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Biochemistry

Erythrocyte protein 4.1 binds and regulates myosin

(membranes/cytoskeleton)

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ABSTRACT **Myosin was recently identified in erythrocytes and was shown to partition both with membrane and cytosolic fractions, suggesting that it may be loosely bound to membranes [Fowler, V. M., Davis, J. Q. & Bennett, V. (1985) *J. Cell Biol.* 100, 47–55, and Wong, A. J., Kiehart, D. P. & Pollard, T. D. (1985) *J. Biol. Chem.* 260, 46–49]; however, the molecular basis for this binding was unclear. The present studies employed immobilized monomeric myosin to examine the interaction of myosin with erythrocyte protein 4.1. In human erythrocytes, protein 4.1 binds to integral membrane proteins and mediates spectrin–actin assembly. Protein 4.1 binds to rabbit skeletal muscle myosin with a K_d = 140 nM and a stoichiometry consistent with 1:1 binding. Heavy meromyosin competes for protein 4.1 binding with K_i = 36–54 nM; however, the S1 fragment (the myosin head) competes less efficiently. Affinity chromatography of partial chymotryptic digests of protein 4.1 on immobilized myosin identified a 10-kDa domain of protein 4.1 as the myosin-binding site. In functional studies, protein 4.1 partially inhibited the actin-activated Mg^{2+}-ATPase activity of rabbit skeletal muscle myosin with K_i = 51 nM. Liver cytosolic and erythrocyte myosins preactivated with myosin light-chain kinase were similarly inhibited by protein 4.1. These studies show that protein 4.1 binds, modulates, and thus may regulate myosin. This interaction might serve to generate the contractile forces involved in Mg^{2+}-ATP-dependent shape changes in erythrocytes and may additionally serve as a model for myosin organization and regulation in non-muscle cells.**

The mammalian erythrocyte, normally a biconcave disc, can undergo complex, energy-dependent shape changes (1–5). These changes seem to exert force upon both the cell membrane and its underlying membrane skeleton and can sometimes entail transmembrane signal transduction (6, 7). Early observations that these changes involved Mg^{2+}-ATPase activity suggested that a form of myosin might mediate the contractile processes involved in discocyte–echinocyte conversion (1–3, 8, 9). In 1985, two groups successfully isolated and characterized a specific isoform of myosin from human erythrocytes (8, 9). Monomeric erythrocyte myosin resembles skeletal muscle myosin in rotary-shadowed and negatively stained preparations. The molecule is ≈150 nm long, with two globular heads at the end of a rod-like tail. In both size and function, the light chains more closely resemble those found in non-muscle myosins. The ATPase activity of erythrocyte myosin is stimulated by Ca^{2+} or EDTA but is inhibited by Mg^{2+} and is refractory to actin stimulation unless the 19.5-kDa light chain is first phosphorylated by myosin light-chain kinase. In erythrocytes, the estimated concentration of 50 μg/ml is sufficiently high for myosin to form bipolar filaments; this led to the suggestion that erythrocyte shape changes might be mediated by an ATP-dependent actin–myosin contractile mechanism. In erythrocytes, myosin is distributed in a punctate fashion and seems to be loosely bound to membranes during fractionation. This further led to the suggestion that myosin might act at specialized foci such as spectrin–actin–protein 4.1 complexes, wherein force might be transmitted to the membrane skeleton and thus to the overlying membrane (8).

The present studies describe an interaction between protein 4.1 and myosin that extends the molecular basis for a myosin-based contractile mechanism in erythrocytes. The studies show that protein 4.1 binds to myosin and can regulate its actin-activated Mg^{2+}-ATPase, further suggesting spectrin–actin–protein 4.1 complexes as the locus of myosin action in erythrocytes.

MATERIALS AND METHODS

Purification of Proteins. Protein 4.1 was purified from human erythrocytes as described (10). Rabbit skeletal muscle myosin was purified according to Margossian and Lowey; C protein was removed by DEAE-cellulose chromatography as described (11). Liver myosin was purified according to Park *et al.* (12). Briefly, this method entailed homogenization of fresh rabbit liver in 0.3 M KCl/10 mM imidazole/10 mM EDTA/2 mM $CaCl_2$/1 mM ATP/ 1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/0.5 mM diisopropyl fluorophosphate at pH 7.2, with a Brinkman Polytron, removal of material sedimenting in 0.3 M KCl, precipitation by a 5-fold dilution, resolubilization in 0.6 M KCl, clarification by centrifugation, ammonium sulfate precipitation, and gel filtration chromatography on Sephacryl S-300. Erythrocyte myosin was purified from human erythrocytes precisely as described (8, 13). Actin was purified from chicken muscle acetone powder (a gift of James Casella, Department of Pediatrics, The Johns Hopkins University School of Medicine) by the method of Spudich and Watt (14), with subsequent gel filtration (15). Heavy meromyosin (HMM) and S1 fragment (myosin head) were prepared by chymotryptic cleavage as described (16). All purifications were monitored by SDS/PAGE (17).

