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The actin binding site in the tail domain of *Dictyostelium* myosin IC (myoC) resides within the glycine- and proline-rich sequence (tail homology region 2)

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Abstract

The majority of protozoan myosins I possess tail domains composed of three distinct and conserved regions of sequence, referred to as tail homology regions 1, 2 and 3 (TH·1, TH·2 and TH·3). While the N-terminal ~half of the tail (corresponding to TH·1) has been implicated in membrane binding, all or some portion of the C-terminal ~half of the tail (corresponding to TH·2 plus TH·3) has been implicated in binding to F-actin in a nucleotide-insensitive fashion. Here we show, using fusion proteins containing portions of the *Dictyostelium* myosin IC (myoC) tail domain and F-actin sedimentation assays, that the ability of the myoC tail to bind to actin resides entirely within the glycine- and proline-rich TH·2 domain. The src-like TH·3 domain does not bind to actin, nor does it augment the binding properties of the TH·2 domain. In addition to defining more precisely the location of the actin binding site in the tail domain of a protozoan myosin I, these results have implications for the function of the src-like TH·3 domain in myosins I and other proteins.

Key words: Myosin I; Actin binding; SH3 domain; *Dictyostelium*

1. Introduction

The myosin superfamily contains, in addition to conventional two-headed myosins (myosin II), a growing family of unconventional myosins (for reviews, see [1–5]). All of these myosins share an ~80 kDa motor domain, which corresponds to the globular head or subfragment 1 of muscle myosin. For the unconventional myosins, this motor domain is attached to a variety of carboxyl-terminal domains (in one case there is also an N-terminal, kinase-like extension [6]). The functional specialization of these unconventional myosins presumably resides within their distinct C-terminal domains, which serve to link the motor domain to various cellular structures (i.e. cargo). The identification of these tail domain ligands, and the correlation of the biochemical properties of tail domains (e.g. the binding of actin, membranes, calmodulin) with their primary structure, will be very important aspects of myosin structure–function analysis.

In the case of the protozoan myosins I, the majority of isoforms sequenced to date (the so called 'classic' myosins I) possess three distinct and conserved regions of sequence within their ~45 kDa tail domains (tail homology regions 1, 2 and 3; TH·1, TH·2 and TH·3, respectively [7–10]). TH·1 spans the N-terminal ~210 residues of the tail, is ~60% conserved (exact matches plus conservative substitutions) between the various isoforms, and possesses a strong net positive charge (+15 to +30). TH·2 spans the central ~180 residues of the tail and is striking in its high content of glycine, proline and either alanine or glutamine residues (these residues usually comprise ~75% of the total residues in TH·2). TH·2 domains also have a strong net positive charge. While the sequences of TH·2 domains from different isoforms can not be aligned with each other in any unique way, they all share this unusual composition and net positive charge. TH·3 spans the C-terminal ~53 residues of the tail[1], is ~75% conserved between isoforms, and is also found in a large and diverse family of proteins, including all non-receptor tyrosine kinases (like pp60 src, where this sequence is known in the literature as src homology region 3 or SH3), several actin binding proteins (e.g. yeast ABP1 and fodrin), and many proteins involved in signal transduction (eg. phospholipase C_γ and SH2/SH3 adaptor proteins like Grb2) (for review see [11–13]).

Biochemical analyses of proteolytic fragments generated from *Acanthamoeba* myosin IA [14] and fragments of *Acanthamoeba* myosin IC expressed in *E. coli* as β-galactosidase fusion proteins [15] have implicated the N-terminal ~half of the tail, corresponding to TH·1, in

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[1] In *Acanthamoeba* myosin IC and *Dictyostelium* myoD the TH·3 domain is slightly internal, splitting the TH·2 domain into two portions.

