The GPQ-Rich Segment of *Dictyostelium* Myosin IB Contains an Actin Binding Site†

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ABSTRACT: Myosin I has been implicated as the motor that drives protrusion of the leading edge of motile cells. This function requires a close association with the plasma membrane and the cytoskeleton. Association with the actin cytoskeleton is mediated by an ATP-dependent binding site in the motor-containing myosin head, as well as by a second, ATP-independent actin binding site. In myosin IC from *Acanthamoeba*, the ATP-independent actin binding site is located in the carboxy-terminal tail, in a domain composed of two segments. The first segment is basic and is referred to as the *GPA-rich segment*. The second is a highly conserved sequence called *src homology region 3* (SH3), found in a variety of cytoskeletal-associated proteins. We have used bacterially-expressed fusion proteins containing portions of *Dictyostelium* myosin IB to determine if the tail of this myosin I isoform also binds to actin and to establish precisely where the actin binding site is located. We have determined that the carboxy-terminal portion of the tail of *Dictyostelium* myosin IB can bind to actin in an ATP-independent manner and that the actin binding site is contained within residues 922–1059, corresponding to the GPA-rich segment of *Acanthamoeba* myosin IC. We conclude that this region contains a specific actin binding site which may be responsible for the cytoskeletal association of this myosin I isoform.

The myosins are a diverse collection of molecular motors that drive a wide variety of cellular movements. They share in common an amino-terminal, globular "head" domain that possesses an actin-activated ATPase activity and can generate motility in *in vitro* assays (Warrick & Spudich, 1987; Korn & Hammer, 1988; Pollard *et al.*, 1991). They differ most markedly from each other in the sequences of their carboxyl-terminal "tail" segments. In the case of myosin I, a family of single-headed myosin molecules, the tail segments do not allow formation of oligomers or bipolar filaments, but instead have been implicated in localizing this family of molecular motors to the plasma membrane and the membrane-associated cytoskeleton (Endow & Titus, 1992; Pollard *et al.*, 1991). This is likely to be central to the functions of myosin I isoforms, which have been proposed to include the development and maintenance of microvilli, the protrusion of pseudopodia, and the transport of membrane vesicles (Endow & Titus, 1992; Fukui *et al.*, 1989; Pollard *et al.*, 1991).

Among the most intensively studied myosin I isoforms are those from protozoans. Sequence studies of myosins IB and IC from *Acanthamoeba* and of myosin IB from *Dictyostelium* reveal that the carboxy-terminal tail consists of two domains (Figure 1). The first, located on the amino-terminal side of the tail, consists of a highly basic sequence of 250 amino acids which is found in other myosin I isoforms of *Acanthamoeba* and *Dictyostelium* (Doberstein & Pollard, 1992; Jung *et al.*, 1989). The second, on the carboxy-terminal side of the tail, consists of two segments. The first segment consists of a sequence of approximately 135 amino acids which is basic, is rich in glycine, proline, and alanine or glutamine, and is referred to as the "GPA-rich" or "GPQ-rich" segment (Doberstein & Pollard, 1922; Jung *et al.*, 1989; Pollard *et al.*, 1991). The second segment consists of a sequence of approximately 50 amino acids which is referred to as "SH3", for *src homology region 3*.[1] This conserved region is found in a variety of cytoskeleton-associated proteins, including several oncogenes (c-src, v-src, c-abl, v-yes, v-crk), α-spectrin, phospholipase Cγ, two neutrophil NADPH oxidase-associated proteins, a yeast actin binding protein, and a putative human transcription factor called HS1 (Drubin *et al.*, 1990; Koch *et al.*, 1991). By contrast, the GPA(Q)-rich sequences from *Acanthamoeba* and *Dictyostelium* myosin I cannot be aligned convincingly with each other or with any other protein. This has led to the suggestion that what is conserved is the unusual amino acid composition, net positive charge, and tertiary structure (Korn & Hammer, 1990).

The functions of the tail domains in *Acanthamoeba* myosin I isoforms have been investigated by proteolytic and bacterial expression studies (Lynch *et al.*, 1986; Brzeska *et al.*, 1988; Doberstein & Pollard, 1992). Bacterially-expressed fusion proteins containing the amino-terminal 250 amino acid domain, designated in Figure 1 as the "membrane binding segment", were found to bind to NaOH-stripped plasma membranes and to phospholipid vesicles, but not to actin, while a proteolytic fragment or bacterially-expressed fusion protein containing the carboxy-terminal domain, comprising both the GPA and SH3 segments, bound to actin filaments but not to phospholipid vesicles or stripped membranes. Actin binding was found to be independent of ATP, in contrast to that seen in the "head" domain, and this feature would allow

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[1] Abbreviations: GPQ, glycine-, proline-, and glutamine-rich segment of the carboxy-terminal tail from *Dictyostelium* myosin IB; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SH3, *src* homology region 3.

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these myosin I isoforms to cross-link actin filaments and translocate one set of filaments relative to another. The relative contributions of the GPA(Q) and SH3 segments to actin binding were not elucidated in these studies. The fact that many SH3-containing proteins are associated with the cytoskeleton had led to the suggestion that this segment represents a unique actin binding region (Drubin *et al.*, 1990). Bacterially-expressed fusion proteins containing the SH3 segments from *Acanthamoeba* myosin IC and the c-abl protooncogene were found not to bind to actin (Doberstein & Pollard, 1992; Cichetti *et al.*, 1992); however, one study found that only a limited repertoire of SH3 segments bound to the c-abl SH3 ligand 3BP-1, thus raising the possibility that other SH3 segments might indeed bind to actin (Cichetti *et al.*, 1992).

