MIIA^{F46} (A) and MIIB^{F47} (B) by PK C. Heavy chain fragments (0−70 μg, 0−1.5 nmol) were incubated for 10 min with PK C (0.1 μg) and liposomes of PS, PS/PC (1:4), PS/PC (3:2), or PS/PC/DG (1:3.84:0.16) in a final volume of 0.1 mL as described in the legend to Figure 5, except for using a final lipid concentration of 0.5 mM: insert, double reciprocal plots of the data; ○, PS; △, PS/PC (3:2); □, PS/PC (1:4); ●, PS/PC/DG (1:3.84:0.16). The values are the averages of duplicate samples. Analyses in A and B were performed by simultaneous assay using the same liposome preparations.

liposomes and gave a V_{max} value approximately 4−5 times higher than MIIA^{F46} did. When the phosphorylation reactions were performed with PS/PC (3:2) liposomes, rates of phosphorylation were lower than those obtained with PS liposomes even at higher concentrations of lipids, giving significant decreases in V_{max} values along with large increases in K_{lipid} values (Table 2). With PS/PC (1:4) liposomes, the rate of phosphorylation was very low over the entire range of lipid concentrations, giving a further decrease in V_{max} and an increase in K_{lipid} values from those with PS/PC (3:2), for both heavy chain isoforms. Thus, mixing PS with the neutral phospholipid PC decreased phosphorylation efficiencies by PK C by changing both the affinity of lipids to heavy chains and the phosphorylation velocity. In contrast, the K_{lipid} values obtained for PS/PC/DG were similar to those for pure PS liposomes; DG reversed the K_{lipid} values increased by PC. However, DG did not reverse the PC-mediated decrease in V_{max} values; the V_{max} values obtained in the presence of PS/PC/DG liposomes were one-seventh to one-eighth of those with pure PS and were not different from those obtained with PS/PC (1:4).

Effect of Phosphorylation on Filament Formation. We next measured the effects of PK C phosphorylation on filament formation. The extent of filament formation was determined by measurement of the recovery of proteins in the supernatants after dilution of the fragments into buffers containing various NaCl concentrations (Figure 8A,B). Microcentrifugation was sufficient for sedimentation of the proteins at low salt concentrations in the presence of EDTA, indicating that MIIA^{F46} and MIIB^{F47} formed large filaments