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the binding of myosins I to anionic phospholipid membranes. For these same two myosin I isoforms, data have also been presented which show that the C-terminal ~half of the tail binds to F-actin in a nucleotide-insensitive fashion. In the first study [16], a chymotryptic fragment of *Acanthamoeba* myosin IA that corresponds to the C-terminal ~30 kDa of the heavy chain was found to bind to F-actin at low ionic strength with a K_D of ~200 nM, both with and without Mg^{2+}ATP. The sequence of the myosin IA heavy chain has not been determined, however, so it is not known what primary sequences form this actin binding site. Nevertheless, it should be noted that amino acid analysis of this 30 kDa fragment revealed a high content of glycine and proline residues [16], suggesting that it probably contained a significant portion of the TH·2 domain. In the second study [15], a β-galactosidase fusion protein containing the TH·2 and TH·3 domains of *Acanthamoeba* myosin IC was found to bind to F-actin, although the quantitation of this binding was made difficult by the high background binding by β-galactosidase alone, and by the tetrameric nature of the fusion protein. This study also showed that a fusion protein containing just TH·3 did not bind to F-actin, suggesting that the binding site resided solely within TH·2. Fusion proteins containing just the TH·2 domain also did not bind to actin, however, leading to the equally plausible conclusion that TH·2 and TH·3 are both required to form a functional actin binding site. Alternatively, either or both of the TH·2 and TH·3 domains might bind to F-actin in the context of native myosin IC, but when these domains are expressed as separate fusion proteins, they might not fold properly.

In this study we have expressed portions of the *Dictyostelium* myoC tail domain (another of the 'classic' myosins I) as glutathione-*S*-transferase (GST) fusion proteins and analyzed their interaction with F-actin by sedimentation assay. The results show that a fusion protein containing TH·2 and TH·3 binds to F-actin in a nucleotide insensitive fashion with a K_D of ~250 nM (in low ionic strength), that a fusion protein containing just TH·3 does not bind to actin, and that a fusion protein containing just TH·2 binds to actin with essentially the same affinity as the fusion protein containing both TH·2 and TH·3. In addition to localizing the actin binding site to the glycine- and proline-rich TH·2 domain, this study sheds light on the possible functions of src-like TH·3 domains and SH3 domains in general.

2. Materials and methods

2.1. Vector constructions

A genomic clone that contains ~90% of the coding sequence for the *Dictyostelium* myoC heavy chain (beginning at a position 11 amino acids N-terminal of the G in GESGAKT and ending 3′ to the stop codon), together with the corresponding DNA/protein sequence, was a generous gift of Dr. Margaret A. Titus (Duke University, Durham, NC). MyoC tail domain fusion proteins were expressed using the vector, pGEX-3X (Pharmacia LKB Biotechnology), in which the myoC tail fragments are fused to the C-terminus of glutathione *S*-transferase (GST; ~26 kDa). To simplify the numbering system (since the complete sequence of myoC is not available), we have numbered the myoC tail domain residues 1–444, beginning at the head/tail junction. Based on this numbering system, TH·1 corresponds to residues 1–213, TH·2 to residues 214–391, and TH·3 to residues 392–444. To express a fusion protein containing both TH·2 and TH·3 (GST–TH·2/TH·3), a 745 bp *Dra*I fragment which encodes the C-terminal 248 amino acids of the myoC tail (from residue 197 to 444) was converted to *Eco*RI ends by linker addition and subcloned into *Eco*RI-cut pGEX-3X. This fusion protein contains the entire TH·2 and TH·3 domains, as well as the last 16 residues (or ~7%) of the TH·1 domain. To express a fusion protein containing just TH·2 (GST–TH·2), a 598 bp *Dra*I–*Rsa*I fragment encoding tail residues 197–395 was converted to *Eco*RI ends by linker addition and cloned into *Eco*RI-cut pGEX-3X. This fusion protein contains the entire TH·2 domain plus the C-terminal 16 residues (or ~7%) of TH·1 and the N-terminal 3 residues (or ~6%) of TH·3. To express a fusion protein containing just the TH·3 domain (GST–TH·3), PCR was used to amplify this region of the myoC tail. Specifically, a 5′ primer containing a *Bam*H1 restriction site (CTAGGATCCTCGCTCTTTACGAGTACGACC) and a 3′ primer containing and *Eco*RI site and the stop codon (CTAGAATTCTTAAATTTGTTGAACATAATTTGAAGG) were used to amplify a plasmid subclone containing the entire tail of myoC. The fragment obtained, which encodes the C-terminal 53 residues of the heavy chain (392–444) (i.e. the entire TH·3 domain), was authenticated by DNA sequencing, cut with *Bam*H1 and *Eco*RI, and cloned into pGEX-3X that had been cut with these two enzymes.