In this study, we have made bacterially-expressed recombinant proteins containing defined segments of the putative ATP-independent actin binding domain from myosin IB of *Dictyostelium discoideum*, using the polymerase chain reaction (PCR). We have determined that ATP-independent actin binding occurs only in those recombinant proteins which contain the GPQ segment and conclude that this segment contains an actin binding site. These results thus precisely map the site responsible for ATP-independent actin binding activity in this class of myosin I.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides used in the polymerase chain reaction (PCR) were synthesized by Oligos Etc. (Guilford, CT). Restriction enzymes, modifying enzymes, and Pfu polymerase were obtained from Stratagene, Inc. (La Jolla, CA). Taq polymerase was supplied by Perkin-Elmer Corp. (Norwalk, CT). Media components were obtained from Difco Laboratories (Detroit, MI). Actin was prepared from rabbit back and leg muscles as described (Spudich & Watt, 1971). Chemicals used for buffers and agarose gel electrophoresis, glutathione–agarose beads, phenylmethanesulfonyl chloride, phosphatidylserine, and thrombin (human plasma) were obtained from Sigma Chemical Co. (St. Louis, MO). Chemicals used for SDS–PAGE were from Bio-Rad Laboratories (Hercules, CA). Desalting of proteins was carried out on prepoured Sephadex G-25 columns (PD-10, Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

Myosin I Tail Expression in Escherichia coli and Purification of Recombinant Proteins. A genomic clone of *Dictyostelium* myosin IB was used as the starting material and was generously provided by Dr. M. A. Titus (Duke University). Four fusion proteins were generated in this study—*myo 2*, *myo 3*, *myo 5*, and *myo 6* (Figure 1). They were produced from DNA inserts generated from PCR amplification of selected regions of the *Dictyostelium* myosin IB genomic clone. The following oligonucleotide primers were used: *N-terminal primer for myo 5* and *6*: 5′-GCGGGATCCCCAAAGAATTATAATCCAAACTC-3′, containing a *Bam*HI restriction site; *N-terminal primer for myo 2*, 5′-GCGGATCCGGCGGTGGCCCAGCAG-3′, containing a *Bam*HI restriction site; *N-terminal primer for myo 3*, 5′-GCGGATCCAAAGCACTCTACGATTATG-3′, containing a *Bam*HI restriction site; *C-terminal primer for myo 6*, 5′-CCGAATTTCTTATGCAGTTGGTCTTGATGGTTG-3′, containing an *Eco*RI restriction site; *C-terminal primer for myo 2, 3, and 5*, 5′-GCTGGATCCCCAAAGAATTATAATCCAAACTC-3′, containing an *Eco*RI restriction site. These inserts were ligated into the vector pGEX-2T (Pharmacia LKB Biotechnology, Inc.). This vector is designed to generate a fusion protein in which the amino terminus of the appropriate myosin I region is fused to the carboxy terminus of glutathione *S*-transferase (GST) from *Schistosoma japonicum* (Smith & Johnson, 1988). The vector codes for a thrombin cleavage site which separates the GST from the insert. DNA from the clones which were generated was prepared by a modification of the alkaline lysis minipreparation method (Ish-Horowicz & Burke, 1981), restriction-digested, and analyzed on agarose gels. Positive clones were used to transform *E. coli* DH5αF′ as described (Sambrook *et al.*, 1989). Transformants were selected on plates of LB with 100 μg/mL ampicillin. For purification of proteins, 2 L of culture was grown at 37 °C and 300 rpm in LB with 100 μg/mL ampicillin to an absorbance at 595 nm of 0.6. The cultures were induced for 3 h by the addition of 0.5 mM IPTG. The cells were harvested by centrifugation and stored at −70 °C. The frozen cells were thawed and resuspended in 30 mL of lysis buffer (PBS, 0.05 M EDTA, 1% Triton X-100, and 1 mM PMSF) and lysed by sonication. The clarified extract was incubated with 2 mL of glutathione–agarose beads for 30 min at 4 °C on a rotator. The beads were washed 5 times with lysis buffer, and bound protein was eluted with two washes of freshly prepared 50 mM Tris/10 mM glutathione, pH 8.00, at 4 °C for 30 min. Yields of fusion proteins averaged 25–30 mg of soluble protein per liter of culture.

Thrombin Digestion of Fusion Proteins. Separation of the myosin IB tail fragment from the GST moiety in myo 2, myo 5, and myo 6 was accomplished by thrombin digestion. This was performed by washing glutathione–agarose-immobilized myo 2, myo 5, or myo 6 with digestion buffer (150 mM NaCl, 50 mM, Tris, and 2.5 mM $CaCl_2$, pH 7.50) followed by addition of thrombin (1:500 w/w). Digestion was allowed to proceed at room temperature for 1 h on a rotator and stopped by addition of phenylmethanesulfonyl chloride to 0.25 mM. Thrombin-cleaved myo 2, myo 5, and myo 6 were separated from bound GST and uncleaved myo fusion proteins by centrifugation. The supernatants, containing cleaved myo 2, myo 5, and myo 6, were desalted into the appropriate buffers using chromatography on PD10 columns.