Partial Digestion of Protein 4.1. Limited chymotryptic digests of ^{125}I-labeled erythrocyte protein 4.1 (10) were prepared at enzyme-to-substrate ratios of 1:200 and 1:1000 (wt/wt) by using α-chymotrypsin (Worthington); digestion was performed at 4°C for 30 min in 10 mM Tris·HCl (pH 8.0) and was stopped by adding diisopropyl fluorophosphate to 0.2 mM at 30 min and again at 12 hr, after which the digests were pooled to assure an even distribution of fragments. The resultant mixture was dialyzed into 145 mM KCl/5 mM potassium phosphate, pH 7.5/0.1 mM phenylmethylsulfonyl fluoride prior to affinity chromatography.

Iodination of Proteins. Protein 4.1 was iodinated by the Iodogen method (18). Generally, 20 μg of Iodogen (Pierce) dissolved in CCl_4 was plated into the bottom of a 1.5-ml

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Abbreviations: HMM, heavy meromyosin; SMPB, succinimidyl 4-(*P*-maleimidophenyl)butyrate.

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microcentrifuge tube, and 200–800 μg of protein 4.1 in 10 mM sodium phosphate/20 mM KCl, pH 7.6, was added, followed by 500 μCi (1 Ci = 37 GBq) of carrier-free ^{125}I as NaI. Unincorporated isotope was removed by dialysis. The specific activities were within the range of 50,000 dpm/μg.

Preparation of Myosin-Sepharose. Ten to 25 ml of EAH-Sepharose CL-4B (Pharmacia), a resin derivatized with hexane diamine by means of an epoxide linkage, was washed in three changes of dimethyl sulfoxide. Following the final wash, the resin, which contained 6–10 μmol of amine per ml of gel, was resuspended in an equal volume of dimethyl sulfoxide containing 100 mg of succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) (Pierce) and slowly stirred at room temperature for 60 min. After reaction, the resin was washed with 500 ml of 40 mM sodium pyrophosphate (pH 7.6) in a sintered glass funnel and resuspended in 20 ml of buffer containing rabbit skeletal muscle myosin at 6 mg/ml. After overnight incubation at 4°C, 2-mercaptoethanol was added to 10 mM and the resin was incubated a further 1 hr. Coupling efficiency was 97% as determined by protein assay (19) of the supernate.

Binding Assays. Binding assays were carried out under conditions indicated in the text in 75-μl volumes. Parafilm (American Can, Greenwich, CT) was used to create 8- × 8-mm wells in the plastic grid from a pipette tip box (no. RT-96, Rainin, Woburn, MA). The buffer required for each incubation was first pipetted into each well; following this, a 1- × 1-cm square of 41-μm mesh Nytex nylon fabric (Tetko, Elmsford, NY) was moistened in the buffer and pressed to the bottom of the well. The remaining reagents were then added with mixing, the assembly was covered, and the mixtures were incubated at 4°C for 1 hr; preliminary studies showed that binding was constant after 1 hr of incubation. Bound and free ligand were separated by carefully lifting one corner of the Nytex screen and inserting one prong of a 2- × 15-cm Whatman 3Chr filter paper strip with a V-shaped notch under the screen into the liquid. The screen, bearing the beads, was drawn up the filter paper until all liquid had been wicked off and then was reserved for γ counting. The other prong of the filter paper was used to swab any remaining supernate from the well, and the filter paper was similarly reserved for γ counting. Finally, the Parafilm wells were excised and similarly counted. One to 2% of the total counts remained with the Parafilm excised from the wells. This assay permitted rapid separation of the myosin-Sepharose beads from the supernate, with quantitative recovery of each.