2.2. Expression and purification of fusion proteins

E. coli strain DH5α, carrying either the vector alone (for expression of unfused GST) or the expression plasmids described above, was grown to saturation, diluted 1:10 in LB media (with 50 μg/ml ampicillin), and grown at 30°C to OD ~0.8 (600 nm). At this point, IPTG was added to a final concentration of 1 mM to induce protein expression. After incubation for 3 h at 30°C , the cells were pelleted and the pellets were resuspended at 4°C in PBS (150 mM sodium chloride, 20 mM sodium phosphate, pH 7.3) containing 2 mM DTT, 1% (v/v) Triton X-100, and protease inhibitors (pepstatin and leupeptin (1 μg/ml each), aprotinin and TAME (2 μg/ml each), 1 mM PMSF, 10 μg/ml AEBSF) at a ratio of 5 ml buffer per g of cells. The cells were then lyzed by sonication (three times 20 s, 50% duty cycle, 3.5 W) using a Branson sonicator and a model C3 horn. The lysates were clarified by centrifugation (10,000 × *g*, 15 min, 4°C) and the supernatants were applied to a GST–Sepharose 4B column (Pharmacia LKB). The column was washed extensively with PBS and fusion proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 8 mM reduced glutathione. The peak of fusion protein (as identified by SDS-PAGE) was dialyzed against the appropriate actin binding buffer (see below). Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as a standard, and molarities were calculated based on molecular weights estimated from SDS-PAGE mobilities. Rabbit skeletal muscle actin, which was a generous gift of Dr. Shannon Holliday (NHLBI, NIH, Bethesda, MD), was purified according to Spudich and Watt [17], followed by gel-filtration on Sephadex G-200. The concentration of actin was determined from its molar extinction coefficient.

2.3. Actin binding assays

The binding of GST, GST–TH·2/TH·3, GST–TH·2, and GST–TH·3 to F-actin was measured using an F-actin sedimentation assay. All binding assays were performed using a constant final concentration of F-actin (2 μM) and varying final concentrations of fusion protein (0.5 to 7 μM). The standard binding assay condition was 10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 0.2 mM ATP, and 0.1 mM DTT. To prevent the loss of fusion proteins by adsorption onto the walls of the centrifuge tubes, the tubes were siliconized and fatty acid-free BSA (at a final concentration of 0.2 mg/ml) was included in all binding assay buffers. Some binding assays also contained either KCl or ATP at final concentrations of 30 mM and 2 mM, respectively. After mixing F-actin and the fusion protein, the samples were incubated for 30 min at room temperature and centrifuged at 65,000 rpm (~160,000 × *g*) for 20 min at 20°C in a TL-100 ultracentrifuge (Beckman). Following centrifugation, the supernatant was removed to a separate tube and the pellet was

