The regulatory protein 14-3-3β binds to the IQ motifs of myosin-IC independently of phosphorylation

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Running Title: Interaction of Myosin-IC with 14-3-3β

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ABSTRACT

Myosin-IC (Myo1c) has been proposed to function in the delivery of glucose transporter type 4 (GLUT4)-containing vesicles to the plasma membrane in response to insulin stimulation. Current evidence suggests that upon insulin stimulation, Myo1c is phosphorylated at Ser-701, leading to binding of the signaling protein 14-3-3β. Biochemical and functional details of the Myo1c–14-3-3β interaction have yet to be described. Using recombinantly expressed proteins and Mass Spectrometry-based analyses to monitor Myo1c phosphorylation, along with pulldown, fluorescence binding, and additional biochemical assays, we show here that 14-3-3β is a dimer and, consistent with previous work, that it binds to Myo1c in the presence of calcium. This interaction was associated with dissociation of calmodulin (CaM) from the IQ motif in Myo1c. Surprisingly, we found that 14-3-3β binds to Myo1c independently of Ser-701 phosphorylation in vitro. Additionally, in contrast to previous reports, we did not observe Myo1c Ser-701 phosphorylation by Ca2+/CaM-dependent protein kinase-II (CaMKII), although CaMKII phosphorylated four other Myo1c sites. The presence of 14-3-3β had little effect on the actin-activated ATPase or motile activities of Myo1c. Given these results, it is unlikely that 14-3-3β acts as a cargo adaptor for Myo1c-powered transport; rather, we propose that 14-3-3β binds Myo1c in the presence of calcium and stabilizes the calmodulin-dissociated, non-motile myosin.

Myosin-I isoforms are single-headed members of the myosin superfamily that link plasma- and intracellular-membranes to the actin cytoskeleton (1-3). Cell biological experiments have revealed a range of cellular processes in which myosin-I isoforms participate, including endosomal trafficking, endocytosis, cell-cell and cell substrate adhesion, and membrane tension (2). Myosin-I isoforms are localized to subcellular regions by motor-domain binding to tropomyosin-free actin filaments, and by direct binding to the headgroups of negatively charged lipids and phosphoinositides through the pleckstrin-homology (PH) domain containing tail (2,4-6). Numerous proteins that bind myosin-I isoforms have also been identified (reviewed in (2)), but how these proteins participate in the regulation, targeting, and/or anchoring of motors is unclear.

A widely expressed myosin-I isoform, myosin-IC (Myo1c), participates in insulin-stimulated translocation of GLUT4-containing vesicles to the plasma membrane in both adipocytes (7,8) and muscle cells (9). Myo1c associates directly with both mobile and tethered GLUT4 vesicles, and it is required for insulin-induced vesicle tethering (9). It has been reported that Myo1c involved GLUT4 transport is affected by
myosin’s interaction with an adaptor protein, 14-3-3β (10). Additionally, it was suggested that 14-3-3β binding is activated by Ca²⁺/CaM-dependent protein kinase-II (CaMKII) -mediated phosphorylation of Myo1c S701. 14-3-3 proteins are a family of dimeric adaptors that predominantly bind phosphorylated proteins and mediate interactions with many different proteins (11). Thus, the interaction between Myo1c and 14-3-3β is an intriguing possible mechanical link between contractile and signaling proteins.

The proposed 14-3-3β binding site at S701 of Myo1c (10) directly precedes the first calmodulin-binding IQ motif near the fulcrum on the myosin lever arm (Fig.1A; (12)). This is a mechanically important region of the motor that links ATPase activity to the force-generating working stroke, and would be an unexpected location for an adaptor that links cellular cargos to transport. Thus, we investigated the biochemical and functional details of the interaction of Myo1c with 14-3-3β. We found that 14-3-3β binding to Myo1c is regulated by Ca²⁺, and this association correlates with calmodulin (CaM) dissociation from the IQ motif, as suggested previously (10,12). We also found that Myo1c bound to 14-3-3β does not support actin gliding in motility assays, and that phosphorylation did not appear to affect 14-3-3β’s ability to bind to the target sequence. These results support a calcium-dependent tethering role for the interaction of 14-3-3β with Myo1c, rather than as an adaptor for a mechanically active motor.