ATPase Assays. ATPase activity was assessed by measurement of released inorganic phosphate as phosphomolybdate, as described by Pollard and Korn (20). The assays were carried out in 25 mM imidazole, pH 7.0/150 mM NaCl/1 mM dithiothreitol in 0.5-ml volumes. HMM or myosin concentrations were 0.5–5 μg/ml; actin, 5–10 μg/ml; ATP, 0–15 mM; KCl, 5 mM; $MgCl_2$, 0.3 mM. In experiments involving liver and erythrocyte myosin, 5 μg of myosin was first incubated for 30 min at room temperature with 1 mM $CaCl_2$/0.2 mM ATP/0.05 μg of myosin light-chain kinase (gift of Thomas Pollard, Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine)/0.2 μM calmodulin. EGTA and $MgCl_2$ were then each added to 2 mM, ATP was adjusted to 2 mM as indicated, and actin was added to 20 μg/ml. Protein 4.1 was used as an inhibitor over the ranges of 0–700 nM. Incubations for determination of ATPase activity were at 35°C for 30 min.

RESULTS

Protein 4.1 Binds to Myosin. Protein 4.1 associates with myosin filaments *in vitro*; a preliminary, qualitative velocity sedimentation experiment showed that protein 4.1 could interact with native myosin under conditions where it forms filaments. Purified human erythrocyte 4.1 was incubated with purified rabbit skeletal muscle myosin in an isotonic salt buffer and then was applied to a 5–20% sucrose gradient in the same buffer. Analysis of the resultant gradient fractions by SDS gel electrophoresis showed that protein 4.1 and myosin cosediment (data not shown), although the wide range of buoyant densities of the myosin species made further analysis difficult in this system. Proteolytic fragments of myosin, HMM and S1, do not assemble into filaments and therefore could potentially have been used to study this interaction, but carried the risk that their binding properties might be altered. These considerations led to the development of a binding assay employing immobilized monomeric myosin as a ligand. Briefly, myosin was attached to Sepharose beads through the reactive thiol groups located in the S1 fragment, or myosin head region (21). EAH-Sepharose, a Sepharose resin commercially derivatized with hexane diamine by means of an epoxide linkage, was reacted with excess SMPB. Following removal of excess SMPB, the resin was then reacted with monomeric rabbit skeletal muscle myosin in 40 mM sodium pyrophosphate (pH 8.0) at room temperature, and the residual reactive groups were capped with 2-mercaptoethanol, resulting in a typical coupling of 6.1 mg of myosin per ml of resin. The ability of erythrocyte protein 4.1 to bind to immobilized myosin was then studied using freshly prepared ^{125}I-labeled protein 4.1. In control experiments, unlabeled protein 4.1 freely exchanged with its iodinated counterpart. Myosin beads were incubated with labeled protein 4.1. After incubation, immobilized myosin with bound protein 4.1 was separated from unbound reagents by filtration through nylon mesh screens (mesh size = 41 μm); supernates and washes were collected on filter paper applied to the underside of the screens, which permitted separation of bound protein 4.1 from unbound within 15 sec. Fig. 1 shows the results of a typical binding study measured in 145 mM KCl/5 mM potassium phosphate, pH 7.5/0.1 mM phenylmethylsulfonyl fluoride. The 75-μl samples were incubated 60 min at 4°C prior to separation. Fig. 1*A* shows the resultant binding isotherm, and Fig. 1*B* shows the related Scatchard plot (22). The binding appeared saturable. Scatchard analysis yielded a linear plot with K_d = 140 nM and saturation at 0.8 mol of protein 4.1 per mol of myosin, most consistent with 1:1 stoichiometry. The salt-dependence curve (data not shown) showed maximal binding at physiologic salt concentrations, suggesting that the observed binding involves more than simple electrostatic interaction.

Interaction of HHM and S1 With Protein 4.1. Fig. 2*A* represents an experiment that tested the ability of HMM to compete with immobilized monomeric myosin for protein 4.1 binding. The experiment was performed (*i*) to determine whether HMM retained the ability to bind protein 4.1 and (*ii*) if it did bind, to ascertain whether the affinity, measured as a K_i, differed from that obtained for immobilized myosin. A Dixon plot (23) of the data yielded a K_i in the range of 36–54 nM, suggesting either that HMM binds protein 4.1 more avidly or that attachment of myosin to beads subtly alters its ability to bind protein 4.1. In comparison to S1 (Fig. 2*B*), HMM competed with myosin for protein 4.1 binding more effectively on a molar basis. This suggests that the protein 4.1 binding site lies in the neck region of myosin, involving structural elements in the head as well as in the most proximal regions of the tail; alternatively, it is possible that both myosin heads participate in binding.