Actin Binding Assays. Determination of which myo fusion proteins bound to actin was made by means of a sedimentation assay. 10 μM myo fusion protein was mixed with 40 μM actin in 10 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM NaN_3, and 0.5 mM ATP, pH 7.50, and sedimented in the Beckman Airfuge at 120000*g* for 20 min at room temperature. SDS–PAGE on a 10% acrylamide gel was performed on the supernatants, and the gel was stained with 0.1% coomassie blue. Measurement of the dissociation constants of the various myo fusion proteins for actin was by a modification of this sedimentation assay. Myo fusion proteins or GST was mixed with actin at final concentrations of 0.2–0.5 μM fusion protein or GST and a 20–150-fold molar excess of actin in 10 mM HEPES, 5 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 1 mM NaN_3, and 0.5 mM ATP, pH 7.50. Samples were sedimented in a Beckman Airfuge at room temperature at 120000*g* for 20 min. The supernatants were transferred to 1.5-mL tubes and evaporated to dryness in a Savant Speed-Vac. Each sample was resuspended in 30 μL of SDS sample buffer and analyzed by SDS–PAGE. The relative content of unbound myo fusion protein was determined by quantitative densitometry of silver-stained polyacrylamide gels (Silver Stain Plus, Bio-Rad Laboratories). Gels were scanned using a Bio-Rad Laboratories Model 620 video densitometer. The concentration of unbound fusion protein was then determined by comparison to a standard curve produced by SDS–PAGE of supernatants of 0.1–0.5 μM fusion protein sedimented in

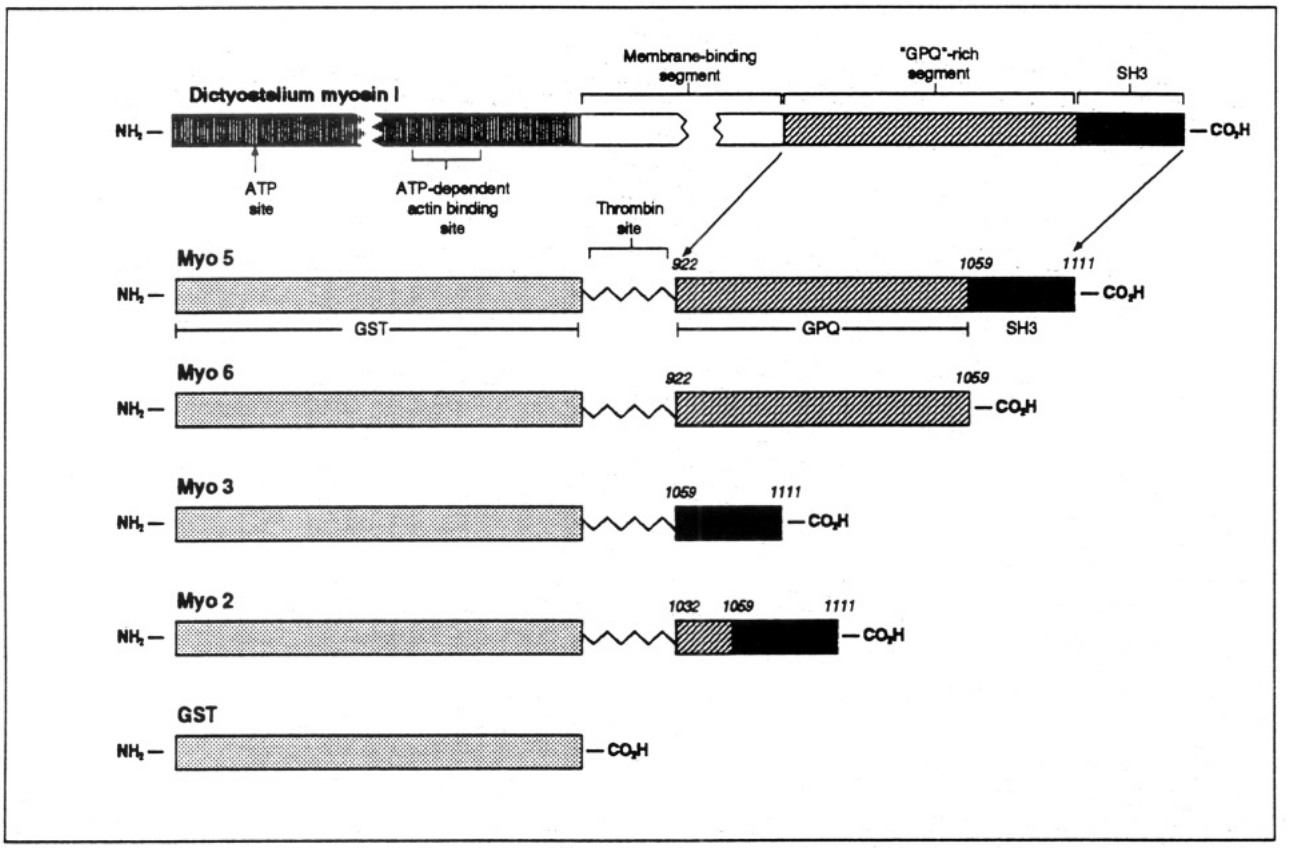

FIGURE 1: Domain structure of *Dictyostelium* myosin IB and myo fusion proteins. Each of the myo proteins represents fusion between glutathione *S*-transferase (stippled bar) and a portion of the actin binding tail domain from myosin IB, which are separated from each other by a thrombin cleavage site (serrated line). The myosin I-derived portion of each fusion protein contains the SH3 segment (black bar), the GPQ segment (diagonally hatched bar), or a mixture of the two. *GST*, glutathione *S*-transferase. *SH3*, c-*src* homology region 3. *GPQ*, glycine-, proline-, and glutamine-rich tail segment. NH_2, amino terminus. CO_2H, carboxy terminus.

the absence of actin. The standard curve remained linear over this concentration range (data not shown). Binding data were fit to a double-reciprocal plot using a linear regression analysis program (DeltaGraph Professional V.20.1, Macintosh).

Binding of myo 5 and myo 6 to actin was also measured at two ionic strengths (10 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM NaN_3, and 0.5 mM ATP ± 20 mM KCl, pH 7.50) using a colorimetric protein concentration assay. Actin at a fixed final concentration of 20 μM was mixed with 6–40 μM myo 5 or myo 6 and sedimented in the Beckman Airfuge at 120000*g* for 20 min at room temperature. The concentration of protein in the supernatant was measured using a colorimetric protein assay (Bio Rad Protein Assay, Bio-Rad Laboratories). The concentration of unbound myo 5 or myo 6 was calculated by subtracting the absorbance of the supernatant of an equimolar actin solution that was sedimented, and comparing the corrected absorbance to that from a standard curve of myo 5 or myo 6 over the same concentration range. This curve remained linear over this range (data not shown). The concentration of bound myo 5 or myo 6 was then calculated from the total, and binding data were fit to a Scatchard plot using a linear regression analysis program (DeltaGraph Professional, V.20.1, Macintosh).