RESULTS

14-3-3β binding to Myo1c is enhanced by calcium and calmodulin dissociation

We investigated the interaction of 14-3-3β with a Myo1c construct that includes the motor domain, regulatory domain with bound CaM, and a carboxy-terminal Avi Tag for site-specific biotinylation (Myo1c-3IQ; Fig. 1A). By analytical ultracentrifugation (see Methods), we determined that 14-3-3β is a dimer under experimental conditions, so dimer concentrations are reported below. Biotinylated Myo1c-3IQ (0.8 μM) was mixed with 2.5 μM 14-3-3β and pulled down by centrifugation of streptavidin-coated beads. 14-3-3β binds to Myo1c-3IQ, and this binding was enhanced in the presence of free calcium. Inclusion of 1 mM MgATP had no detectable effect on the binding (Fig. 1B).

Previous work showed that calcium binding to CaM weakens CaM affinity for Myo1c (13-15), and given that calcium appears to enhance 14-3-3β binding (Fig. 1B), we investigated the relationship between calmodulin and 14-3-3β binding. We mixed biotinylated Myo1c-3IQ with 0 - 10 μM 14-3-3β, and pulled down the proteins with streptavidin beads in the absence and presence of 100 μM free calcium. In the absence of calcium, 14-3-3β binds weakly to Myo1c-3IQ (Fig. 2A). Addition of calcium resulted in increased binding with increased 14-3-3β concentration (0 – 10 μM) until binding approached saturation at an apparent ratio of one 14-3-3β dimer per Myo1c-3IQ. The increased 14-3-3β binding correlated with decreased calmodulin binding, with one 14-3-3β dimer displacing one calmodulin (Fig. 2A). Thus, 14-3-3β and CaM appear to compete for Myo1c binding.

To further explore the possibility that 14-3-3β competes with calmodulin for Myo1c binding, we treated Myo1c-3IQ with 0.5 mM trifluoperazine (TFP), a calmodulin agonist known to dissociate light chains from the IQ motifs of myosins (16). Upon pull-down of 0.5 μM Myo1c-3IQ in the presence of 1.25 μM 14-3-3β, TFP-mediated dissociation of calmodulin allows 14-3-3β to bind Myo1c robustly in the absence of calcium (Fig. 2B). In the presence of TFP, the molar ratios of 14-3-3β dimer and CaM to heavy chain in the absence of calcium are 1.7 and 0.9, respectively, as determined by densitometry. In the presence of calcium, the ratios of 14-3-3β dimer and CaM to Myo1c are 1.9 and 0.6, respectively. Thus, 14-3-3β competes with calmodulin for Myo1c binding.

S701 phosphorylation does not affect 14-3-3β binding to Myo1c

Phosphorylation of S701 of Myo1c by CaMKII was reported to be important for myosin’s association with 14-3-3β (10); thus, we examined the effect of CaMKII on Myo1c-3IQ under phosphorylation conditions (see Methods). Mass spectrometry (MS) analysis of sf9 purified Myo1c showed partial phosphorylation of S373 and S572. To dephosphorylate the protein, we pre-incubated the myosin with lambda protein phosphatase and subsequently purified the protein. Dephosphorylated Myo1c-3IQ was incubated with...
activated CaMKII, and MS analysis showed S73, S104, S332, S501 of Myo1c were phosphorylated; however, no S701 phosphorylation was detected (Fig. S1), in contrast to previous investigations (10). Given the weak affinity of CaM for IQ1 in the presence of calcium (13), it is unlikely that CaM would sterically hinder CAMKII from interacting with S701 under the calcium-containing reaction conditions.

It is possible that an unknown kinase participates in the phosphorylation of S701 of Myo1c, which was not captured in our in vitro assay. Therefore, we expressed unphosphorylatable (S701A) and phosphomimic (S701E) mutants of Myo1c-3IQ, and we examined 14-3-3β binding using two different pull-down assays. Soluble 14-3-3β was incubated with Myo1c-3IQ-S701A or S701E attached to streptavidin-coated beads (Fig. 3A), and soluble Myo1c-3IQ-S701A or S701E was incubated with GST-14-3-3β attached to glutathione beads (Fig. 3B). Although mutation of serine to glutamic acid does not always mimic phosphorylation (17), we found in both assays that both mutants bound 14-3-3β, with enhanced binding in the presence of calcium. We also treated both mutants with 0.5 mM TFP, and again found 14-3-3β binding (Fig. 3C), supporting the finding that phosphorylation is not required for 14-3-3β binding.