resuspended in a volume of binding buffer equal to that of the supernatant. Both samples were mixed with an equal volume of 2 × SDS-PAGE sample buffer and boiled for 5 min. The amount of fusion protein bound was determined by SDS-PAGE analysis of both supernatants and pellets. The actual amount of fusion protein in these samples was calculated based on quantitative densitometry of gels stained with Coomassie blue and scanned using a laser densitometer (LKB). Purified fusion proteins were used as internal standards. For the data presented in Fig. 3, the amount of fusion protein bound was determined based on the appearance of fusion protein in the pellet (as opposed to depletion of fusion protein from the supernatant). Calculation of the amount bound based on depletion of fusion protein from the supernatant gave, as expected, very similar values. Less than 5% of each fusion protein sedimented in the absence of F-actin, and the binding data were corrected for the exact amount. The binding data were directly fitted using a non-linear least squares fitting routine (DEC 10 MLAB program, DCRT, NIH). It was assumed that the GST fusion proteins are monomeric, which is consistent with previous reports [18] and with the lack of obvious cooperativity in the binding data, and that the fusion proteins bind to actin in a 1:1 molar ratio (i.e. single site equilibrium).

3. Results

3.1. Expression and purification of GST–myoC tail domain fusion proteins

The tail domain of *Dictyostelium* myoC shows a high degree of similarity to the tail domains of other 'classic' myosins I, possessing all three tail homology regions (TH·1, TH·2 and TH·3) described previously for myosins IB and IC from *Acanthamoeba* and myoB and myoD from *Dictyostelium* (Fig. 1). Like these myosins, the TH·2 domain of myoC is rich in glycine, proline and alanine residues (11%, 28%, and 16%, respectively), and possesses a strong net positive change (+21). Furthermore, the src-like TH·3 domain of myoC is 70% and 73% similar to the TH·3 domains of *Dictyostelium* myoB and myoD, respectively. Previous biochemical studies of other classic protozoan myosin I isoforms have implicated all or some portion of the TH·2 plus TH·3 domains in binding to F-actin in a nucleotide-insensitive fashion [15,16]. To further dissect this actin binding site, GST–myoC tail domain fusion proteins containing TH·2 plus TH·3 (GST–TH·2/TH·3), TH·2 alone (GST–TH·2), and TH·3 alone (GST–TH·3) (Fig. 1) were expressed and purified. Fig. 2 shows a Coomassie blue-stained gel of these fusion proteins (along with unfused GST) following their purification on glutathione–Sepharose 4B. The estimated molecular masses of GST, GST–TH·2/TH·3, GST–TH·2, and GST–TH·3 were 26, 58, 52 and 32 kDa, respectively. All of these proteins were greater then 98% pure as judged by SDS-PAGE. The GST–TH·3 fusion protein routinely appeared as a close doublet with the upper band corresponding in size to the full-sized fusion protein. Efforts to separate the intact fusion protein from the apparent proteolytic degradation product (which varied from 10–35% of the total) were unsuccessful, so binding assays were done with the mixture of both peptides.

3.2. Actin binding assays

The interaction of these GST–myoC tail domain fusion proteins with F-actin was determined using F-actin sedimentation assays. Since GST alone showed no significant binding to actin, binding assays were done using the intact fusion proteins, i.e. no effort was made to cleave off the GST portion of the fusion proteins. As described in section 2, all binding assays were performed using a constant final concentration of F-actin (2 μM) and varying final concentrations of fusion proteins (0.5–7.0 μM). Fig. 3 shows the binding curves for GST–TH·2/TH·3, GST–TH·2, and GST–TH·3, plotted as the concentration of free fusion protein vs. the fractional saturation of F-actin in the assay (a value of 1.0 equals 2 μM