Gel Electrophoresis. Proteins were run on SDS–PAGE as described (Laemmli, 1970). Polyacrylamide gels were stained either with 0.1% Coomassie blue in 40% methanol/10% acetic acid or with a silver stain kit (Silver Stain Plus, Bio-Rad Laboratories). DNA samples were run on 0.8% agarose gels in Tris–acetate buffer and stained with 0.5 μg/mL ethidium bromide. Bands were visualized on a UV transilluminator.

Protein Concentration Assays. Concentrations of the various myo fusion proteins were determined from the calculated molar extinction coefficients and the amino acid compositions (Mihalyi, 1963). Protein concentrations were also determined by a colorimetric assay (Bio-Rad Protein Assay) using bovine serum albumin as a standard. Agreement between these two assays was within 20%.

RESULTS

The various myo fusion proteins are depicted in Figure 1. Each fusion protein contains a portion of the C-terminal, putative actin binding region attached to the C-terminus of GST (grey strippled bar) *via* a thrombin recognition site (serrated line). Myo 5 and myo 6 both contain the GPQ segment, while myo 5 also contains the SH3 segment. Myo 3 contains only the SH3 segment, while myo 2 contains the SH3 segment plus a 27 amino acid long segment from the C-terminal portion of the GPQ domain. A Western blot of the various fusion proteins using a rabbit polyclonal anti-*Dictyostelium* myosin IB antibody (kindly provided by Dr. M. A. Titus, Duke University) revealed that the antibody specifically reacted with myo 3 (M_r 31 000), myo 2 (M_r 34 000), myo 6 (M_r 40 000), and myo 5 (M_r 45 000).

In the experiment illustrated in Figure 2a, 10 μM fusion protein or GST was sedimented at 120000*g* in a Beckman Airfuge in the presence and absence of 40 μM F-actin. Under these conditions, F-actin will sediment, but the various myo fusion proteins by themselves will not. The supernatants from this centrifugation were run on SDS–PAGE using a 10% acrylamide gel. Samples sedimented in the absence of actin

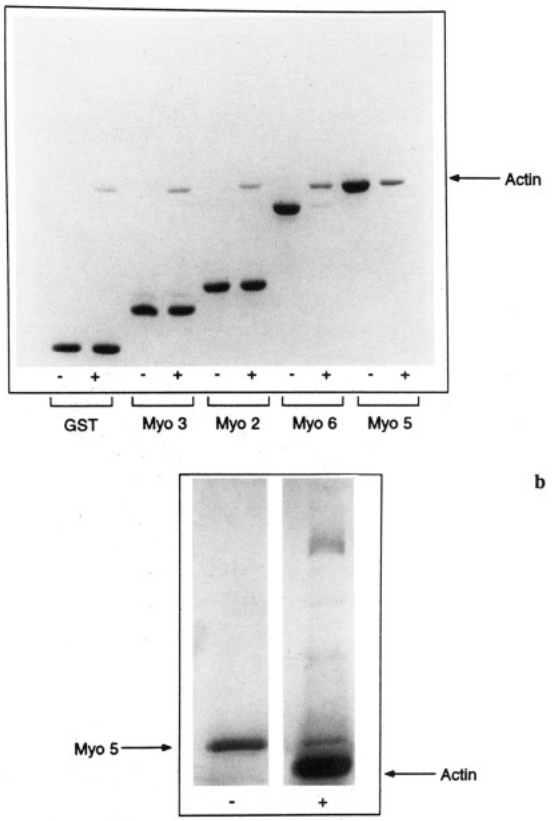

FIGURE 2: Binding of myo fusion proteins to actin. (a) Samples containing 10 μM myo fusion in 10 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM NaN_3, and 0.5 mM ATP, pH 7.50, were sedimented in the Beckman Airfuge at 120000*g* for 20 min in the presence or absence of 40 μM F-actin. SDS–PAGE on a 10% acrylamide gel was performed on the supernatants, and the gel was stained with 0.1% Coomassie blue. Lanes marked "–" are from samples sedimented in the absence of actin, while lanes marked "+" are from samples sedimented in the presence of actin. The upper faint band in lanes marked "+" represents unsedimented actin. Binding of myo 6 and myo 5 is clearly demonstrated, although quantification of myo 5 binding is hampered by its similar mobility to actin in this gel system. (b) 0.5 μM myo 5 was sedimented in the absence (lane "–") or presence (lane "+") of 60 μM actin in 10 mM HEPES, 20 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM NaN_3, and 0.5 mM ATP, pH 7.50. Supernatants were run on 12% SDS–PAGE, and the gel was silver-stained as described under Experimental Procedures. Separation of myo 5 from the remaining, unsedimented actin allows the extent of myo 5 binding to be appreciated.