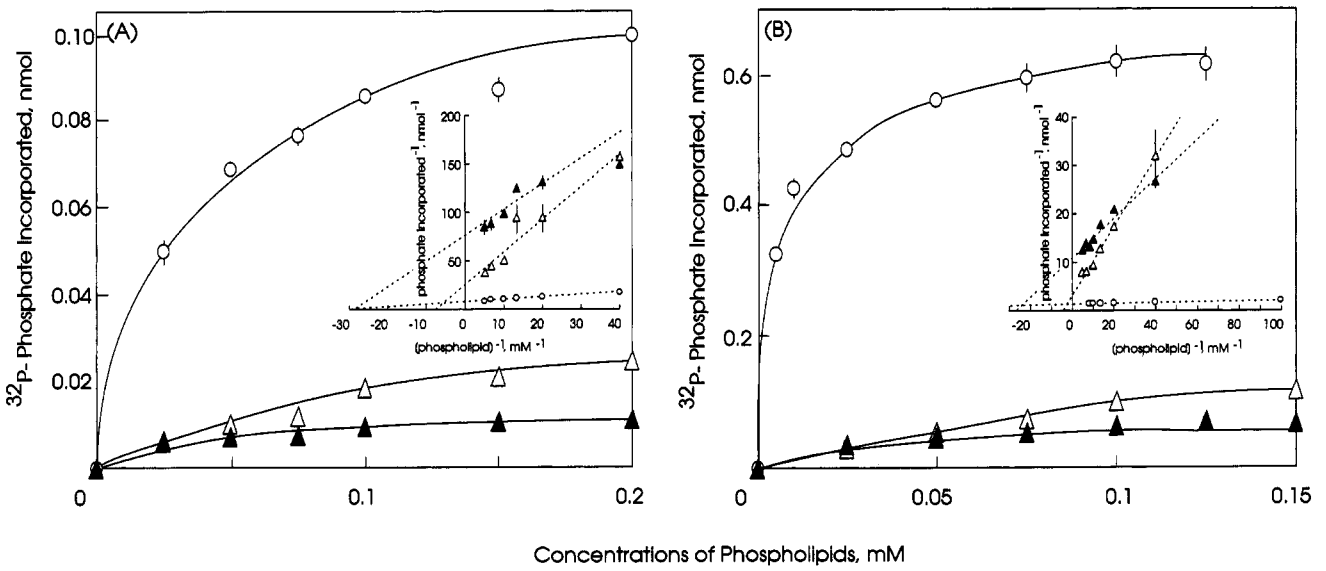

FIGURE 7: Phosphorylation of MIIA^{F46} (A) and MIIB^{F47} (B) by PK C in the presence of various concentrations of liposomes. Heavy chain fragments (30 μg) were phosphorylated by PK C (0.26 μg) for 10 min in the presence of various concentrations of liposomes: ○, PS; △, PS/PC (3:2); ▲, PS/PC/DG (1:3.84:0.16). The values shown are the averages of duplicate samples.

Table 2: Kinetic Parameters for the PK C-Mediated Phosphorylation of MIIA^{F46} and MIIB^{F47} Determined by Using Various Concentrations of Liposomes[a]

liposome	K_{lipid} [μM]	V_{max} [nmol of phosphate min^{-1} (mg of PK C)$^{-1}$]
MIIA^{F46}		
PS	32 ± 5	47 ± 4
PS/PC (1:4)	159 ± 36	5 ± 2
PS/PC (3:2)	102 ± 37	16 ± 3
PS/PC/DG (1:3.84:0.16)	32 ± 13	6 ± 1
MIIB^{F47}		
PS	11 ± 5	210 ± 30
PS/PC (1:4)	160 ± 9	9 ± 3
PS/PC (3:2)	81 ± 23	73 ± 16
PS/PC/DG (1:3.84:0.16)	26 ± 14	32 ± 8

[a] Heavy chain fragments (0.3 mg/mL) were incubated with PK C (0.1−0.26 μg) at 30 °C for 10 min in the presence of various concentrations of liposomes between 0.005 and 0.2 mM. The apparent K_m values for lipid (K_{lipid}) and V_{max} values were calculated from double reciprocal plots of the data. Numbers listed are the average of four independent measurements.

even in the absence of divalent cations. For both nonphosphorylated and phosphorylated MIIA^{F46}, recovery of the fragments in the supernatant increased with increasing salt concentrations. Recovery was essentially the same for both samples (Figure 8A), and thus, phosphorylation by PK C did not seem to affect MIIA^{F46} assembly. The recoveries of nonphosphorylated MIIB^{F47} in the supernatant were constant (less than 20% of total protein) up to 0.15 M NaCl and were lower than those of MIIA. At 200 mM NaCl, MIIB^{F47} was recovered completely in the supernatant. In contrast, the phosphorylated MIIB^{F47} was completely recovered in the supernatant over the entire range of salt concentrations (Figure 8B). Aliquots of each sample were also ultracentrifuged at 40 000 rpm (TL45 rotor) for 30 min by using a Beckman TL-100 ultracentrifuge. Although the overall recovery of fragments in the supernatants decreased slightly, especially in the presence of NaCl below 0.1 M, the recovery patterns of fragments were very similar to those shown in Figure 8 for both the nonphosphorylated and the PK C-phosphorylated forms (data not shown). This may suggest that the PK C-mediated phosphorylation inhibits assembly of monomeric MIIB^{F47} into small filaments rather than assembly of minifilaments into thick filaments. Our present study demonstrates that MIIB^{F47} is a better substrate for PK C than MIIA^{F46}, MIIB^{F47} forms filaments at higher salt concentrations than MIIA^{F46}, and its assembly is regulated by PK C phosphorylation.