Localization of Myosin Binding to a 10-kDa Domain of Protein 4.1. Affinity chromatography of limited digests of protein 4.1 on myosin beads localized the myosin binding site to a 10-kDa domain of protein 4.1. This domain is biologically active in other systems; it promotes spectrin–actin association (24, 25) and contains a cAMP-dependent phosphorylation site. Limited chymotryptic digests of ^{125}I-labeled eryth-

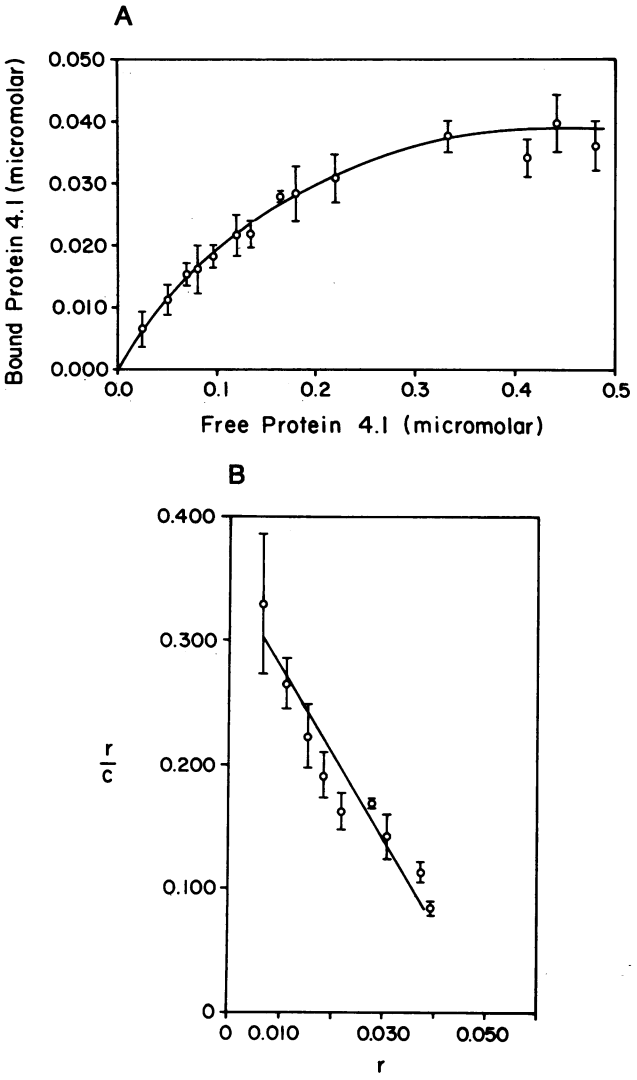

FIG. 1. Binding of protein 4.1 to myosin. (*A*) Isotherm obtained for the binding of protein 4.1 to immobilized myosin. The error bars represent the standard deviations obtained from three separate experiments, each performed in duplicate. (*B*) Data from the initial portion of the binding isotherm plotted according to Scatchard (22) using the conventional definitions of *r* and *c* to denote the bound and free concentrations of protein 4.1, respectively.

