A crosslinked and ribosylated actin trimer does not interact productively with myosin

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Abstract

A purified F-actin derived actin trimer that interacts with end-binding proteins did not activate or bind the side-binding protein myosin under rigor conditions. Remodeling of the actin trimer by the binding of gelsolin did not rescue myosin binding, nor did the use of different means of inhibiting the polymerization of the trimer. Our results demonstrate that ADP-ribosylation on all actin subunits of an F-actin derived trimer inhibits myosin binding and that the binding of DNase-I to the pointed end subunits of a crosslinked trimer also remolds the myosin binding site. Taken together, this work highlights the need for a careful balance between modification of actin subunits and maintaining protein-protein interactions to produce a physiologically-relevant short F-actin complex.

Keywords: actomyosin, conformational change, protein crosslinking, polymerization inhibition,

Abbreviations: GS: gelsolin, ANP: N-(4-azido-2-nitrophenyl) putrescine, pPBM: para-phenylenebismaleimide, S1: myosin subfragment-1, ADPr--; ADP-ribosylated
Introduction

Actin is a highly regulated cytoskeletal protein. Rarely do actin proteins exist on their own in cells, unbound by an actin binding protein (ABP) modulating where and when actin functions (for reviews, see (Dos Remedios et al. 2003, Winder and Ayscough 2005)). Actin binding proteins fall into two main categories: monomer-binding or filament-binding. We call these G-ABPs and F-ABPs to follow the tradition of G-actin and F-actin in the field. Capping proteins bind filament ends, either the pointed end (e.g. tropomodulin) or the barbed-end (e.g. gelsolin) while side-binding proteins interact along the side of the filament (e.g. myosin or tropomyosin) (Fig 1).

Current technology is approaching atomic resolution of F-actin complexes using electron microscopy; however, the resolution and quality from this work still lacks important atomic details, such as local conformational differences between neighbouring subunits and flexible regions of the actin molecules when interacting with F-ABPs. X-ray crystallography remains a mainstay for determining atomic resolution interactions between proteins. Our work has been aimed at developing a short F-actin-derived structure by combining chemical crosslinking with polymerization inhibition to serve as a platform for determining the atomic structure of F-ABP complexes. We previously produced an ADP-ribosyl (ADPr)-modified and para-phenylenebismaleimide (pPBM)-crosslinked actin trimer derived from F-actin, referred to as the ADPr-trimer (Perieteanu et al. 2010). We found that DNase-I binds the pointed end and gelsolin binds the barbed end of the ADPr-trimer. Our next step is to study side-binding F-ABPs with the ADPr-trimer, using myosin as an example.

Covalent modification of F-actin has different effects on actin function depending on the chemical nature and location of the modification (reviewed in (Kumar and Mansson 2017)).
interactions between inter- or intrastrand crosslinked F-actin and myosin has been studied (Kim et al. 2002; Mannherz et al. 2008; Morrison et al. 2010). Filaments made up of purified intrastrand crosslinked actin dimers produced 50% myosin activity (Mannherz et al. 2008). When these dimers were bound by G-ABPs to inhibit polymerization, no myosin activity was observed; however, the stoichiometry of the ABP to the subunits may have inhibited myosin activity. Previously, we produced an intrastrand actin dimer containing an actin mutant that was blocked on its pointed end by DNase-I or by ADP-ribosylation on both subunits. We found that these dimers interacted with myosin (Morrison et al. 2010), confirming previous work showing a ternary complex of myosin subfragment-1, actin and DNase-I can be formed (Blanchoin et al. 1995).

Directly relevant to our work, the impact of pPBM crosslinking of F-actin on myosin activity has been studied previously (Kim et al. 2002). In their work, Kim et al crosslinked F-actin randomly, resulting in crosslinking of half of the subunits in the filaments. The crosslinked F-actin inhibited myosin-mediated filament movement in *in vitro* motility assays; however, myosin binding and ATPase activity remained largely unchanged (Kim et al. 1998, 2002). Specifically, the $V_{\text{max}}$ of myosin ATPase activity was comparable between crosslinked and unmodified F-actin, suggesting that crosslinked F-actin subunits retain the ability to bind myosin.