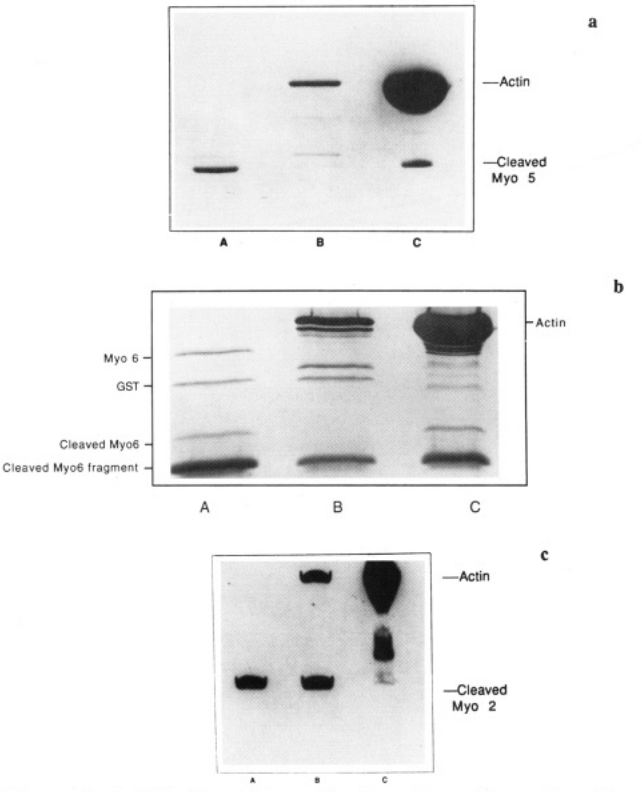

FIGURE 3: (a) Binding of thrombin-cleaved myo 5 to actin. Myo 5 was incubated with thrombin as described under Experimental Procedures in order to generate a fragment ("cleaved myo 5") which contains only the intact GPQ and SH3 segments. Binding to actin was assayed as described in Figure 2a. Sedimentation of 7 μM cleaved myo 5 in the absence of actin (lane A) shows the expected band at M_r 19 500 on 12% SDS–PAGE. Sedimentation in the presence of 30 μM actin leads to its disappearance from the supernatant (lane B) and its appearance in the pellet (lane C). (b) Binding assay of thrombin-cleaved myo 6 to actin. Thrombin cleavage generated small amounts of the expected fragment of M_r 14 050 ("cleaved myo 6") and much greater amounts of a smaller fragment of M_r 11 300 ("cleaved myo 6 fragment"). Silver staining of this preparation reveals small amounts of intact myo 6 and GST (lane A). Cosedimentation with actin leads to the complete disappearance from the supernatant (lane B) of intact myo 6 and cleaved myo 6, along with reduction in the content of the cleaved myo 6 fragment. SDS–PAGE of the pellet reveals the presence of both cleaved myo 6 as well as the cleaved myo 6 fragment. (c) Binding assay of thrombin-cleaved myo 2 to actin. Myo 2 was incubated with thrombin to generate a protein containing the SH3 segment and the 27 carboxy-terminal residues of the GPQ-rich segment ("cleaved myo 2"). Binding assay was performed as in (a). Supernatant from cleaved myo 2 alone (lane A) shows the expected band at M_r 8000 on 15% SDS–PAGE. Sedimentation in the presence of actin shows that all of the cleaved myo 2 remains in the supernatant (lane B) and none is detectable in the pellet (lane C).

were run in lanes marked "–", while the corresponding samples mixed with actin were run in lanes marked "+". It is apparent from inspection that none of the GST, myo 3, and myo 2 cosediment with actin, while nearly all of the myo 6 is removed by cosedimentation with actin. Although Figure 2a demonstrates cosedimentation of myo 5 with actin, the small amount of residual actin in the supernatant and the similar mobilities of myo 5 and actin in this gel system complicate the quantification of binding. SDS–PAGE in 12% acrylamide resolves myo 5 from actin and demonstrates that this fusion protein binds to actin as well (Figure 2b).

The GST moieties from myo 2, myo 5, and myo 6 were removed by thrombin digestion. While thrombin digestion of myo 2 and myo 5 yielded the expected fragments of M_r 8000 and 19 500, respectively, thrombin digestion of myo 6 yielded small amounts of the expected of M_r 14 050 and much larger amounts of a fragment of M_r 11 300 (Figure 3b, lane A). The relative proportion of the M_r 14 050 fragment could be increased by digesting for a shorter time, and both fragments reacted with polyclonal antibody to *Dictyostelium* myosin IB on Western blot (data not shown). Thrombin-cleaved myo 2 and myo 5, at a final concentration of 7 μM, and thrombin-cleaved myo 6, at a final concentration of 80 μg/mL (corresponding to an approximate concentration of 7–10 μM fragment mixture), were mixed with actin at a final concentration of 30 μM. They were sedimented in the Airfuge at 120000*g* for 20 min at room temperature, and the supernatants and pellets were dissolved in SDS sample buffer and analyzed by 12% SDS–PAGE. The disappearance of thrombin-cleaved myo 5 from the supernatant (Figure 3a, lane B) is explained by its quantitative binding to actin, as demonstrated by its appearance in the pellet (Figure 3a, lane C). By contrast, no binding of thrombin-cleaved myo 2 to actin could be demonstrated (Figure 3c). The content of myo 2 in the supernatant was unaffected by cosedimentation with actin, and no myo 2 could be demonstrated in the pellet. The preparation of thrombin-cleaved myo 6 used in this experiment contained