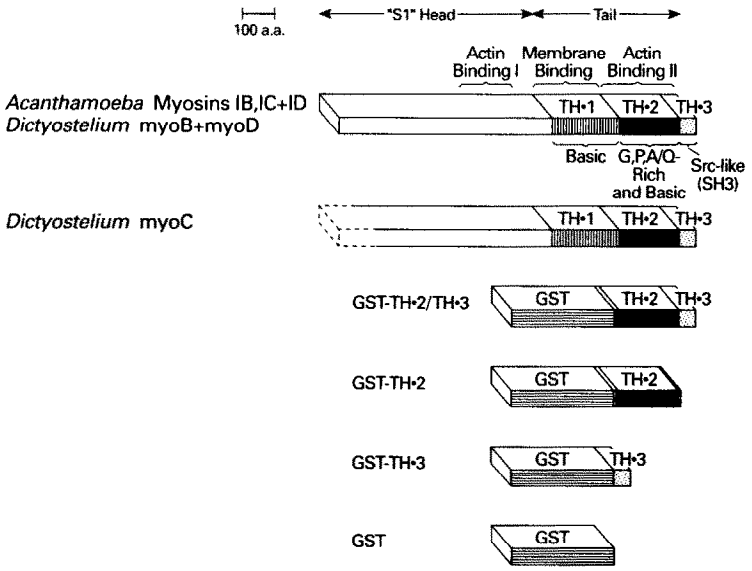

Fig. 1. Scheme depicting the structure of the myoC heavy chain, its relationship to previously characterized myosin I heavy chains, and the structure of the GST–myoC tail domain fusion proteins used in the actin binding assays.

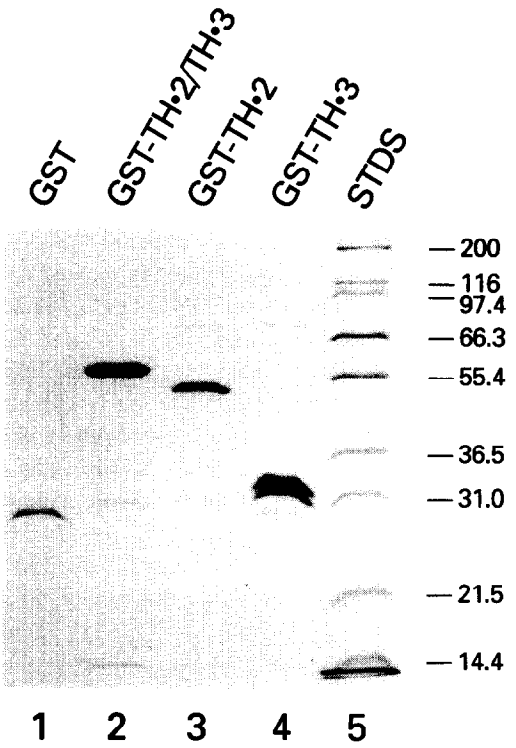

Fig. 2. Coomassie blue-stained gel of purified GST fusion proteins resolved by SDS-PAGE.

bound peptide). Under standard assay conditions, the GST–TH·2/TH·3 fusion protein bound to F-actin with high affinity (K_D = 260 ± 20 nM) (Fig. 3A, squares). The maximal binding observed was 0.95 mol of GST–TH·2/TH·3 per mol of actin subunit. In the presence of 30 mM KCl, the binding of GST–TH·2/TH·3 to actin was slightly weaker (K_D = 570 ± 20 nM) (Fig. 3A, triangles) and the maximal binding observed was 0.9 mol of fusion protein per mol of actin. The addition of 2 mM Mg^{2+}ATP did not significantly alter the affinity of GST–TH·2/TH·3 for F-actin (data not shown).

While GST–TH·2/TH·3 bound tightly to F-actin, GST–TH·3 showed no significant affinity for actin (Fig. 3A, circles), suggesting that the actin binding site resides entirely within TH·2. This was borne out by binding assays performed with GST–TH·2. Specifically, GST–TH·2 bound to F-actin under standard assay conditions with a K_D of 250 ± 10 nM (Fig. 3B, squares), a value which does not differ significantly from that obtained for the GST–TH·2/TH·3 fusion protein. The maximal binding observed was 0.95 mol GST–TH·2 per mol of actin subunit. In the presence of 30 mM KCl, the binding of GST–TH·2 was again slightly weaker (K_D = 530 ± 50 nM) and the maximal binding observed was 0.9 mol GST–TH·2 per mol of actin subunit (Fig. 3B, triangles). Like GST–TH·2/TH·3, the binding of GST–TH·2 to F-actin was insensitive to 2 mM Mg^{2+}ATP (data not shown).