DISCUSSION

We used two isoforms of heavy chain fragments as a model system to study the importance of the tail end region for regulation of myosin filament assembly. Our present study demonstrates that these fragments (1) bind to acidic phospholipids, (2) are phosphorylatable by PK C, and (3) form filaments and that the two isoforms behave differently. Binding of heavy chains to phospholipids was measured by two methods, indirect turbidity measurement and direct sedimentation analysis. Turbidity measurement is a more sensitive and convenient method, while sedimentation analysis permits quantification of heavy chain fragment binding to liposomes. The latter required a much higher liposome concentration (4 mM) than turbidity analysis, and millimolar levels of Mg^{2+} enhanced the sedimentation of heavy chains with liposomes. The fragment−liposome binding was therefore measured by two methods, and both led to the same conclusion that binding of MIIA^{F46} and MIIB^{F47} to phospholipids required liposomes containing at least 70−80 mol % PS.

Heavy chain−phospholipid binding probably occurs through electrostatic interactions between negative charges in liposomes and positive charges in the protein since higher salt concentrations gave smaller increases in turbidity. MIIA and MIIB gave different turbidity increases. Net charge, location, and numbers of PS binding regions, as well as positive amino acids clustered within the PS binding region, all may affect the turbidity changes. MIIA^{F46} and MIIB^{F47} have similar amino acid sequences within the α-helical rod region, but their amino acid sequences differ substantially within the nonhelical tail region. This may account for the differences in the extent of the turbidity increases between MIIA^{F46} and MIIB^{F47}. PK C phosphorylates Ser/Thr in a basic environ-

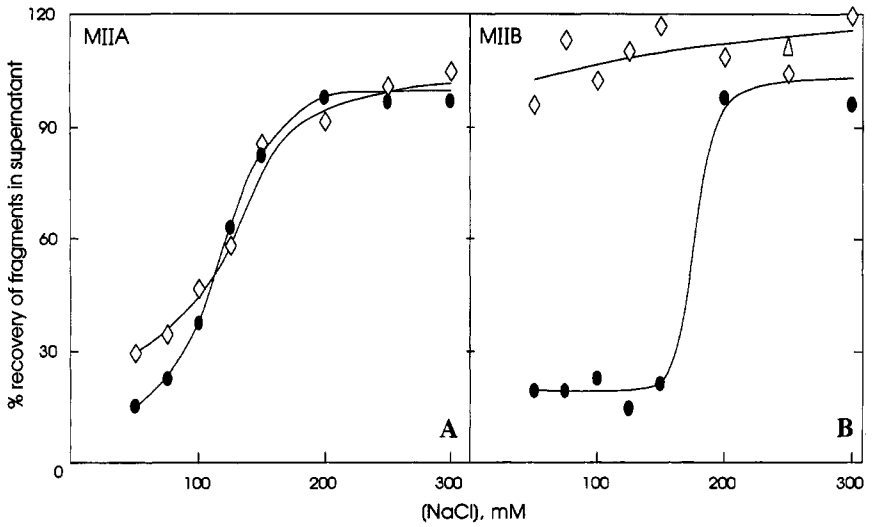

FIGURE 8: Effects of phosphorylation by PK C on assembly of MIIA^{F46} (A) and MIIB^{F47} (B); percent recovery of fragments in the supernatant. The fragments (20 μg, 0.43 nmol) were incubated in the presence of 25 mM NaF, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 mM EGTA, and various concentrations of NaCl in a final volume of 0.3 mL overnight. Aliquots of the mixtures were divided into tubes and microcentrifuged at 13600g for 30 min. The resultant supernatants (65 μL each) were used for the determination of protein. The percentages of the proteins recovered in the supernatant were calculated by taking the amount of protein, for each point, measured before centrifugation as 100%. The protein was measured by the method of Bradford (1976) in a final volume of 0.5 mL using MIIA or MIIB as a standard. The Bradford method gave different values depending upon the NaCl concentrations. This seemed to be due to aggregation of myosin and its heavy chain fragments. Addition of NaOH to 0.05 N prior to addition of the dye solution rectified this problem: ●, control; ◇, PK C.