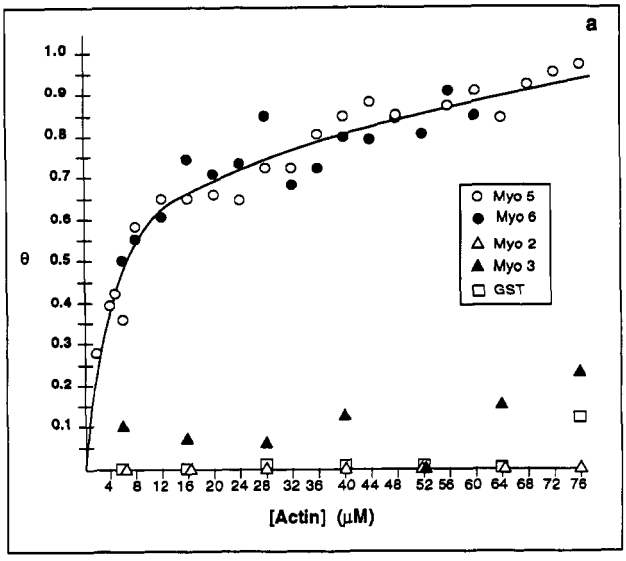

FIGURE 4: Concentration dependence of F-actin binding to myo fusion protein. Conditions: 10 mM HEPES, 5 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 1 mM NaN_3, and 0.5 mM ATP, pH 7.50, at room temperature. The data are depicted as fractional binding (θ) *versus* actin concentration. The solid curve defines a binding isotherm characterized by a K_d = 7.2 μM. (b) Double-reciprocal plot of data from (a) for myo 5 and myo 6. The solid line defines a K_d of 7.2 μM with a maximum degree of association of 0.94.

small amounts of both intact myo 6 and GST, as demonstrated by use of a silver-staining procedure. As Figure 3b demonstrates, intact myo 6 disappeared from the supernatant when cosedimented with actin (lane B), while GST did not. The small amount of thrombin-cleaved myo 6 (labeled "cleaved myo 6") present in this preparation could be completely removed from the supernatant by cosedimentation with actin (lane B) and could be demonstrated in the pellet (lane C). The M_r 11 300 fragment of myo 6 (labeled "cleaved myo 6 fragment") also bound to actin. Approximately 70% of this fragment disappeared from the supernatant when cosedimented with actin (lane B), and could be accounted for quantitatively by its appearance in the actin-containing pellet (lane C).

Dissociation constants for fusion protein binding to F-actin were measured with actin concentration in large excess. For the experiments whose results are depicted in Figure 4, this

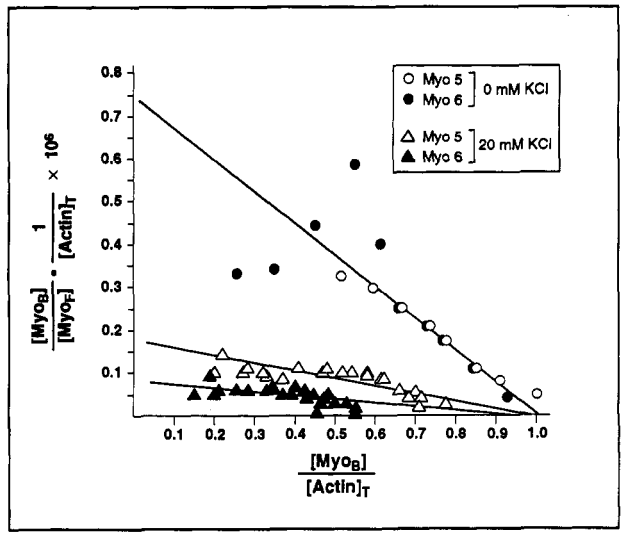

FIGURE 5: Scatchard plot of myo 5 and myo 6 binding to actin in 10 mM HEPES, 5 mM $MgCl_2$, 1 mM DTT, 1 mM NaN_3, and 0.5 mM ATP, pH 7.50, at room temperature ± 20 mM KCl. Data for myo 5 and myo 6 in the absence of KCl fit the same line, defining a K_d of 1.3 μM and a stoichiometry of 1.01. Data for myo 5 at 20 mM KCl fit a line defining a K_d of 6.6 μM and a stoichiometry of 0.98, which the corresponding values for myo 6 are 12.0 μM and 0.96. $[Myo_B]$: concentration of bound fusion protein, expressed as monomers. $[Myo_F]$: concentration of free fusion protein, expressed as monomers. $[Actin]_T$: total actin concentration.

represented a 20–150-fold molar excess over fusion protein. Data for myo 5 and myo 6 fit a binding isotherm defining an apparent dissociation constant of 7.2 μM in the presence of 20 mM KCl (Figure 4a). Myo 3, containing only the SH3 segment, showed a small degree of association (<20%), but the data did not reliably fit a binding isotherm. The data for myo 5 and myo 6 are also depicted on a double-reciprocal plot in Figure 4b, which reveals a maximum degree of association of 0.94. The dissociation constant of myo 5 and myo 6 to actin under the conditions of Figure 4a,b is approximately 10–20-fold higher than that reported for ATP-independent actin binding of *Acanthamoeba* myosin I (Doberstein & Pollard, 1992; Lynch *et al.*, 1986). This difference may reflect the fact that these earlier studies used a lower ionic strength (Lynch *et al.*, 1986) or pH (Doberstein & Pollard, 1992). The net positive charge of the GPQ-rich region of +13 suggests that electrostatic interactions may be important in binding to actin. This was examined in the experiment whose results are depicted in Figure 5. Mixtures of myo 5 or myo 6 and actin were prepared with a fixed actin concentration of 20 μM and with a range of myo 5 or myo 6 concentrations from 6 to 40 μM. Bound and free fusion protein concentrations were calculated from a sedimentation assay, as described above, and the data are depicted on a Scatchard plot in Figure 5. Under conditions of the same ionic strength and pH as those in Figure 4a,b, the dissociation constant of myo 5 to actin as measured in this assay is 6.6 μM, while that for myo 6 is 12.0 μM. By contrast, lowering the ionic strength by deleting KCl from the binding assay decreased the dissociation constants for both myo 5 and myo 6 to 1.3 μM. At both of these ionic strengths, the binding stoichiometry was approximately 1:1. Given the difference in the method of measuring unbound fusion protein between this and the previous experiment (Figure 4a,b), the agreement in the values of the dissociation constants at 20 mM KCl is reasonable. Raising the ionic strength to 100 mM KCl raised the dissociation constant of myo 5 to 49 μM (data not shown). This is similar to the dissociation constant of *Acanthamoeba* myosin I to actin in

the presence of ATP at this ionic strength (10–40 μM; Miyata *et al.*, 1989).

