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Phosphorylation of Myosin-I from Rat Liver by Protein Kinase C Reduces Calmodulin Binding

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Three isoforms of the cytoskeletal-associated, mechanochemical enzymes known as myosin-I have been purified from rat liver; each coisolates with calmodulin. Incubation of the purified myosin-I's with protein kinase Cγ and ^{32}P-ATP results in phosphorylation of the myosin-I heavy chains. After phosphorylation, the myosin-I isoforms bind less radiolabeled calmodulin in binding assays than observed for control samples. Since the purified isoforms are phosphoproteins as determined by immunoblotting with monoclonal antibodies which recognize phosphoamino acids, these results indicate that phosphorylation might play a role in regulation of myosin-I.

A number of cellular events including cytokinesis, locomotion, intracellular transport, and the extension of pseudopodia are mediated by microfilaments. Although conventional two-headed myosin is required for cytokinesis [1,2], other cellular processes might rely on any of a number of unconventional myosins now known to be present in non-muscle cells (for a review, see [3]).

Myosin-I is a class of single-headed, actin-associated, mechanoenzymes. In higher organisms, biochemical and molecular biological approaches have

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Abbreviations used: ATP, adenosine triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EGTA, ethyleneglycol tetraacetic acid; IgG, immunoglobulin; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol-12-myristate-13-acetate; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

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demonstrated that myosin-I isoforms are present in a variety of tissues including intestine [4,5,6,7], kidney [8], adrenal gland [9], liver [10,11] and brain [12,13,14,15]. Localization studies indicate that in intestinal cells myosin-I connects the microvillar membrane to the cytoskeleton [16,17,18] and is present on cytoplasmic vesicles [19,20]. Some forms of intracellular transport are presumed to be mediated by this class of protein.

Myosin-I isoforms of 130-kDa, 110-kDa, and 105-kDa have been purified from rat liver [10,11]. Partial peptide sequence analysis [11,21] indicates that the 130-kDa myosin-I corresponds to an *MM1α* or *myr1* gene product; the 110-kDa corresponds to the *MM1β* or *myr2* gene product; and the 105-kDa corresponds to an *MM1γ* or *myr4* gene product. The *MM1α-γ* and *myr* genes represent myosin-I's identified in mouse and rat brain, respectively [12,13,14,15]. Each of the myosin-I isoforms co-purifies with calmodulin. The 130-kDa polypeptide co-purifies with 6 molecules of calmodulin whereas the 110-kDa and 105-kDa polypeptides each co-isolates with two molecules of calmodulin [11]. These values correspond to the number of putative calmodulin binding domains predicted from the deduced amino acid sequences for the isoforms [12,13,14,15].

Since other myosins are known to be regulated in vitro by phosphorylation (see [22]), the interesting possibility exists that phosphorylation also regulates myosin-I activity. In this study we demonstrate that each of the myosin-I isoforms from rat liver are in vitro substrates for phosphorylation by protein kinase C, a regulator of numerous cellular activities [23,24,25]; phosphorylation is regulated by the calcium ion concentration and by calmodulin. The purified myosin-I isoforms are phosphorylated as determined by immunoblotting using antibodies recognizing phosphoamino acids. In addition, this is the first report that binding of calmodulin to myosin-I is reduced after phosphorylation by protein kinase C.

Materials and Methods

Male rats (150 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Protein kinase Cγ was purchased from Boehringer Mannheim (Indianapolis, IN) or Calbiochem (San Diego, CA). Type III-S histone from calf

thymus was purchased from Sigma Chem. Co. (St. Louis, MO) Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Phorbol-12-myristate-13-acetate was purchased from Bolton-Hunter reagent and γ-[^{32}P]-ATP were obtained from New England Nuclear/Dupont (Wilmington, DE). Proteins used as molecular weight markers for SDS-PAGE were obtained from Sigma. Antibodies to phosphoamino acids and secondary antibodies conjugated to alkaline phosphatase were purchased from Sigma. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate were purchased from Promega Biotech (Madison, WI). Buffers, salts and other reagents were of the best quality commercially available.

Preparation of proteins

Myosin-I isoforms were isolated from rat liver as previously described [10, 11] and used within 1-2 weeks. Rabbit skeletal muscle actin was purified as described by Spudich and Watt [26] with an additional gel-filtration step [27]; the actin was stored as G-actin at -80°C until needed. Calmodulin was isolated from bovine brain according to Lin et al [28] and stored at -80°C.