ADPr modification of actin on Arg-177 has been reported to inhibit actin polymerization (Wegner and Aktories 1988) and the structure of ADPr-actin has been solved (Margarit et al. 2006). In their work, Ballweber et al. (Ballweber et al. 2001) examined the impact of ADP-ribosylation of actin monomers on the binding of actin binding proteins, including myosin. They found that G-actin binding proteins and cofolin and gelsolin all interacted with ADPr-monomers. Significantly, the fluorescence of pyrene-labeled ADPr-actin increased in the presence of myosin.
and electron micrographs revealed short filaments of ADPr-actin in the presence of myosin, confirming an interaction of myosin with ADPr-actin.

Recent electron microscopy reconstructions have produced high resolution structures of the myosin bound to F-actin (Behrmann et al. 2012; Wulf et al. 2016; Banerjee et al. 2017; Fujii and Namba 2017a; Gurel et al. 2017; Mentes et al. 2018). These structures suggest that the location of the pPBM crosslink and the position of ADP-ribosylation on Arg-177 of actin do not sterically block the myosin binding site (Fig 1).

Based on the previous biochemical work with pPBM-crosslinked F-actin, ADPr-actin and electron microscopy-based structures of actomyosin, we tested the hypothesis that a pure ADPr-trimer will bind myosin. Our results show that myosin does not interact with the ADPr-trimer, ADPr-trimer capped with gelsolin, or DNase-I capped trimer. Comparing with the recent work of Qu et al (Qu et al. 2018), our results suggest that complete ribosylation of the actin trimer remodels the myosin binding site to inhibit myosin interactions and that the capping of the pointed end of a crosslinked actin trimer with DNase-I also blocks myosin binding. Future work will involve determining a balance of actin modifications that support myosin activity and studying the variety of mechanisms for different F-ABP binding to short F-actin complexes.

Materials and methods

Reagents

Unless otherwise stated, all buffer reagents and media were obtained from Fisher Scientific (Mississauga, ON) or Sigma–Aldrich (St. Louis, MO). All chromatography columns were obtained from GE Healthcare (GE Healthcare, Piscataway, NJ).
Protein Purification

Actin protein was purified from turkey breast tissue as described (Perieteanu et al. 2010). Photox ADP-ribosyltransferase enzyme was purified from inclusion bodies from *E. coli* Rosetta cells as described (Visschedyk et al. 2010). *E. coli* cells expressing recombinant full-length gelsolin (GS) were lysed in a French Press and the clarified lysate was developed on an Affigel Blue column with a linear gradient of 1 M NaCl in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) as described (Morrison and Dawson 2007). Fractions containing GS were pooled and dialysed against TE buffer with β-mercaptoethanol (β-ME). Pure myosin and myosin subfragment-1 (S1) was obtained from rabbit *soleus* muscle following established methods (Margossian and Lowey 1982).

**ADPr-oligomers** – Chemical crosslinking of F-actin with *p*-phenylenebismaleimide (pPBM), depolymerization and ADP-ribosylation (ADPr-) catalyzed by Photox enzyme was performed as described (Perieteanu et al. 2010). F-actin-derived ADPr-oligomers of crosslinked actin were purified with iterative Superdex-200 (26/600) chromatography in G-10 buffer (10 mM Tris-HCl, pH8.0, 0.2 mM CaCl₂, 0.2 mM ATP, 0.2 mM β-ME, 0.2 mM NaN₃, 0.25 mM phenylmethanesulfonyl fluoride (PMSF)) with 50 mM NaCl, following the previously published method (Perieteanu et al. 2010). This previously published method results in a stoichiometry of one ADP-ribosylation on Arg-177 of each actin subunit in the crosslinked trimer. Mass spectrometry confirmed the presence of the chemical crosslink between Lys-191 and Cys-374 (data not shown).

**GS:ADPr-trimer** – Gelsolin-bound ADPr-trimer (GS:ADPr-trimer) was produced by incubating GS and ADPr-trimer in 1.4 molar ratio overnight at 4°C. The resulting reaction
mixture was purified with Superdex-200 (10/300) column chromatography developed in G-10 buffer with 50 mM NaCl. The elution fractions were run on a native gel with GS alone or ADPr-trimer alone as controls. The fractions containing GS:ADPr-trimer were pooled, stored at 4°C and studied within a week of purification.