Given the calcium sensitivity of 14-3-3β binding and its ability to compete with calmodulin, we assessed its ability to bind a peptide with the sequence of the first Myo1c IQ motif (IQ1). Peptides were synthesized without phosphorylation (IQ1-WT) and with S701 phosphorylation (IQ1-pS701; Fig. 4). We determined the stoichiometry of 14-3-3β binding to IQ peptide by titrating both 1 µM and 5 µM IQ1-WT with 0–10 µM 14-3-3β and monitoring changes in intrinsic tryptophan fluorescence. The peptide-14-3-3β complex has a higher fluorescence level than the sum of the individual proteins (Fig. 4A). In both cases, we found two peptides bind one 14-3-3β dimer, which suggests a tight affinity with dissociation constants well below experimental protein concentrations. We compared the stoichiometry of IQ1-WT and pS701 interacting with 14-3-3β. Titrations showed that both IQ1-WT and IQ1-pS701 bound 14-3-3β with a similar stoichiometry of two peptides per 14-3-3β dimer (Fig. 4B). This results differs from 14-3-3β binding experiments with Myo1c-3IQ, which suggest that one Myo1c-3IQ binds one 14-3-3β dimer, which may be the result of steric constraints.

**14-3-3β does not affect Myo1c motor function**

To determine if 14-3-3β affects Myo1c motor function, we measured the ATPase activity of Myo1c in the presence of 50 µM actin and calcium. Although micromolar concentrations of calmodulin slightly activate the actin-activated ATPase (< 2 fold; (13)), we found no effect of 0–7.5 µM 14-3-3β on this activity (Fig. 5A).

We determined if modification of S701 affects the speeds of actin gliding. Because calcium inhibits actin gliding (13), Myo1c-3IQ, WT, S701A, and S701E were assayed in EGTA conditions. The three different myosin constructs powered actin gliding at the same speed, and 0–15 µM 14-3-3β did not affect this gliding rate (Fig. 5B). Although micromolar concentrations of calmodulin are able to restore actin gliding in the presence of calcium (13), 10 µM 14-3-3β was not able to rescue the inhibited motility of Myo1c (Fig. 5B).

**DISCUSSION**

Our results show that in vitro binding of dimeric 14-3-3β to Myo1c is independent of phosphorylation, and it is enhanced by calcium-mediated dissociation of calmodulin from the motor’s lever arm. We also found that 14-3-3β does not affect the ATPase activity of Myo1c in the presence of calcium, and 14-3-3β-bound to myosin in the presence of calcium does not support actin filament gliding.

**14-3-3β binding to non-phosphorylated IQ motifs**

Pull-down assays show that 14-3-3β does not compete effectively with CaM for binding to Myo1c-3IQ in the absence of calcium, but displaces a single CaM in the presence of calcium. Previous work with this Myo1c-3IQ construct showed that the IQ motif nearest the motor domain (IQ1) has the weakest affinity among the three for CaM in the presence of calcium (13). Taken together with the result that 14-3-3β has a very tight affinity for the IQ1 peptide (Fig. 4B), we propose that one 14-3-3β dimer is binding to IQ1 in the presence of calcium. However, we note that when two CaMs are dissociated from Myo1c-3IQ by the agonist TFP,
two 14-3-3β dimers are able to interact with the myosin.

Dimeric 14-3-3 proteins largely interact with phosphorylated ligands, forming a symmetric bowl-shaped structure consisting of helices that create an amphipathic ligand binding groove, in which a positively charged cluster and a hydrophobic “roof” coordinate an interaction with phosphate (11,18). Mechanisms of phosphorylation independent binding of ligands to 14-3-3 appear to be more variable, with some proteins showing similar binding interactions with phosphorylated ligands (18-20) while others have unique modes of interaction, e.g., the α2 helix of carbohydrate response element-binding protein (ChREBP; (21)). Comparison of the amino acid sequence of the α2 helix of ChREBP with Myo1c shows that the sequence of the key interacting amino acids of the α2 helix is similar to IQ1 of Myo1c (Fig. S2). Thus, Myo1c-3IQ may bind to 14-3-3β in a similar manner as ChREBP (Fig. S2). Finally, we note that residue S701 does not overlap with the calmodulin binding site on IQ1 (6,12), and it points away from the conserved positively charged cluster (R58-R129-Y130), again suggesting that this residue does not have a role in 14-3-3β binding.