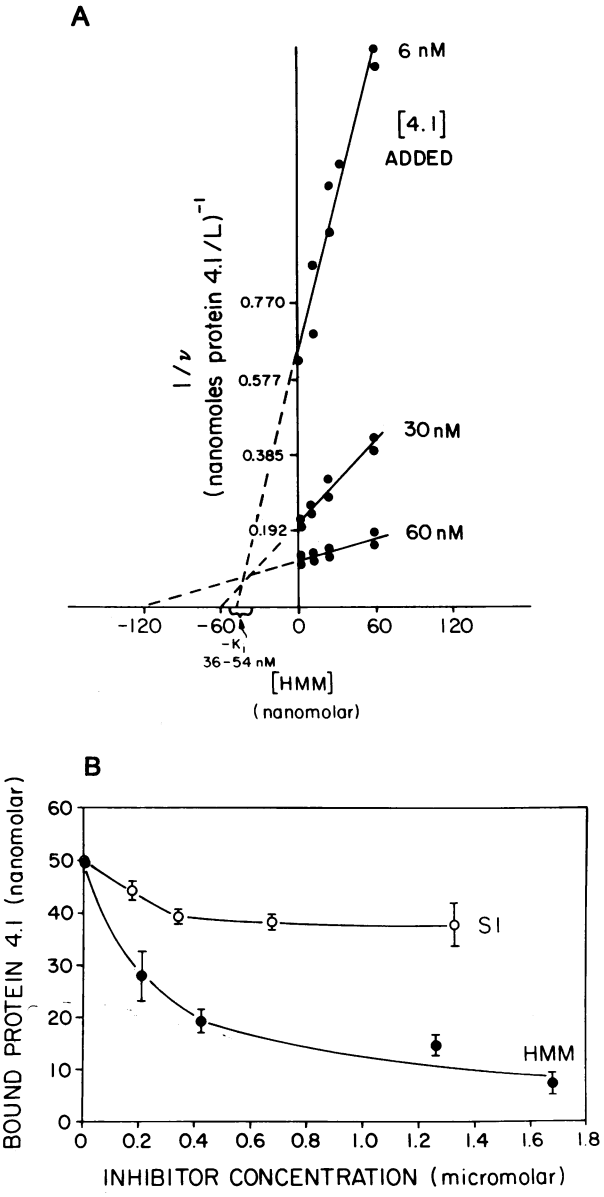

FIG. 2. Effect of myosin fragments on protein 4.1–myosin interaction. (*A*) Inhibition of protein 4.1 binding to immobilized myosin by HMM. Reagents were added in the following order: protein 4.1, HMM, and myosin beads. The other conditions were the same as those for Fig. 1. The data are plotted according to Dixon (23). (*B*) Effect of S1 and HMM. The error bars represent the standard deviations obtained for two separate experiments, each performed in triplicate.

rocyte protein 4.1 were loaded onto an affinity column of the previously described myosin beads in 145 mM KCl/5 mM potassium phosphate, pH 7.5/0.1 mM phenylmethylsulfonyl fluoride. Fig. 3 shows the experiment analyzed by SDS/PAGE and autoradiography. The distributions of unbound and retained fragments differ markedly; the boxes on either side illustrate the peptide composition according to the scheme of Leto and Marchesi (10). The only region shared by retained fragments is the 10-kDa region; conversely, the only region absent from the fragments flowing through the column is the 10-kDa region. All fragments possessing the 10-kDa region seemed to behave in a similar manner. The likely conclusion is therefore that the myosin binding site of protein 4.1 lies in the 10-kDa region and that other domains lying N- or C-terminal to the 10-kDa domain do not influence its binding under these conditions.

Protein 4.1 Modulates Myosin's Actin-Activated ATPase Activity. The binding studies described above suggested that under physiologic conditions protein 4.1 could interact with myosin in a specific manner. To determine whether this binding resulted in a functional modification of myosin, actin-activated ATPase was examined. In the experiment shown in Fig. 4, 5 μg of HMM, 10 μg of actin, ATP as indicated, and protein 4.1 as indicated were incubated in 0.5 ml of 25 mM imidazole, pH 7.0/150 mM NaCl/1 mM dithiothreitol/2 mM $MgCl_2$/2 mM EGTA, and the resultant released inorganic phosphate was measured (20). The data, plotted according to Dixon (23), show that increasing concentrations of protein 4.1 cause commensurate reductions in skeletal muscle myosin's actin-activated ATPase activity, saturating at an ≈50% overall reduction in activity. The K_i of 51 nM is in reasonably good agreement with the binding constants measured directly for immobilized myosin (140 nM) and indirectly by HMM inhibition, (≈45 nM). Erythrocyte and liver cytosolic myosins are also inhibited by protein 4.1, as shown in Fig. 5. In this experiment, erythrocyte and liver myosin were first phosphorylated by incubation with 0.1