**D:trimer** – To produce DNase-I capped pPBM-crosslinked trimer (D:trimer), F-actin was crosslinked with pPBM using the conditions described above for making ADPr-trimer. Crosslinked F-actin was incubated with DNase-I (Worthington) at a 1:1 actin subunit:DNase-I molecular ratio in F-buffer (25 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM MgCl$_2$, 1 mM EGTA, 0.1 mM ATP, 1 mM β-ME) overnight at 4°C. The reaction was centrifuged at 392 000 g for 20 min and the resulting supernatant was resolved on a Superdex-200 (10/300) column in G-10 buffer.

**Dynamic Light Scattering**

ADPr-trimer and myosin S1 were clarified through 0.22 um filters. Molar concentrations were adjusted using filtered G-10 buffer with 50 mM NaCl. Dynamic light scattering measurements were performed on Nano S Zetasizer (Malvern, U.K.) equipped with a 633 nm red laser and a 173° backscatter detector. Experimental Stokes radii of the samples were determined by a normal distribution fit of volume distribution measurements.

**Acrylamide Gel electrophoresis**

SDS-PAGE was performed as described previously (Laemmli, 1970) with acrylamide gels comprised of 10% resolving and 5% stacking gel. Native PAGE was performed with non-denaturing acrylamide gels comprised of 8% resolving and 5% stacking gel supplemented with
0.2 mM ATP and 0.2 mM CaCl$_2$. Native PAGE was run at a low voltage (< 60V) on ice. SDS-PAGE and Native gels were visualized with either Coomassie Brilliant Blue R-250 (Sigma Aldrich) or Silver Stain (Pierce$^\text{TM}$ Silver Stain Kit).

**Myosin Actin-activated ATPase assay**

The myosin ATPase activity assay used was based on a colorimetric assay based on the production of a blue by-product between released inorganic phosphate (Pi) and molybdate, modified from Trybus (2000) for a 96-well format as described (Liu et al. 2017). Single concentration assays (5 uM actin on a per-subunit basis for ADPr-trimer) in the presence of 0.25 mg/mL myosin were performed in triplicate with released Pi measured at 10-minute time intervals for 30 minutes. Owing to small amounts of pure GS:ADPr-trimer and D:trimer, myosin ATPase assays were performed with a single concentration of 1.5 uM actin on a per-subunit basis with released Pi measured at 40 and 60 minutes for GS:ADPr-trimer and 20, 40 and 60 minutes for D:trimer. The average ATPase activity was plotted using Prism 7.0c software (GraphPad, LaJolla, Calif.).

**Results**

**Myosin does not bind ADPr-trimer under rigor conditions**

Since the ADPr-trimer contains one potential myosin site per complex (Mentes et al. 2018) (**Fig 1**), we tested the binding of myosin subfragment-1 (S1) to ADPr-trimer under rigor conditions and found little evidence of interaction. No significant increase in hydrodynamic radius was seen between protein alone controls and a mixture of ADPr-trimer and myosin S1 (**Fig 2**) and no shift in the mobility of ADPr-trimer was observed in the presence of myosin S1.
on native PAGE gels (Fig 3A). The presence of a single band in the trimer sample on SDS-
PAGE shows that all subunits in the trimers are covalently crosslinked to each other (Fig 3B).
Since myosin S1 alone was not observed on the native gels, a new band might not run on the gel,
or the myosin S1 might be found in the band present on the gel. We performed mass
spectrometric analysis on the ADPr-trimer-containing bands from the native PAGE and found no
myosin peptides (data not shown). Likewise, no shift was seen in analytical gel filtration
chromatography peak elution volumes for ADPr-trimer in the absence or presence of myosin S1
(Fig 4); rather, a peak of myosin S1 protein eluted in different fractions than the ADPr-trimer
protein.