DISCUSSION

Extensive *in vitro* studies, utilizing electron microscopy, turbidity, sedimentation, and ATPase kinetic data, have shown that myosin I from *Acanthamoeba* can bind to actin in an ATP-independent manner [reviewed in Polland *et al.* (1991)]. Experiments utilizing proteolytic fragments and bacterially-expressed fusion proteins established that ATP-independent actin binding was mediated by a portion of the carboxy-terminal tail, encompassing a region that contained the GPA and SH3 segments (Lynch *et al.*, 1986; Brzeska *et al.*, 1988; Doberstein & Polland, 1992). Proteolytic fragments or fusion proteins that included these two segments bound to actin with dissociation constants in the range of 100–350 nM at low ionic strength or pH. However, these studies did not determine which of these two segments contained the actin binding site. Fusion proteins containing only the SH3 or only the GPA segment did not bind to actin (Doberstein & Pollard, 1992). These results could be taken to mean that both segments contribute to actin binding. However, in *Acanthamoeba* myosin IC, the SH3 sequence is nested within the GPA segment, and splits the latter nearly in half. Therefore, an additional possibility is that the separation of these two domains might have interfered with the proper folding of an actin binding site in the GPA segment, as suggested by the authors of that study. Alternatively, any actin binding by the 57-residue-long SH3 segment might have been sterically inhibited by the much larger β-galactosidase moiety of the fusion protein.

We have been able to take advantage of the fact that the SH3 segment of *Dictyostelium* myosin IB is at the extreme carboxy terminus (Figure 1), allowing expression of fusion proteins containing the SH3 segment, the GPQ segment, or combinations of both. This has allowed us to identify the GPQ segment as responsible for ATP-independent actin binding, as only myo 5 and myo 6—the fusion proteins that contain the complete GPQ segment—bound to actin with any appreciable affinity. It seems likely that the SH3 segment does not contribute to actin binding, as the dissociation constants of myo 5 and myo 6 to actin are nearly identical. The finding that both thrombin-cleaved myo 5 and thrombin-cleaved myo 6 bind to actin can only be explained by concluding that the GPQ segment alone accounts for all of the actin binding activity of the intact fusion proteins. This is further supported by the lack of binding of thrombin-cleaved myo 2. Specificity of binding of the GPQ segment to actin is also supported by the results of Scatchard plots (Figure 5), which demonstrates a 1:1 stoichiometry for binding of myo 5 or myo 6 monomers to actin at two ionic strengths and imply that actin has one binding site for the GPQ-rich segment. Finally, gel filtration chromatography of mixtures of anionic phospholipids with myo 5 or myo 6 demonstrated no evidence of interaction (unpublished observations). This assay, which previously demonstrated specific phospholipid binding only by those fragments of *Acanthamoeba* myosin IC which contained the membrane binding domain (Adams & Pollard, 1989), provides further support for the specificity of GPQ segment binding to actin.

Further insight into the structural basis for GPQ segment binding to actin is provided by studies with thrombin-cleaved myo 6. As Figure 3b demonstrates, thrombin not only separates the GPQ segment from GST in the myo 6 fusion but also cleaves the GPQ segment internally to generate a fragment that is 2.75 kDa smaller than the parent GPQ segment. The location of this second cleavage site was not unequivocally determined in this study. However, it is possible to deduce the most likely site given the amino acid sequence requirements for thrombin cleavage (Leavis *et al.*, 1978). Thrombin cleaves preferentially at arginine–glycine or arginine–valine bonds, and requires the presence of an acidic residue or its amide at a postion 6–8 residues on the carboxy side of the cleavage site. These requirements are met by only one site—between residues 947 and 948 in the primary sequence (Jung *et al.*, 1989). Cleavage at this site would generate a fragment approximately 2.7 kDa small than the parent GPQ segment, in excellent agreement with the observed result. Lack of thrombin cleavage at this site in myo 5 may be due to the steric blocking effect of the SH3 segment, which is present in myo 5 but not in myo 6. Figure 3b further demonstrates that although this smaller fragment still binds to actin, it does so with a lower affinity than that for the full GPQ segment. The sequence of the GPQ-rich segment contains six copies of the consensus repeat PQQGGG and seven copies of the consensus repeat GRPMP, which together constitute 52% of the entire GPQ-rich segment (Jung *et al.*, 1989). Cleavage between positions 947 and 948 would eliminate one of the PQQGGG consensus repeats. Further work will be required to determine the minimum sequence in the GPQ-rich segment which is responsible for actin binding, but one possibility is that these consensus sequences contribute together to constitute such a binding site.

The affinity of myo 5 and myo 6 for actin decreased with increasing ionic strength (Figure 5). This finding, along with the fact that the GPQ-rich segment has a net charge of +13 with no acidic residues, suggests that its binding to actin is mediated at least in part through electrostatic interactions with acidic residues on actin. This conclusion explains in part the higher affinities for ATP-independent actin binding by *Acanthamoeba* myosin I and myosin I tail fragments, which were performed at a lower ionic strength or pH (Lynch *et al.*, 1986; Doberstein & Pollard, 1992). However, reducing the ionic strength to a level similar to previous work (Lynch *et al.*, 1986) still resulted in a dissociation constant that is 2–6-fold higher than that for *Acanthamoeba* (Figure 5). This difference may reflect intrinsic differences in actin affinities between myosin I isoforms from *Dictyostelium* and *Acanthamoeba*. Raising the ionic strength to a more physiologic 100 mM KCl increased the dissociation constant to 48 μM. Given that intracellular concentrations of actin are in the range of 100–250 μM (Pollard, 1981), at least 67% of the ATP-independent actin binding sites of *Dictyostelium* myosin IB would still be bound to actin under these conditions. Several examples exist of highly basic polymers and proteins that can bind to actin filaments. These include poly(L-lysine) (Fowler & Aebi, 1982), myelin basic protein (Barylko & Dobrowolski, 1984), and MARCKS (Hartwig *et al.*, 1992). The latter is a cytoplasmic protein involved in leukocyte motility which is particularly rich in glycine and alanine, and has a highly basic segment—residue 155–173—which contains at least one of the actin binding sites.