Cellular role of the 14-3-3β-Myo1c interaction

Although it has been demonstrated that Myo1c and 14-3-3β affect GLUT4 translocation in response to insulin stimulation (10), the molecular and functional details of these interactions are not clear. 14-3-3β binds to Myo1c in the presence of calcium, and it has been proposed that calcium signaling beneath the plasma membrane in both muscle and adipocyte cells is important for insulin-stimulated GLUT4 transport (22,23). However, Myo1c-powered transport of GLUT4 in response to an increase in calcium concentration is unlikely. Calcium binding to Myo1c-bound CaM results in CaM dissociation, which structurally compromises the motor’s lever arm. Although the actin-activated ATPase activity increases in the presence of calcium, motility is inhibited (1,13-15,24). Micromolar concentrations of CaM can rescue motility in the presence of calcium in vitro; however, the free calmodulin concentration in cells is < 200 nM in the presence of calcium (25,26). IQ1 would be calmodulin-free under these conditions (13), and our results show that 14-3-3β does not rescue this calcium-inhibited Myo1c motility (Fig. 5B). Thus, our in vitro results indicate that it is unlikely that 14-3-3β acts as a cargo adaptor for Myo1c-powered transport of membranes.

14-3-3β bound to the first IQ motif may act as an adaptor that links Myo1c to other proteins, allowing the motor to act as a tether. Notably, several proteins other than calmodulin have been identified to bind to the IQ regions of Myo1c (27-30) and myosin-V (31), suggesting that this region not only acts as myosin’s lever-arm, but is also an important signaling hub.

Finally, it has been shown in vitro that dissociation of light chains from IQ motifs within the lever arm results in aggregation of myosin-I isoforms (32-34). Thus, we suggest the possibility that 14-3-3β acts as a stabilizing protein to prevent myosin aggregation during the calcium regulated state. This role is consistent with the a chaperone-like activity that has been proposed for some 14-3-3 isoforms (35).

EXPERIMENTAL PROCEDURES

Expression and purification of Myo1c-3IQ

A Myo1c construct (Myo1c-3IQ; isoform b, NP_032685.1) containing the N terminal motor domain and three CaM-binding IQ motifs, and C-terminal Avi-(GLNDIFEAQKIEWHE) and FLAG-tags (DYKDDDDK), was expressed and purified with CaM as described previously (5,36). Biotinylation of Myo1c-3IQ was performed as described previously (37). The protein was stored in liquid nitrogen after dialyzing in KMg25 buffer (10 mM Mops, pH 7.0, 25 mM KCl, 1 mM MgCl2, 1 mM EGTA and 1 mM DTT). The plasmids of Myo1c-3IQ mutants, Myo1c-3IQ-S701A and S701E were generated via a QuikChange kit (Stratagene), and the proteins were expressed and purified as described for Myo1c-3IQ.

Expression and purification of 14-3-3β

A plasmid (pGEX-2TK) containing human 14-3-3β originally generated by Yaffe et al. (38), was purchased from Addgene (Plasmid number: 13276), and transformed into BL21 (DE3) pLysS cells. To express GST tagged 14-3-3β, the cultured cells were induced with 0.2 mM IPTG and cultured at room temperature for ~10 hrs. Cell pellets from 1L cultures were suspend with 25 ml 1x Bugbuster protein Extraction Reagent (MilliporeSigma) containing 1 mM PMSF, 0.01 mg/ml leupeptin and
aprotinin, 0.01 mg/ml lysozyme, mixed and stayed at room temperature for 15 min. The lysate was centrifuged at 43,000 g for 30 min at 4 °C after sonication, 6 times (15 seconds on, 15 seconds off). 1 mL Glutathione sepharose 4B was mixed with the supernatant, and rotated at 4 °C for 1 hr. The sepharose was washed with 10 mL 1xPBS 5 times, loaded onto a column, and washed with 5 mL 1x thrombin buffer (20 mM Tris-HCl, pH8.4, 150 mM NaCl, 2.5 mM CaCl₂) twice. The sepharose was resuspended with ~3 mL 1x thrombin buffer containing ~30U thrombin (Heametech), and rotated at 4 °C overnight. The cleaved protein was dialyzed in KMg25 buffer overnight, centrifuged and loaded on to a MonoQ 5/50 GL column (GE healthcare), which was prewashed with buffer A (10 mM Tris-HCl, pH7.5, 50 mM KCl, 1 mM EGTA, 1 mM DTT). A gradient of buffer A (0 - 0.6 M KCl) was flowed through the MonoQ column with FPLC (Amersham Biosciences) at 1 mL / min. The purified 14-3-3β protein was collected, dialyzed in KMg25 buffer overnight, aliquoted, flash frozen, and stored in liquid nitrogen.