To confirm that S1 does not bind ADPr-trimer, we found no significant increase in
myosin ATPase activity in the presence of ADPr-monomer, -dimer, or –trimer (Fig 3C), whereas
myosin ATPase activity is activated in the presence of F-actin, as expected.

Remodeling by gelsolin does not restore myosin binding.

Previously, we showed that ADPr-modified actin oligomers do not nucleate actin
polymerization (Perieteanu et al. 2010). The binding of gelsolin (GS) to ADPr-trimer restored
nucleation of polymerization from the pointed end, suggesting that GS remolds the ADPr-trimer
into a native-like state. We therefore tested whether gelsolin-bound ADPr-trimer (GS:ADPr-
trimer) would bind myosin S1 under rigor conditions.

Our results showed no significant interactions between GS:ADPr-trimer and myosin.

Native PAGE (Fig 5) and analytical gel filtration chromatography (Fig 6) analyses of mixtures
of purified GS:ADPr-trimer and myosin S1 showed no significant shifts in either band mobility
or peak elution volumes. The minimal myosin ATPase activity measured in the presence of
GS:ADPr-trimer was not significantly different than the activity seen with ADPr-monomer as substrate ($p = 0.18$) (Fig 5C). Therefore, GS remodeling does not restore myosin binding to the ADPr-trimer.

DNase-I capped pPBM-trimers do not bind myosin

While myosin has been shown to bind free ADPr-monomer (Ballweber et al. 2001), the inhibition of S1 binding may be due to the presence of the ADPr moiety on each actin subunit, inhibiting native intersubunit interactions needed for productive myosin binding. Since earlier work reported that DNase-I does not block myosin binding to actin (Blanchoin et al. 1995), we produced pPBM-crosslinked actin trimers capped at the pointed end by DNase-I (D:trimer) using gel filtration chromatography (Fig 7) to test the possibility that ADP-ribosylation inhibits actomyosin interactions. We found no evidence of interactions between D:trimer and myosin in native gel electrophoreograms or myosin ATPase assays (Fig 8).

Discussion and conclusions

The ADPr-trimer interacts with barbed-end and pointed-end F-ABPs. Perhaps overlooked as an F-ABP, actin monomers bind ADPr-trimer at its pointed end when gelsolin is present on the trimer (Perieteanu et al. 2010). In this paper, we asked whether the ADPr-trimer provides a binding surface for myosin as an example of a side-binding F-ABP. Our work shows that myosin does not bind the ADPr-trimer, nor does the ADPr-trimer activate myosin actin-activated ATPase. We also found that neither gelsolin-bound ADPr-trimer nor crosslinked actin trimer capped with DNase-I (D:trimer) interacts with myosin.
Since previous work shows that pPBM crosslinking inhibits force generation and movement but does not inhibit myosin binding to F-actin and ATPase activity (Knight and Offer 1980; Kim et al. 2002), we reasoned that the crosslinking of the ADPr-trimer would permit myosin binding. Similarly, crosslinking F-actin with glutaraldehyde does not inhibit myosin binding (Prochniewicz and Yanagida 1990), but results in a conformational state of actin that does not generate movement (Kozuka et al. 2007).

Past research shows that myosin binds to ADPr-monomers (Ballweber et al. 2001); however, the presence of an ADP-ribose on each subunit of the F-actin-derived trimer might disrupt intersubunit contacts necessary for myosin binding. To test this model, we blocked the polymerization of F-actin-derived trimer by binding DNase-I to the pointed end rather than by ADP-ribosylation. We found that myosin also did not bind DNase-I bound crosslinked actin trimer, suggesting that either ADP-ribosylation on each subunit does not inhibit myosin binding, or that DNase-I binding to the pointed end remolds the trimer into a state that blocks myosin.