Phosphorylation reactions

Myosin-I (or calf thymus histone in control samples) was phosphorylated in vitro as described in Swanljung-Collins and Collins [29] with minor modifications. Total reaction volumes were typically 100-200 µl and contained from 30-50 µg/ml of the appropriate myosin-I isoform. Prior to phosphorylation, myosin I was dialyzed into buffer containing 20 mM imidazole, pH 7.5; 130 mM NaCl; 6 mM $MgCl_2$; 0.5 mM EGTA; 1 mM DTT; 1 mM PMSF; 1 µM PMA; 4% (w/v) sucrose; 0.005% Triton X-100; and 0-2 mM $CaCl_2$. Free calcium concentrations were calculated using a program developed by R. E. Godt (Medical College of Georgia, Augusta, GA). To initiate the phosphorylation reaction the following were added to the dialyzed myosin-I: 40 µg/ml phosphatidylserine vesicles (prepared as described in Hayden et al., 1990 [30]), 0.1 mM γ-^{32}P-ATP (20-50 µCi/reaction; 50,000-100,000 cpm/mmol), and 1-2 µg/ml protein kinase C. Unless otherwise specified, after 30 min at 37°C, the reactions were stopped by boiling for 3 min in SDS-sample buffer then subjected

to SDS-PAGE. After staining with Coomassie blue, the gels were dried onto filter paper, and used to produce autoradiographs. Incorporation of radioisotope was quantitated by phosphorimaging (Molecular Dynamics) and/or excision of the acrylamide portion of the stained bands followed by liquid scintillation counting with correction for background radiation levels. Calculations were performed using a Microsoft Excel spreadsheet and were based on total estimated molecular masses (including light chains) of 232-kDa, 144-kDa, and 140-kDa for the 130-kDa, 110-kDa and 105-kDa isoforms, respectively. These values are obtained based on estimates of calmodulin stoichiometry as described in Coluccio [11].

SDS-polyacrylamide gel electrophoresis

Proteins were separated on 7.5%/15% mini-polyacrylamide gels [5] according to Laemmli [31].

^{125}I-calmodulin overlays

Bovine brain calmodulin was radiolabeled with Bolton-Hunter reagent [32].

After separation by SDS-PAGE, the proteins were transferred electrophoretically to nitrocellulose in Tris-glycine buffer [33]. The transferred proteins were visualized by Ponceau S staining. The nitrocellulose was incubated for 3 h with 3% bovine serum albumin; 10 mM Tris, pH 7.5; 150 mM KCl then overnight with ^{125}I-calmodulin in the same buffer containing 1 mM EGTA. Unbound radiolabeled calmodulin was removed by washing in 10 mM Tris, pH 7.5; 150 mM KCl; 0.05% Tween 20 after which the nitrocellulose was dried. The amount of bound calmodulin was determined by phosphorimaging (Molecular Dynamics, Sunnyvale, CA) and normalized.

Immunoblotting

Proteins were separated by SDS-PAGE on 7.5%/15% mini-gels and transferred electrophoretically to nitrocellulose according to Towbin et al [33]. The transferred proteins were visualized by staining with Ponceau S then incubated in 3% BSA; 10 mM Tris, pH 7.5; 150 mM KCl for 3 h before incubation in the same

buffer containing one of the monoclonal anti-phosphoamino acid antibodies at the manufacturer's recommended dilutions. After washing, the nitrocellulose was incubated with goat-anti-mouse IgG-alkaline phosphatase. Color development was with 5-bromo-4-chloro-3-indoyl phosphate and nitrobluetetrazolium per the manufacturer's instructions.

ATPase assays

The actin-activated ATPase activity of the myosin-I isoforms was determined largely as described in Matsumura et al [34]. Typically, reaction mixtures of 100 µl contained myosin-I at a concentration of 30- 50 µg/ml in buffer containing 10 mM imidazole, pH 7.5; 2 mM $MgCl_2$; 20 mM KCl; 5 mM DTT; 0.7 mM ATP; 0.7 µM PMA; 30 µg/ml PS vesicles; 1 mM PMSF; 1.5 mM benzamidine; and 0.42 mg/ml F-actin. To some samples protein kinase C was added to a final concentration of 1 µg/ml. The reaction was allowed to proceed for 60 min and terminated by the addition of 50 µl 3N H_2SO_4 in 4% silicotungstic acid. Inorganic phosphate was measured by the method of Pollard [35].