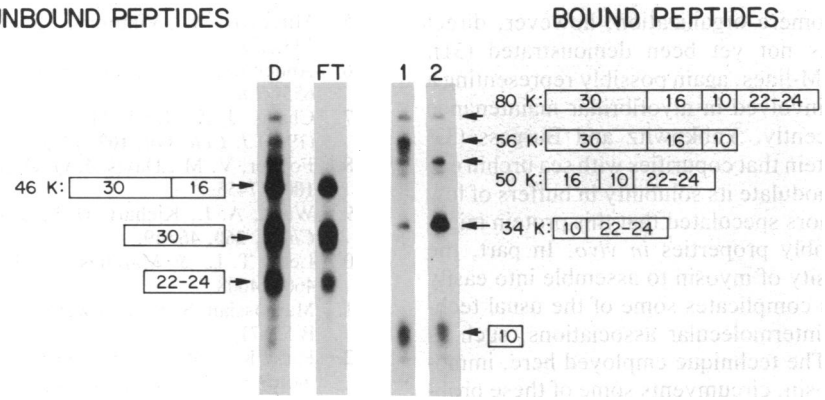

FIG. 3. Myosin affinity chromatography of protein 4.1 digest. Lanes: D, total digest; FT, flow-through; 1, 195 mM KCl eluate; 2, 395 mM KCl eluate. The figure represents an autoradiograph of a 10–15% polyacrylamide gradient gel. Intact protein 4.1 (80 kDa) and its chymotryptic cleavage products are designated numerically according to the scheme of Leto and Marchesi (10).

μM myosin light-chain kinase (from turkey gizzard, a gift of Thomas Pollard) in the presence of 0.2 mM calcium, 200 nM calmodulin, and 0.2 mM ATP. After incubation, EGTA was added to 2 mM, followed by actin, additional ATP in some cases, and protein 4.1. Under these conditions, both erythrocyte and liver myosin display actin-dependent ATPase activity; the addition of protein 4.1 resulted in substantial dose-dependent inhibition of the ATPase activity of both of these myosins at levels well below the 480 nM actin concentration. In the absence of actin and protein 4.1, the basal values were 0.23 nmol of P_i per mg per sec for erythrocyte myosin at 0.2 mM ATP, 0.55 nmol of P_i per mg per sec for erythrocyte myosin at 2 mM ATP, 0.09 nmol of P_i per mg per sec for liver myosin at 0.2 mM ATP, and 0.31 nmol of P_i per mg per sec for liver myosin at 2 mM ATP.

DISCUSSION

The results of these manipulations of myosin and protein 4.1 *in vitro* support the notion that there is a physiologic interaction between them. This suggestion is supported by quantitative binding studies, by a physiologic salt and pH dependence of the interaction, by localization of binding to a discrete domain of protein 4.1, and by the demonstration that the binding of protein 4.1 to myosin modulates actin-activated Mg^{2+}-ATPase activity, a physiologic property of myosin. The quantitative binding studies were carried out with skeletal muscle myosin because of its abundance and its well-characterized properties, including the ability to generate well-characterized subfragments. Although binding was not quantitated for erythrocyte or liver myosins, binding was demonstrated qualitatively for all three myosins by using radiolabeled protein 4.1 in a ligand blotting assay (data not shown), further suggesting that the myosin light chains are not required for binding, since they were resolved in the SDS/PAGE gel prior to transfer.

Focusing upon myosin, these data provide evidence for regulation of myosin function by a myosin-binding protein. Myosin function is regulated by phosphorylation of heavy and light chains, by calcium, and by actin (reviewed in refs. 26 and 27). Only a handful of myosin-binding proteins have been described, in marked contrast to actin, where many functionally important properties are governed by a now numerous family of actin-binding proteins (reviewed in refs. 28 and 29). For example, C protein is a 135- to 150-kDa polypeptide, found in white and red skeletal muscles and in cardiac muscle (30), that copurifies with myosin up to the stage of anion-exchange chromatography. By binding to both actin and myosin, C protein both inhibits myosin's actin-activated ATPase activity at low ionic strengths and stimulates it in under isotonic conditions. Two unusually large myofibrillar proteins, titin and nebulin, are thought to play a