ment (Ferrari et al., 1985; Turner et al., 1989), and the PK C-mediated phosphorylation of the fragments was highly correlated with this heavy chain-PS binding (Figures 2, 4, and 5); therefore, we assume that heavy chains probably bind to PS via basic amino acids near the PK C phosphorylation site. PK C phosphorylated both platelet myosin and MIIA at the same site, at a serine residue located near the end of the predicted α-helical region (Conti et al., 1991; Murakami et al., 1994), while at least one PK C site was also located in the nonhelical region of MIIB^{F47} (manuscript in preparation). Binding to PS may be a prerequisite for the PK C-mediated phosphorylation of these heavy chains. There appear to be three possible mechanisms by which heavy chain−liposome binding could increase turbidity: (1) fusion of liposomes, as is seen with divalent cations, such as Ca^{2+} and Mg^{2+} (McLaughlin et al., 1981), and myelin basic protein (Lampe & Nelsestuen, 1982); (2) cross-linking of liposomes via multiple PS binding sites in the heavy chains; or (3) stimulation of heavy chain aggregation by binding to liposomes. The fragments and the liposomes are probably similar in size, about 40 nm in diameter for unilamellar liposomes (Tien, 1974) and about 50 nm for the fragments estimated from the known 150 nm length of a 140 kDa rod. On the basis of the sizes, the magnitude of the turbidity increases, and at the ionic strength used for the analyses, we consider (1) to be more likely.

Substrates for PK C generally bind to PS-containing liposomes and micelles (Bazzi & Nelsestuen, 1987, 1992). The major differences between their results (Bazzi & Nelsestuen, 1987, 1992) and ours, however, exist in the requirements of phospholipids in a liposome form, not micelles (PS micelles did not work in our system), and of a higher mole percent of PS; most PK C substrates studied by them bound not only to liposomes but also to micelles with PS of 30 or less mol % and were phosphorylatable by PK C. The other difference is an inhibitory effect of Mg^{2+} on phosphorylation efficiency. In our case, preincubation of heavy chain with PS liposomes without Mg^{2+} was required before the phosphorylation reaction was started. If the reaction was started by addition of ATP, i.e. fragments were first mixed with PS and Mg^{2+}, the heavy chain phosphorylation was less effective than when the reaction was started by addition of Mg^{2+}. Mg^{2+} itself in millimolar levels binds to acidic phospholipids, especially to PS, and again requires a high mole percent of PS for its binding (Bazzi & Nelsestuen, 1992). In addition to this, millimolar levels of Mg^{2+} also affected the structure of the tail end region of myosin heavy chains; the nonhelical tail domain was easily cleaved by proteases such as papain and chymotrypsin, but Mg^{2+} prevented this (Murakami et al., unpublished data). Mg^{2+} in millimolar levels is also known to change the conformation of myosin rod and to stimulate aggregation of minifilaments into thick filaments (Pollard, 1982; Sinard & Pollard, 1989). Phosphorylation analyses were performed at 0.15 M NaCl since apparent phosphorylation efficiencies by PK C were the highest under this condition. It is likely, therefore, that Mg^{2+} causes its inhibitory effect on the rate of heavy chain phosphorylation by accelerating filament formation.