Results

Incubation of the myosin-I isoforms with γ-^{32}P-ATP and protein kinase C in the presence of phospholipids results in phosphorylation of the myosin-I heavy polypeptide chains in a time dependent manner (Figure 1). Most of the labeling of the heavy chain occurred within 40 min; no labeling of calmodulin was observed. Scintillation counting of excised bands indicated a maximum stoichiometry of 1.5 - 2.0 moles phosphate per mole 130-kDa or 110-kDa myosin-I heavy chain.

Extent of phosphorylation of the myosin-I's by protein kinase C was a function of calcium ion concentration (Figure 2; panel A). Phosphorylation of myosin-I by protein kinase C was greater in the presence of calcium ion than in buffer containing EGTA. In the presence of EGTA, the addition of exogenous calmodulin reduced significantly (by ~50%) the extent of phosphorylation of each of the three myosin-I isoforms. Similarly, in the presence of calcium ion, the addition of calmodulin caused a reduction in the extent of phosphorylation of the myosin-I

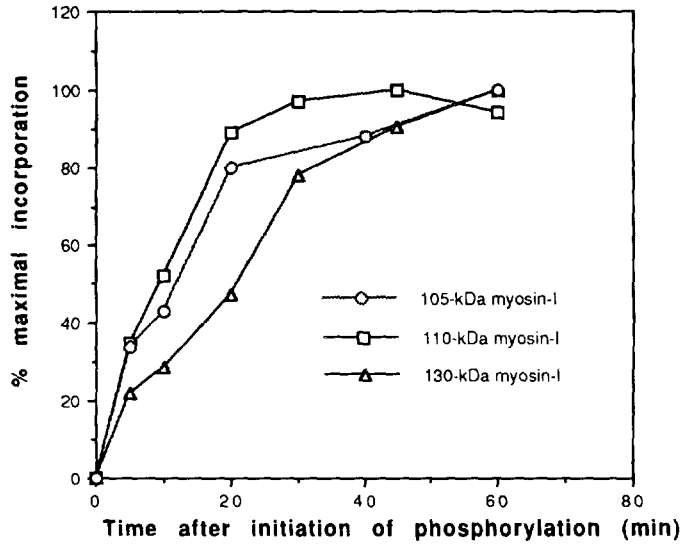

Figure 1. Phosphorylation of myosin-I heavy chains by protein kinase C as a function of time. Following the addition of protein kinase C in the presence of γ-^{32}P-ATP to solutions containing myosin-I, aliquots were removed at several time intervals, separated by SDS-PAGE and analyzed by phosphorimaging. Values are normalized to 100% of the maximal incorporation observed for that isoform.

isoforms by ~60-80%. Control samples in which myosin-I was replaced with histone indicate that the calcium ion or calmodulin-mediated changes in the extent of phosphorylation of the myosin-I isoforms could not be due to a direct effect on protein kinase C activity (Figure 2; panel B). In fact, phosphorylation of histone by protein kinase C was slightly enhanced in the presence of calcium ion and significantly enhanced in the presence of calmodulin.

When immobilized on nitrocellulose the three myosin-I isoforms will bind iodinated calmodulin [10,11]. Treatment of the myosin-I's with protein kinase C before separation by electrophoresis, electrophoretic transfer to nitrocellulose, and probing with ^{125}I-calmodulin decreased the amount of radiolabeled calmodulin bound by the myosin-I isoforms (Figure 3). The greatest change was a 70% reduction in calmodulin binding observed for the 110-kDa myosin-I heavy chain.