4. Discussion

The results presented here indicate that the tail domain of *Dictyostelium* myoC contains a nucleotide-insensitive actin binding site and that this site resides entirely within the glycine- and proline-rich sequence (TH·2). It is unlikely that the lack of affinity for F-actin exhibited by the GST–TH·3 fusion protein is due to improper folding of the TH·3 domain or to shielding of this domain by the GST moiety, since the fusion protein containing both TH·2 and TH·3 did not bind actin any more tightly than did the fusion protein containing TH·2 alone.

The existence of this actin binding site in the tail, together with the nucleotide-sensitive site within the head, should allow myoC to crosslink actin filaments, as has been shown directly for purified *Acanthamoeba* myosins I [19]. It is almost certain that *Dictyostelium* myoB and myoD also possess this second actin binding site, not only because they possess a glycine- and-proline-rich TH·2 domain, but because their Mg^{2+}ATPase activities display a triphasic dependence on F-actin concentration [20]. This complex kinetic behavior is thought to be a direct consequence of actin crosslinking by myosin I [19]. Light immunofluorescence performed with isoform-specific antibodies reveals that myoB [21],

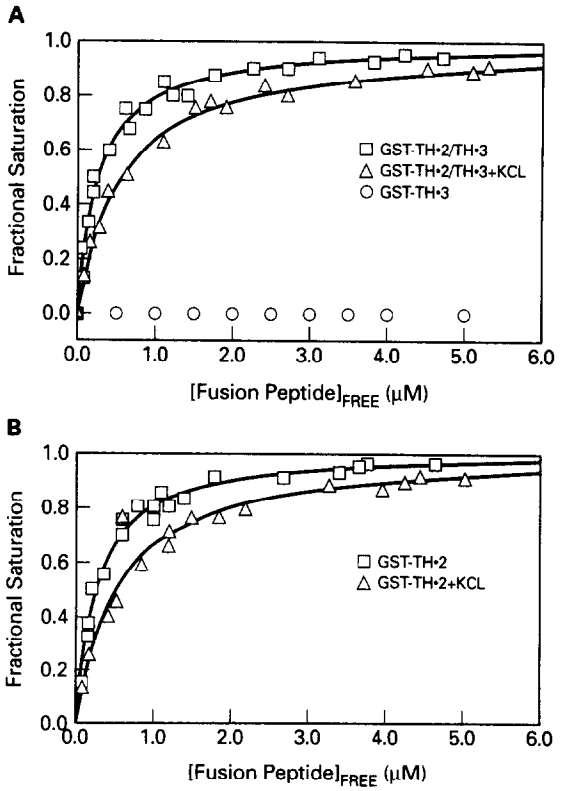

Fig. 3. Actin binding data plotted as the fractional saturation of F-actin vs. the concentration of free fusion protein. All assays contained a constant amount of F-actin (2 μM) and varying amounts of fusion protein. At a value of 1.0, there is 2 μM bound fusion protein in a 1:1 molar complex with actin subunits.

myoC[2], and myoD [10] are concentrated in actin-rich cortical regions such as pseudopodia and lamellopodia. In these regions, actin exists as short filaments arranged in an isotropic network or meshwork [22,23]. In this organizational state, activated (i.e. heavy chain phosphorylated) myosins I should produce a contractile or collapsing force on the actin gel, as has been shown in vitro [19]. Therefore, the likelihood that these myosin I isoforms can act as ATP-dependent actin crosslinkers, and that they can generate a contractile tension in the cortical actin meshwork, should allow them to play a crucial role in cell shape change, cell locomotion, and a host of other membrane/cytoskeletal interactions (e.g. phagocytosis and pinocytosis) (for review, see [24]).