Consistent with our previous work using native myosins (Murakami et al., 1994), PK C phosphorylated MIIB^{F47} more efficiently than MIIA^{F46}. This difference in the phosphorylation efficiency seemed to be due to a larger V_{max} for MIIB^{F47} than for MIIA^{F46}, not to different affinities for the two heavy chain isoforms (K_m). Mixing PS with a neutral phospholipid, PC, reduced the V_{max} values without affecting the K_m values for substrates, for both MIIA^{F46} and MIIB^{F47}. However, the apparent affinity of heavy chains to phospholipids (K_{lipid}) was clearly reduced when PS was mixed with PC. When DG was added to the PS/PC, the reduction of K_{lipid} values caused by PC was reversed; however, the decrease in V_{max} values was not reversed. In our previous study, it was shown that PS/PC/DG liposomes did not bind heavy chains and native myosins (Murakami et al., 1994). DG, therefore, might work to increase the affinity of PK C, but not myosin heavy chains, to phospholipids. On the other

hand, PK C would be more active when it binds to PS/PC/DG than when it binds to PS/PC liposomes and DG has no effect on heavy chain−phospholipid binding.

In higher vertebrates, multiple heavy chain isoforms are often distributed within different parts of cells in an isoform specific manner (Borrione et al., 1990; Cheng et al., 1992; Maupin et al., 1994), and cells must therefore have a mechanism(s) by which to accomplish this. The nonhelical regions of the heavy chains of all nonmuscle and smooth muscle myosins have isoform specific amino acid sequences and contain phosphorylation sites for CK II (both smooth muscle and nonmuscle myosins) and PK C (for nonmuscle myosins), and deletion studies have revealed that the tail end region has a key role in assembly of all muscle and nonmuscle myosins (Tashiro et al., 1985; Maeda et al., 1991; Ikebe et al., 1991; Hodge et al., 1992). Our results obtained by using the myosin heavy chain fragments demonstrated clearly, for the first time, that the phosphorylation of myosin heavy chain by PK C at the tail end region affects filament formation of vertebrate myosin II and that this effect is isoform specific. Phosphorylation by PK C strongly inhibited assembly of $MIIB^{F47}$ while we did not detect any obvious effects of phosphorylation on assembly of $MIIA^{F46}$. Thus, assembly of nonmuscle myosin II can be regulated through phosphorylation of not only the light chains but also the heavy chains near the tail ends.

Since the average PS concentration in plasma membranes is around 20 mol %, a requirement of more than 70 mol % PS might seem unphysiologically high. Most of the PS seems to locate within the cytoplasmic region of the plasma membranes [review, Rathman and Lenard (1977)]. If we assume that *in vivo* PS exists unevenly within the cytoplasmic side of the cellular membranes, local PS concentrations (mole percent of PS) may be high enough to allow heavy chains to bind. In fact, platelet myosin is phosphorylated *in vivo* by PK C (Kawamoto et al., 1989; Conti et al., 1991) at the same site as seen in $MIIA^{F46}$. Nonmuscle myosins are found near the plasma membranes (Cheng et al., 1992; Miller et al., 1992; Maupin et al., 1994). In addition, tight association of the brain type myosin to neuronal plasma membranes has been reported (Li et al., 1994). Taken together, the accumulated evidence suggests that myosin II heavy chains bind *in vivo* to lipid membranes enriched in PS. In the studies reported here, we have focused primarily on the heavy chain−PS binding, because of the requirement of PS for phosphorylation by PK C. However, as shown in Figure 1, MIIA and MIIB also bind to another acidic phospholipid, PI. *In vivo*, local changes in the concentrations of acidic phospholipids and in the phosphorylation state of phosphatidylinositol compounds such as PI 4-phosphate, PI 4,5-bisphosphate, and PI 3,4,5-trisphosphate could affect the binding of myosin heavy chains to the cellular membrane, which, in turn, could affect the phosphorylation efficiency by PK C and isoform specific cellular redistribution of nonmuscle myosins.

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