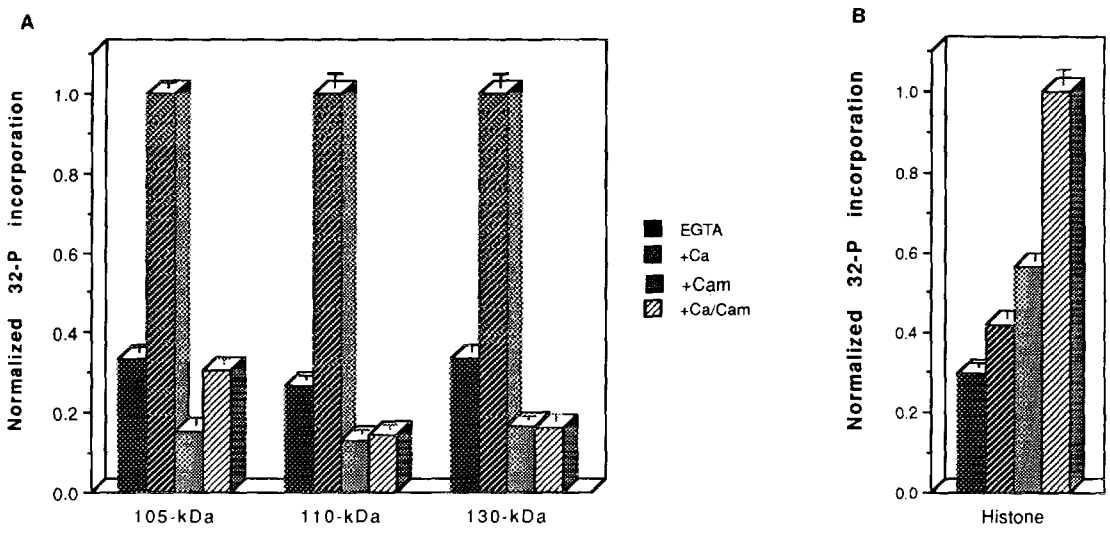

Figure 2. Exogenous calmodulin inhibits phosphorylation of myosin-I by protein kinase C. Panel A: Each of the three rat liver myosin-I isoforms was phosphorylated with protein kinase C under different ionic conditions. Values are normalized to the highest observed incorporation. Solid bar, no added calcium; striped black bar, 0.1 mM free Ca^{2+}; stippled bar, no added calcium, plus 6 μM exogenous calmodulin; striped white bar, 0.1 mM free Ca^{2+} plus 6 μM exogenous calmodulin. All reactions were performed in the presence of 0.5 mM EGTA as a calcium buffer, and the final free calcium ion values were calculated using a computer program. Panel B: Control samples containing histone in place of the myosin-I isoforms.

All myosins exhibit an actin-activated Mg^{2+}-ATPase activity. The ATPase activity exhibited by either the 130-kDa or 110-kDa myosin-I isoforms was unchanged as a result of phosphorylation by protein kinase C (Figure 4).

Immunoblotting, with monoclonal anti-phosphoamino acid antibodies, of the three myosin-I isoforms as isolated from rat liver indicates that the three isoforms are phosphoproteins. All three of the myosin-I's were recognized by anti-phosphoserine (Figure 5; panel B) and anti-phosphothreonine (Figure 5; panel C). The two smaller myosin-I's were recognized by anti-phosphotyrosine to a similar extent (Figure 5; panel D); the 130-kDa polypeptide was recognized to a lesser

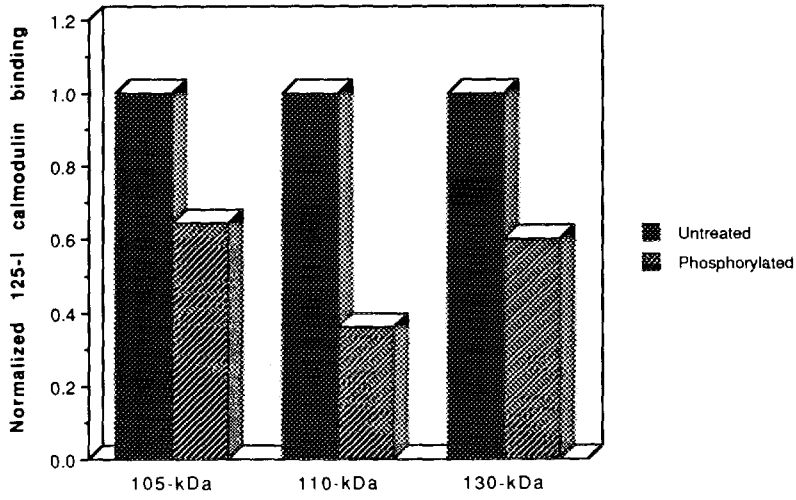

Figure 3. Decrease in calmodulin binding by myosin-I after phosphorylation with protein kinase C. Purified myosin-I was phosphorylated with protein kinase C in the presence of 5 mM (unlabeled) ATP, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was probed using ^{125}I-labeled calmodulin and subjected to autoradiography followed by phosphorimaging. All values are normalized to those of the untreated sample. Solid black bars, unphosphorylated samples; striped black bars, phosphorylated with protein kinase C. All values were normalized to the maximum observed for each myosin-I.