The affinities obtained here for the fusion proteins containing the TH·2 domain of myoC are very similar to the affinity of the C-terminal ~30 kDa chymotryptic fragment of *Acanthamoeba* myosin IA for actin measured under similar conditions (~200 nM). The salt sensitivity of the interaction between this ~30 kDa fragment and actin was not determined. Here we found about a two-fold decrease in the binding constant for actin by the addition of 30 mM KCl. Addition of 100 mM KCl substantially reduced the binding of myoC tail domain fusion proteins to actin (data not shown). While we did not accurately determine the binding under these conditions, we note that in the actin-rich regions where these classic myosins I are concentrated, actin concentrations have been estimated to be of the order of 200–300 μM [25]. At these extremely high concentrations, even relatively weak binding at physiological ionic strength would probably be sufficient to bind much of the myosin I to actin through its nucleotide-insensitive actin binding site.

Further efforts to localize this actin binding site within the TH·2 domain should focus on the numerous, short, glycine- and-proline-rich pseudorepeats that occur in the TH·2 domain of this and other classic myosins I [7–10]. For example, the TH·2 domain of myoC contains two 7-copy repeats the consensus sequences of which are PAPGG and PMMKKPAP. Invariably, TH·2 domains also possess a strong net positive charge, and these charges presumably play a role in binding to actin based on the salt sensitivity of the binding. SH3 domains have recently been shown to bind to certain proline-rich sequences [26,27], so it will be interesting to see if the TH·3 domain of myoC interacts significantly with the TH·2 domains from this or other classic myosin I isoforms.

While the data presented here do not reveal what molecule(s) the SH3-like TH·3 domain of myoC interacts with, they do indicate that actin is not one of these molecules. The SH3 domain of C-abl, a src kinase relative, has also been reported to lack affinity for actin, but the data is largely anecdotal [26]. At the present time, generalizations regarding SH3-domain ligands must be formulated with caution, especially since one recently identified SH3 ligand, the protein 3BP1, demonstrates a strong selectively in its interactions with the SH3 domains from various src-family members [26]. Nevertheless, our results suggest that the localization of a GST fusion protein that contains just the SH3 domain from phospholipase Cγ to the actin cytoskeleton [28], as well as the shift in localization of the adaptor protein Grb2 from actin-rich membranes ruffles to the cytoplasm by SH3-loss-of-function mutations [28], are not due to a direct interaction between SH3 and F-actin. Rather, SH3 domains may interact with a common, highly conserved actin-associated protein. Consistent with this, the SH3 domain from pp60 src was recently shown to bind to paxillin, a protein which interacts with the ubiquitous actin binding protein, vinculin [29].

SH3 domains have recently been linked to signal transduction pathways mediated by ras and other closely-related monomeric GTPases. First, the protein, 3BP1, which binds to the SH3 domain of C-abl, has homology to Bcr, N-chimearin and GAP-rho, all three of which have GTPase activating (GAP) activity for ras-related proteins [26]. Interestingly, rho and its close relative rac are ras-related GTPases that control the organizational state of the actin cytoskeleton [30,31]. Second, the adaptor protein, Grb2, which is composed almost entirely of two SH2 domains and a central SH3 domain, mediates the transference of signals from receptor tyrosine kinases to ras by binding to phosphorylated tyrosine residues on the cytoplasmic tails of the receptors (via SH2) and to Sos, a nucleotide exchange factor for ras (via SH3) (for review, see [32]). These findings point to the exciting possibility that myosins I may play a role in ras-mediated signal transduction and that they may even influence the organizational state of the actin cytoskeleton. The myoC tail domain fusion proteins described in this report would appear to fold properly and should, therefore, be useful for further studies designed to identify other tail domain ligands, such as membrane-associated myosin I docking proteins and proteins that interact with the src-like TH·3 domain.

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