The results discussed above also confirm previous observations that SH3 segments do not directly bind to actin (Doberstein & Pollard, 1992; Cicchetti *et al.*, 1992). The SH3 ligands that have so far been identified are components of GTP-dependent signal transduction pathways (Cicchetti *et al.*, 1992; Rozakis-Adcock *et al.*, 1993). The presence of an SH3 segment on a myosin I species could thus allow a signal-transducing component to be localized to the cytoskeleton and to be transported along an actin filament by

means of the motor-containing head. Another example of a linkage between signal transduction and myosin I is that of the *ninaC* myosins in *Drosophila*, which consist of an amino-terminal protein kinase domain attached to a carboxy-terminal myosin I (Montell & Rubin, 1988).

Thus, this study extends information on ATP-independent actin binding to a second form of myosin I, for which no other biochemical data are currently available. It provides direct evidence that the GPQ segment of *Dictyostelium* myosin IB contains a specific, ATP-independent actin binding site. Its homology to the GPA segment of *Acanthamoeba* myosin I argues that the latter segment also contains the ATP-independent actin binding site, as suggested by previous studies (Lynch *et al.*, 1986; Brzeska *et al.*, 1988; Jung *et al.*, 1989; Doberstein & Pollard, 1992). It is also consistent with previous reports that the SH3 segment does not bind to actin (Doberstein & Pollard, 1992; Cichetti *et al.*, 1992). ATP-independent cytoskeletal attachment may not be a feature solely of protozoan myosin I isoforms. A recent report indicates that a chymotryptic 32-kDa, carboxy-terminal tail fragment of vertebrate brush border myosin I binds to actin in an ATP-independent manner (Swanljing-Collins & Collins, 1992). The approach used in this and previous studies (Doberstein & Pollard, 1992) should be useful in delineating the mechanisms of cytoskeletal association by vertebrate myosin I isoforms.

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REFERENCES

Adams, R. J., & Pollard, T. D. (1989) *Nature 340* 565–568.
Barylko, B., & Dobrowlski, Z. (1984) *Eur. J. Cell Biol. 35*, 327–335.
Brzeska, H. T., Lynch, T. J., & Korn, E. D. (1988) *J. Biol. Chem. 263*, 427–435.
Cicchetti, P., Mayer, B. J., Thiel, G., & Baltimore, D. (1992) *Science 257*, 803–806.
Doberstein, S. K., & Pollard, T. D. (1992) *J. Cell Biol. 117*, 1241–1249.
Drubin, D. G., Mulholland, J., Zhu, Z., & Botstein, D. (1990) *Nature 343*, 288–290.
Endow, S. A., & Titus, M. A. (1992) *Annu. Rev. Cell Biol. 8*, 29–66.
Fowler, W. E., & Aebi, U. (1982) *J. Cell Biol. 93*, 452–458.
Fukui, Y., Lynch, T. J., Brzeska, H., & Korn, E. D. (1989) *Nature 341*, 328–331.
Greene, L. E., & Eisenberg, E. (1980) *J. Biol. Chem. 255*, 543–548.
Hartwig, J. H., Thelen, M., Rosen, A., Janmey, P. A., Nairn, A. C., & Aderem, A. (1992) *Nature 356*, 618–622.
Ish-Horowicz, D., & Burke, J. F. (1981) *Nucleic Acids Res. 9*, 2989.
Jung, G., Saxe, C. L., III, Kimmel, A. R., & Hammer, J. A., III (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6186–6190.
Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., & Pawson, T. (1991) *Science 252*, 668–674.
Korn, E. D., & Hammer, J. A., III (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 23–45.
Korn, E. D., & Hammer, J. A., III (1990) *Curr. Opin. Cell Biol. 2*, 57–61.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Leavis, P. C., Rosenfeld, S. S., & Lu, R. C. (1978) *Biochim. Biophys. Acta 535*, 281–286.
Lynch, T. J., Albanesi, J. P., Korn, E. D., Robinson, E. A., Bowers, B., & Fujisaki, H. (1986) *J. Biol. Chem. 261*, 17156–17162.
Mannervik, B. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol. 57*, 357–417.
Mihalyi, E. (1963) *J. Chem. Eng. Data 13*, 179–182.
Miyata, H., Bowers, B., & Korn, E. D. (1989) *J. Cell Biol. 109*, 1519–1528.
Montell, C., & Rubin, G. M. (1988) *Cell 52*, 757–772.
Pollard, T. D. (1981) *J. Cell Biol. 91*, 156S–165S.
Pollard, T. D., Doberstein, S. K., & Zot, H. G. (1991) *Annu. Rev. Physiol. 53*, 653–681.
Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., & Bowtell, D. (1993) *Nature 363*, 83–85.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Smith, D. B., & Johnson, K. S. (1988) *Gene 67*, 31–40.
Smith, D. B., Davern, K. M., Board, P. G., Tiu, W. U., Garcia, E. G., & Mitchell, G. F. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8703–8707.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Swanljung-Collins, H., & Collins, J. H. (1992) *Mol. Biol. Cell. 3*, 156a.
Warrick, H. M., & Spudich, J. A. (1987) *Annu. Rev. Cell Biol. 3*, 379–421.