extent. The standards in lane 4 which crossreact with these antibodies are myosin-II, ovalbumin, and soybean trypsin inhibitor; each is known to be phosphorylated in vivo.

Discussion

Depending on the source, actin-binding, ATPase activity, filament formation, and/or motility of conventional myosins can be regulated by phosphorylation. In addition to substrate-specific kinases, conventional myosins can be phosphorylated by general kinases such as protein kinase C and casein kinase II (for a review, see [22]).

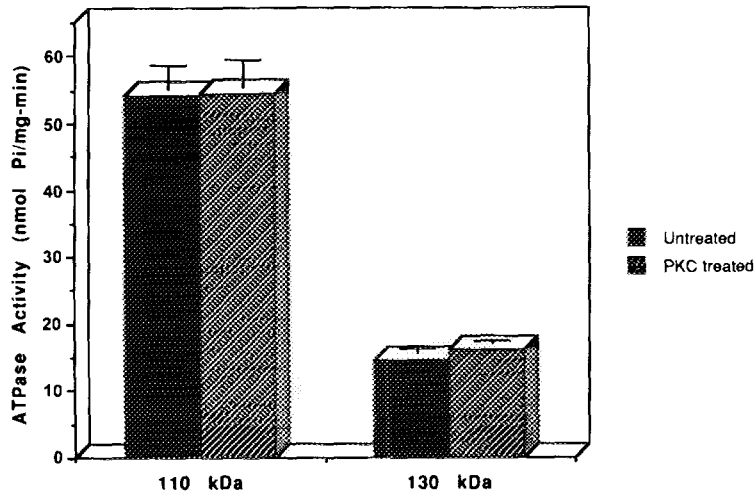

Figure 4. Effect of phosphorylation on ATPase activity. The ATPase activity was determined for the 130-kDa or 110-kDa myosin-I with or without treatment with protein kinase C. Activity was measured in nmol inorganic phosphate released per mg protein per min.

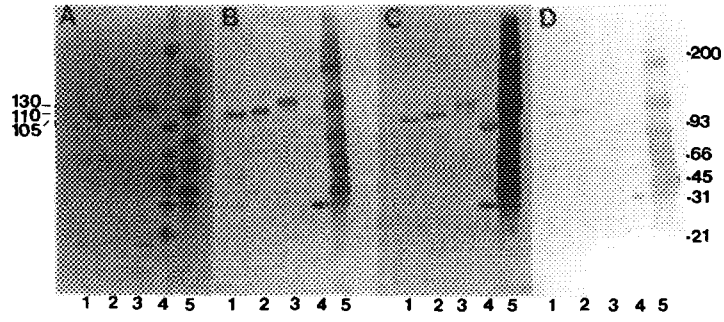

Figure 5. Recognition of myosin-I isoforms with anti-phosphoamino acid antibodies on immunoblots. The three myosin-I isoforms were separated by SDS-PAGE [Panel A], transferred to nitrocellulose, then probed with anti-phosphoserine [Panel B], anti-phosphothreonine [Panel C], and anti-phosphotyrosine antibodies [Panel D]. Lane 1; 105-kDa myosin-I; lane 2, 110-kDa myosin-I; lane 3, 130-kDa myosin-I; lane 4, molecular weight markers; lane 5, prestained molecular weight markers.

Less is known about the effects of phosphorylation on myosin-I's. Phosphorylation of *Acanthamoeba* myosin-I by a heavy chain kinase isolated from *Acanthamoeba* is required for myosin-I-mediated movement of beads in the *Nitella* motility assay [36]; the site phosphorylated is not present in higher eukaryotic myosin-I's. Intestinal brush border myosin-I (BBMI) can be phosphorylated in vitro by protein kinase C [29], with a similar calcium dependence to that seen in our study of the rat liver myosin-I's. Phosphorylation requires that BBMI be associated with phospholipid and is inhibited by addition of exogenous calmodulin [29]. The effect of this modification on BBMI's activity in terms of ATPase activity, calmodulin binding, or motility has not been characterized; moreover, whether phosphorylation of BBMI by protein kinase C occurs in vivo is unknown.