A body of work shows that flexibility and conformational changes in F-actin are essential for function (reviewed in (Blanchoin et al. 2014)). At the single filament level, different conformations within actin subunits depend on the bound nucleotide (Murakami et al. 2010) and blocks of different subunit states can exist along a filament (Galkin et al. 2010). Different subunit states might be the result of, or be bound by, F-ABPs that lead to changes in the twist of F-actin (Mochida et al. 2002; Kozuka et al. 2007; Gurel et al. 2017). Despite the requirement for conformational change in F-actin for function, the literature is clear that pPBM crosslinking permits the binding of myosin to F-actin (Knight and Offer 1980; Kim et al. 2002) and, by extension, the necessary conformational changes in F-actin for binding.
During final preparation of our manuscript, Qu et al reported the isolation of a crosslinked and ADP-ribosylated actin trimer similar to ours that activates myosin ATPase (Qu et al. 2018). To produce their actin trimer, Qu et al first purified PBM-crosslinked actin trimer and then *Iota* toxin to ADP-ribosylate actin on Arg-177. The ADP-ribosylation was not complete, however, since the resulting trimers were partially polymerization competent. To completely inhibit polymerization, gelsolin segment-1 (GS1) was incubated at a 1:1 molecular ratio to the trimer.

The primary difference between the ADPr-trimer we report on here and that described by Qu et al is the stringency of the modifications employed to inhibit polymerization. Our work demonstrates that complete ribosylation of the trimer inhibits myosin binding, while our earlier work shows that binding of all subunits in the trimer with GS1 disrupts F-actin contacts (Dawson et al. 2003). Therefore, our requirement for a homogeneous non-polymerizing actin trimer resulted in treatments that inhibit some F-actin activities, whereas the combination of two modifications at sub-stoichiometric levels likely results in a heterogeneous actin trimer preparation that activates myosin activity.

Comparison of our work and that of Qu et al suggests that ribosylation of all subunits in the PBM-trimer remodels the myosin binding site on actin. Moreover, these finding suggests that capping of trimer with DNase-I must also remodel the trimer such that myosin cannot bind, despite the finding that DNase-I can form a ternary complex with actin and myosin. One speculation is that the binding of DNase-I to both pointed end subunits repositions the two molecules and disrupts the myosin binding site.

Some of the interactions between actin and myosin are being revealed through advances in high-resolution cryo-EM (Behrmann et al. 2012; Ropars et al. 2016; Wulf et al. 2016;
Banerjee et al. 2017; Fujii and Namba 2017b; Gurel et al. 2017); however, not all of the
actomyosin states critical for lever arm movement have been captured (Houdusse and Sweeney
2016), and surface loops on different forms of myosin interact with different groups on actin.
Moreover, myosin binding changes the flexibility, twist and structural states of actin filaments
(discussed in (Mochida et al. 2002; Kozuka et al. 2007; Gurel et al. 2017)), suggesting that
myosin binds to F-actin in a cooperative manner through structural changes communicated
through the actin subunits in the filament. Indeed, the binding of myosin to F-actin displays
cooperative behaviour under specific conditions (Orlova and Egelman 1997; Tokuraku et al.
2009; Ngo et al. 2016). However, the work of Qu et al suggests that an actin trimer, with only
one myosin binding site located between two along-strand actin subunits, is the minimal
structure required for myosin ATPase activity.

Requiring actin to function as a polymer with only a few subunits is a challenging design
goal. The body of work using chemical crosslinking and different means of controlling actin
polymerization illustrates the need for careful balancing between the needs of the design strategy
and the activity of actin. In this work, the complete ADP-ribosylation of crosslinked actin trimer
and capping of the trimer on the pointed end with DNase-I both result in no activation of myosin
ATPase activity. At the same time, other F-ABPs bind to the ADPr-trimer, highlighting the
variety of actin binding modes among the F-ABPs. Future work can study the binding of other
F-ABPs to the ADPr-trimer to test different modes of actin binding and examine the
contributions of conformational flexibility and longer-range contacts along the actin filament for
side-binding F-ABPs. Moreover, it will be important to confirm the activity of
substoichiometrically-modified crosslinked actin trimers and develop further tests to determine
whether an actin trimer can support lever arm movement and force generation coupled with
myosin ATPase activity.

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Author Contributions

J.F.D. supervised the research, obtained the funding, and wrote and revised the
manuscript. N.S. performed all the experiments for the research and revised the manuscript.
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proteins (WIP), may antagonistically regulate type I myosins in Saccharomyces cerevisiae.