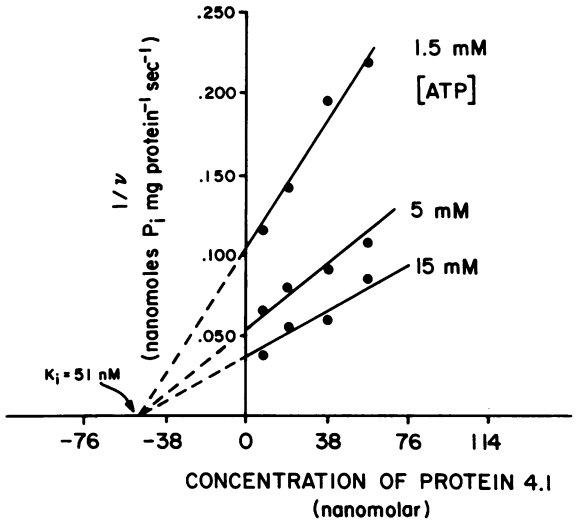

FIG. 4. Inhibition of myosin's actin-activated ATPase activity by protein 4.1. Various concentrations of protein 4.1 were incubated with 5 μg of HMM, 10 μg of actin in 0.5 ml of 25 mM imidazole, pH 7.0/150 mM NaCl/1 mM dithiothreitol/2 mM $MgCl_2$/2 mM EGTA at 35°C for 30 min; ATP concentrations are indicated on the graph.

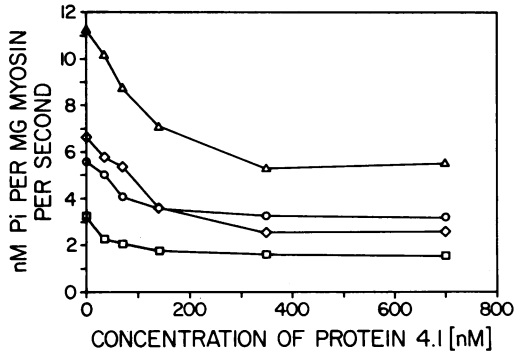

FIG. 5. Effect of protein 4.1 on cellular myosin's actin-activated ATPase activity. Various concentrations of protein 4.1 were incubated with 10 μg of actin and 5 μg of myosin in 0.5-ml volumes. Standard deviations ranged from 0.02 to 0.6 nM P_i per mg of myosin per sec, and were generally smaller than the symbols shown. The figure represents three separate experiments, each performed in duplicate. □, Liver myosin with 0.2 mM ATP; ○, liver myosin with 2.0 mM ATP; ◇, erythrocyte myosin with 0.2 mM ATP; △, erythrocyte myosin with 2 mM ATP.

structural role in sarcomere organization; however, direct binding to myosin has not yet been demonstrated (31). Myomesin localizes to M-lines, again possibly representing a structural component involved in myofibrillar maintenance or assembly (32). Recently, Yabkowitz and Burgess (33) described a 53-kDa protein that copurifies with sea urchin egg myosin and seems to modulate its solubility in buffers of low ionic strength; the authors speculated that this protein might regulate myosin assembly properties *in vivo*. In part, the salt-dependent propensity of myosin to assemble into easily sedimentable filaments complicates some of the usual techniques used to study intermolecular associations, such as immunoprecipitation. The technique employed here, immobilized monomeric myosin, circumvents some of these problems.

Increasingly, erythrocyte protein 4.1 appears to be a multifunctional protein with complex properties (34, 35). In erythrocytes, it associates with several integral membrane proteins (36–38) and promotes spectrin–actin associations. A recent report showed that protein 4.1 can also interact with tubulin (39). Moreover, the observations that it is a substrate for protein kinases A and C (25, 40–43) and that its association with glycophorin A seems sensitive to the phosphorylation state of membrane inositol phospholipids (37) suggest a regulatory role. At a genetic level, several alternatively spliced erythroid forms have been identified, although their physiologic significance is not clear, particularly regarding membrane skeletal assembly and regulation (44). The present study adds to this complexity by demonstrating a biochemical interaction between protein 4.1 and myosin.

If, as seems reasonable, the behavior of protein 4.1 and myosin *in vivo* is similar to that observed with isolated proteins *in vitro*, one may consider the possibility that myosin participates directly in the Mg^{2+}/ATP-dependent shape changes in erythrocytes. A number of important questions, however, remain open. The small amount of myosin in erythrocytes is considered sufficient to form filaments and, with actin, generate contractile forces (8). The present studies, however, provide no direct evidence of involvement, such as inhibition of shape changes by myosin-specific reagents; this must await further work. Moreover, in the erythrocyte, the physiologic interplay between spectrin, actin, protein 4.1, and myosin may be complex, since the same region of protein 4.1 that promotes spectrin–actin interaction (24) also binds myosin; moreover, this region possesses a cAMP-dependent phosphorylation site (25) whose role in the interaction is presently unknown. Nevertheless, it is intriguing to speculate that protein 4.1 may serve a dual role, on one hand anchoring myosin to the membrane in close proximity to spectrin–actin complexes, and, on the other, serving to regulate myosin function.

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