In an effort to determine if phosphorylation might affect higher eukaryotic myosin-I activity we have examined the effects of phosphorylation by protein kinase C on the rat liver myosin-I's. The study presented here indicates that, like brush border myosin-I [29], the three myosin-I isoforms are also in vitro substrates for protein kinase C. That the myosin-I isoforms are phosphorylated in vivo is supported by immunoblots using commercially available antibodies to the phosphoamino acids, which indicate that the myosin-I isoforms are phosphorylated as purified. Although the affected amino acids have not been identified, and the relevant kinases are as yet unknown, sequence analysis indicates that there are multiple possible serine/threonine phosphorylation sites for protein kinase C in each of the myosin-I heavy chains. Since the 105-kDa and 110-kDa react with the antibodies recognizing phosphotyrosine, these isoforms might be substrates for tyrosine kinases.

Phosphorylation of the myosin-I heavy chains is greater in buffers containing calcium ions than in those containing the calcium ion chelator, EGTA. The activation of protein kinase C activity which occurs in the presence of Ca^{2+} can only be partially responsible for this observation, since phosphorylation of control samples containing histone was only slightly enhanced in the presence of excess calcium ion. A second possibility is that in excess Ca^{2+}, calmodulin dissociates from the complex and exposes new sites to the kinase. This is supported by experiments

in which extent of phosphorylation decreases further when exogenous calmodulin is added. These results are in contrast to those observed for control samples containing histone in which phosphorylation was observed to increase with the addition of calmodulin.

Previous studies on brush border myosin-I indicate that in the presence of excess Ca^{2+}, a subset of calmodulin molecules dissociates from the complex [5], and that this dissociation might lead to enhanced phosphorylation by PKC [29]. It has been postulated that calmodulin dissociation is required for association of the myosin-I molecules with anionic phospholipids [29].

The myosin-I polypeptides will bind radiolabeled calmodulin after separation by SDS-PAG electrophoresis and renaturation. In a recent study, we have learned that the ability of the three isoforms to bind radiolabeled calmodulin in overlays does not correspond directly with the number of calmodulin molecules associated with the polypeptides in solution [11]. This is presumably due to differences in the heavy chains' abilities to renature after treatment with detergent. Nevertheless, the overlays can provide qualitative information concerning the ability of the isoforms to associate with calmodulin. The ability of the myosin-I isoforms to bind calmodulin is compromised after phosphorylation with protein kinase C. This suggests that phosphorylation of myosin-I might in turn serve to reduce the affinity of the molecule for calmodulin.

One possible model would be that dissociation of calmodulin due to a transient increase in calcium might lead to increased association of the myosin-I with phospholipid membranes, as suggested by Swanljung-Collins and Collins [29]. Phosphorylation by PKC (or another kinase) could then serve to stabilize this membrane binding by decreasing the affinity of the molecule for calmodulin, rebinding of which would presumably result in dissociation from the membrane. This would allow for longer term localization of the myosin-I in response to a transient calcium signal.

Analysis of fragments generated by controlled proteolysis of the intestinal myosin-I polypeptide [37] and organization of the major functional domains as predicted by the deduced amino acid sequence for the rat liver myosin-I's [12,13] indicate that the amino terminus of myosin-I contains the nucleotide binding and

actin binding domains whereas the carboxyl end is required for calmodulin binding. Phosphorylation does not affect the ability of the 130-kDa or 110-kDa polypeptides to hydrolyze ATP. Furthermore, in preliminary experiments with PKM, the proteolytic Ca^{++} and lipid independent subfragment of PKC , no effect on the ability of myosin-I to translocate actin filaments in vitro has been observed. That calmodulin binding but neither ATPase activity nor motility is affected by phosphorylation might indicate that the site of phosphorylation by protein kinase C is present in the tail region. This, together with the finding that the isoforms are phosphoproteins as isolated, allows for speculation that phosphorylation might be involved in other aspects of myosin-I regulation such as intracellular targeting.

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