Prochniewicz, E., and Yanagida, T. 1990. Inhibition of sliding movement of F-actin by


Figure captions

Figure 1. An Actin Trimer. (A) An actin trimer consisting of two subunits from one strand of the two-start long pitch F-actin helix (green and pink subunits labelled A3 and A1) and one subunit of the counterpart strand (light blue, A2) with the A3 subunit at the pointed end. Circled in red are the rigour-state myosin interaction sites on the surface of the actin trimer, adapted from (Fujii and Namba 2017a) and the outline of myosin (dotted violet line) in its bound position from the actomyosin structure of Mentes et al (Mentes et al. 2018). (B) Butterfly representation of the trimer with subunit A2 in the same position as in panel A and subunits A1 and A3 rotated 180˚ to show the internal ADP-ribosylated Arg-177 residues (in blue) and the location of the residues participating in the interstrand pPBM crosslinks indicated by a dotted black line: Lys-191 (in red) and Cys-374 (in orange). Note that Cys-374 of subunits A3 and A2 are located behind the surface representation shown. These structures were generated with chains B – D and P from PDB 6C1D (Mentes et al. 2018) and visualized using PyMol.

Figure 2: Dynamic Light Scattering measurement of ADPr-trimer and myosin S1. The hydrodynamic radii of different samples of ADPr-trimer (4 uM trimer = side binding sites) and myosin S1 (1.5 uM) were measured using a Nano S Zetasizer. The derived radii of the different samples were: (A) ADPr-trimer alone, 4.33 nm; (B) S1 alone, 4.34 nm; and (c) ADPr-trimer and S1, 4.62 nm. Error bars represent the standard deviation for three readings per sample. No significant increase in the radii seen in the reaction mix with ADPr-trimer and S1.

Figure 3. Subfragment-1 binding and myosin ATPase assays in the presence of purified ADPr-actin oligomers. (A) Native gel of reactions containing 2 uM of ADPr-monomer, ADPr-
dimer, or ADPr-trimer incubated for 20 min on ice in the presence of 0.87 μM myosin S1 in G-10 buffer containing 50 mM NaCl with 60 μg/mL each of antipain, leupeptin, TLCK, and TPCK protease inhibitors. Note that 6.1 μg of S1 was run in lane as a standard, while only 0.95 μg of S1 was run in the reaction mixtures. Since S1 tends to run as a smear on native gels, the resulting diffuse signal at lower S1 concentrations is difficult to visualize with Coomassie staining. (B) SDS-PAGE of the samples in A above, showing the presence of S1 in all reactions. (C) The ATPase hydrolase activity of 0.25 mg/ml myosin was measured in the presence of 5 μM F-actin or purified ADPr-actin oligomers. The average activity for triplicate measurements is plotted ± SEM. The ADPr-actin-containing complex activities were significantly different than that in the presence of F-actin (p-values; ADPr-monomer: 0.003, ADPr-dimer: 0.005, ADPr-trimer: 0.001) ADPr-trimer did not activate myosin ATPase activity beyond that seen with ADPr-monomer (p = 0.679).

**Figure 4. Analytical size exclusion chromatography of ADPr-trimer and myosin S1 reactions.** (A) A mixture of 2 μM ADPr-trimer and 0.87 μM myosin S1 in G-10 buffer containing 50 mM NaCl or 2 μM ADPr-trimer alone was incubated for 1 hour at room temperature and then developed on a Superdex-200 (10/300) analytical gel filtration column developed in G-10 buffer containing 50 mM NaCl. The bar above the absorbance profile indicates the fractions analyzed in panel (B). (B) Silver-stained SDS-PAGE of 10 μL sample of fractions the chromatographic analysis of the mixture of ADPr-trimer and S1 in (A). The peaks of S1 and ADPr-trimer do not appear to in the same fractions, suggesting no interaction.

**Figure 5. Subfragment-1 binding and myosin ATPase assays in the presence of purified**
**GS:ADPr-trimer.** (A) Native gel of reactions containing 0.23 μM of ADPr-monomer, or Gelsolin:ADPr-trimer complex (GS:ADPr-trimer) incubated for 15 min on ice in the presence of 0.20 μM myosin S1 in G-10 buffer containing 50 mM NaCl with 24 μg/mL each of antipain, leupeptin, TLCK, and TPCK protease inhibitors. (B) SDS-PAGE of the samples in A, showing the presence of all components in the reactions. Some contaminating full-length myosin is present in the S1 preparation. (C) The ATPase hydrolysis activity of 0.25 mg/ml myosin was measured in the presence of 1.5 μM F-actin, purified ADPr-monomer, GS:ADPr-trimer. The average activity for triplicate measurements is plotted ± SEM. GS:ADPr-trimer did not activate myosin ATPase activity beyond that seen with ADPr-monomer (p = 0.18).

**Figure 6. Analytical size exclusion chromatography analysis of GS:ADPr-trimer-S1 binding reactions.** (A) Chromatograms of a GS:ADPr-trimer-S1 binding reaction containing 0.9 umol of GS:ADPr-trimer and 0.8 umol S1 (dotted line) and 0.9 umol GS:ADPr-trimer alone (solid line) run on a Superdex-200 (10/300) column. Both samples also contained 150 μg/mL antipain, 150 μg/mL leupeptin, 60 μg/mL TLCK, and 60 μg/mL TPCK. A major peak at an elution volume of about 11.8 mL is observed, along with a minor peak eluting at just after 15 mL. (B) SDS-PAGE of fractions from the GS:ADPr-trimer-S1 reaction in (A). Fractions B4 to C2 are across the major peak indicated by the top bar in (A) while fraction D9 is from the minor peak. While S1 is present in the loaded sample (Load), S1 is not present as a major band in the peak fractions of the elution profile. (C) Silver stained native gel of major peak fractions indicated by the bottom bar in (A). Note that S1 is not a major band in the peak fractions.

**Figure 7. Purification of DNase I-bound PBM-trimer (D:trimer).** (A) Chromatogram of a
sample of crosslinked F-actin incubated with DNase-I in a 1:1 molecular ratio developed on a
Superdex S-200 (10/300) column as described in Methods and Materials. The identity of DNase-
I bound actin oligomers is indicated on the chromatogram. The range of elution fractions
analyzed by SDS-PAGE (B) or Native PAGE (C) is indicated by the box above the
chromatogram.

Figure 8: DNase-I bound crosslinked actin trimer (D:trimer) interactions with Myosin S1.

(A) Silver stained Native PAGE of binding reactions including 0.42 uM DNase-I capped actin
oligomers in the presence or absence of 0.37 uM S1 and 33 ug/mL anitpain, leupeptin, TLCK
and TPCK protease inhibitors. DNase-I was also run alone to identify that band. No new bands
were seen suggesting S1 bound to the DNase-I bound actin oligomers. (B) Myosin ATPase
activity assays including 0.25 mg/mL myosin in the presence of 1.5 uM F-actin or DNase-I
bound crosslinked actin oligomers. The average activity for triplicate measurements is plotted ±
SEM. No significant activity was observed for DNase-I bound actin trimers.
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Figure 3. Subfragment-1 binding and myosin ATPase assays in the presence of purified ADP-actin oligomers. (A) Native gel of reactions containing 2 μM of ADP-monomer, ADP-dimer, or ADP-trimer incubated for 20 min on ice in the presence of 0.87 μM myosin S1 in G-10 buffer containing 50 mM NaCl with 60 μg/mL each of antipain, leupeptin, TLCK, and TPCK protease inhibitors. Note that 6.1 μg of S1 was run in lane as a standard, while only 0.95 μg of S1 was run in the reaction mixtures. Since S1 tends to run as a smear on native gels, the resulting diffuse signal at lower S1 concentrations is difficult to visualize with Coomassie staining. (B) SDS-PAGE of the samples in A above, showing the presence of S1 in all reactions. (C) The ATPase hidrolase activity of 0.25 mg/ml myosin was measured in the presence of 5 μM F-actin or purified ADP-actin oligomers. The average activity for triplicate measurements is plotted ± SEM. The ADP-actin-containing complex activities were significantly different than that in the presence of F-actin (p-values; ADP-monomer: 0.003, ADP-dimer: 0.005, ADP-trimer: 0.001) ADP-trimer did not activate myosin ATPase activity beyond that seen with ADP-monomer (p = 0.679).
Figure 4. Analytical size exclusion chromatography of ADPr-trimer and myosin S1 reactions. (A) A mixture of 2 μM ADPr-trimer and 0.87 μM myosin S1 in G-10 buffer containing 50 mM NaCl or 2 μM ADPr-trimer alone was incubated for 1 hour at room temperature and then developed on a Superdex-200 (10/300) analytical gel filtration column developed in G-10 buffer containing 50 mM NaCl. The bar above the absorbance profile indicates the fractions analyzed in panel (B). (B) Silver-stained SDS-PAGE of 10 μL sample of fractions the chromatographic analysis of the mixture of ADPr-trimer and S1 in (A). The peaks of S1 and ADPr-trimer do not appear to in the same fractions, suggesting no interaction.
Figure 5. Subfragment-1 binding and myosin ATPase assays in the presence of purified GS:ADPr-trimer. (A) Native gel of reactions containing 0.23 uM of ADPr-monomer, or Gelsolin:ADPr-trimer complex (GS:ADPr-trimer) incubated for 15 min on ice in the presence of 0.20 uM myosin S1 in G-10 buffer containing 50 mM NaCl with 24ug/mL each of antipain, leupeptin, TLCK, and TPCK protease inhibitors. (B) SDS-PAGE of the samples in A, showing the presence of all components in the reactions. Some contaminating full-length myosin is present in the S1 preparation. (C) The ATPase hydrolysis activity of 0.25 mg/ml myosin was measured in the presence of 1.5 uM F-actin, purified ADPr-monomer, GS:ADPr-trimer. The average activity for triplicate measurements is plotted ± SEM. GS:ADPr-trimer did not activate myosin ATPase activity beyond that seen with ADPr-monomer.
Figure 6. Analytical size exclusion chromatography analysis of GS:ADPr-trimer-S1 binding reactions. (A) Chromatograms of a GS:ADPr-trimer-S1 binding reaction containing 0.9 umol of GS:ADPr-trimer and 0.8 umol S1 (dotted line) and 0.9 umol GS:ADPr-trimer alone (solid line) run on a Superdex-200 (10/300) column. Both samples also contained 150 ug/mL antipain, 150 ug/mL leupeptin, 60 ug/mL TLCK, and 60 ug/mL TPCK. A major peak at an elution volume of about 11.8 mL is observed, along with a minor peak eluting at just after 15 mL. (B) SDS-PAGE of fractions from the GS:ADPr-trimer-S1 reaction in (A). Fractions B4 to C2 are across the major peak indicated by the top bar in (A) while fraction D9 is from the minor peak. While S1 is present in the loaded sample (Load), S1 is not present as a major band in the peak fractions of the elution profile. (C) Silver stained native gel of major peak fractions indicated by the bottom bar in (A). Note that S1 is not a major band in the peak fractions.
Figure 7. Purification of DNase I-bound PBM-trimer (D:trimer). (A) Chromatogram of a sample of crosslinked F-actin incubated with DNase-I in a 1:1 molecular ratio developed on a Superdex S-200 (10/300) column as described in Methods and Materials. The identity of DNase-I bound actin oligomers is indicated on the chromatogram. The range of elution fractions analyzed by SDS-PAGE (B) or Native PAGE (C) is indicated by the box above the chromatogram.
Figure 8: DNase-I bound crosslinked actin trimer (D:trimer) interactions with Myosin S1. (A) Silver stained native PAGE of binding reactions including 0.42 uM DNase-I capped actin oligomers in the presence or absence of 0.37 uM S1 and 33 ug/mL anitpain, leupeptin, TLCK and TPCK protease inhibitors. DNase-I was also run alone to identify that band. No new bands were seen suggesting S1 bound to the DNase-I bound actin oligomers. (B) Myosin ATPase activity assays including 0.25 mg/mL myosin in the presence of 1.5 uM F-actin or DNase-I bound crosslinked actin oligomers. The average activity for triplicate measurements is plotted ± SEM. No significant activity was observed for DNase-I bound actin trimers.