**Analytical ultracentrifugation**

Analytical ultracentrifugation experiments were performed with an XL-A analytical ultracentrifuge (Beckman-Coulter) and a TiAn60 rotor with six-channel charcoal-filled epon centerpieces and quartz windows as described (39). Briefly, sedimentation equilibrium data of 14-3-3β were collected at 4 °C with detection at 280 nm for 3 concentrations (0.3, 0.55, 0.8 mg/ml) at three successive speeds (22, 24 and 27,000 rpm) allowing the samples to reach equilibrium (20 hrs). The 14-3-3β migrated as a single component for all conditions, and the acquired data were analyzed using global fits with strict mass conservation using the program SEDPHAT (40). We determined that 14-3-3β is a very tight dimer (Kd < 0.1 nM) by analytical ultracentrifugation. The 14-3-3β protein concentrations reported in this paper refer to the concentration of 14-3-3β dimer.

**Phosphorylation of Myo1c-3IQ and mass spectrometry analysis**

To prepare Myo1c-3IQ for identification of the CaMKII-phosphorylated sites via mass spectrometry, biotinylated Myo1c-3IQ (200 µL of ~5 µM) was incubated in dephosphorylation buffer (50 mM HEPES pH7.5, 100 mM NaCl, 2 mM DTT, 1.5mM MnCl₂, 0.01% Brij35 and 6 µL Lambda Protein Phosphatase (400 U / µL, NEB)) for 2.5 hr at 30 °C, then mixed with 25 µL streptavidin agarose beads and rotated at 4 °C for 1.5 hr. The beads were washed with 200 µL phosphorylation buffer (50 mM Tris-HCl, pH7.5, 10 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA, 0.01% Brij35, 20 mM NaCl, 5 mM Mg²⁺-ATP, 1 mM CaCl₂) 15 times, resuspended with 200 µL phosphorylation buffer containing 3 µL activated CaMKII (500 U / µL, NEB), and incubated at 30 °C for 2 hrs. The supernatant was discarded after brief centrifugation, and 200 µL fresh phosphorylation buffer and 3 µL activated CaMKII were added and incubated for another 2 hrs. The phosphorylation buffer was replaced with another 200 µL fresh buffer containing 3 µL activated CaMKII, and rotated with beads at 4 °C overnight. After washing 5-times with 200 µL KMg25 buffer, the beads were resuspended with 100 µL 1x SDS loading buffer and boiled. Phosphorylated Myo1c-3IQ was subjected to SDS-PAGE, and detected by Pro-Q Diamond Phosphoprotein Gel Stain and SYPRO Ruby Protein Gel Stain (ThermoFisher Scientific), respectively. Non-phosphorylated (~25 µg) and ~60 µg phosphorylated Myo1c-3IQ were subjected to BioRad 4-20% gradient SDS-PAGE, stained with coomassie brilliant blue (CBB)-G250.

The gel band was destained with 100 mM ammonium bicarbonate/acetonitrile (50:50). The band was reduced in 10 mM DTT and 100 mM ammonium bicarbonate for over 60 min in 52°C. The band was then alkylated with 100 mM iodoacetamide in 100 mM ammonium bicarbonate at room temperature for 1 hr in the dark. The protein in the gel band was digested by incubation with trypsin overnight. The supernatant was removed and kept in fresh tubes. Additional peptides were extracted from the gel by adding 50 µL of 50% acetonitrile and 1% trifluoroacetic acid and shaking for 10 min. The supernatants were combined and dried. The dried samples were reconstituted in 0.1% formic acid for mass spectrometry analysis.

Desalted peptides were analyzed with a Q-Exactive HF mass spectrometer (Thermo Scientific) attached to an Ultimate 300 nano UPLC system (Thermo Scientific). Peptides were eluted with a 25 min gradient from 2% to 32% acetonitrile (ACN) and to 98% acetonitrile over 5 min in 0.1% formic acid. Data dependent acquisition mode with a dynamic exclusion of 45 s was enabled. One full
MS scan was collected with scan range of 350 to 1200 m/z, resolution of 70 K, maximum injection time of 50 ms and automatic gain control (AGC) of 1e6. Then, a series of MS2 scans were acquired for the most abundant ions from the MS1 scan (top 15). Ions were filtered with charge 2–5. An isolation window of 1.4 m/z was used with quadruple isolation mode. Ions were fragmented using higher-energy collisional dissociation (HCD) with a collision energy of 28%. Orbitrap detection was used with, resolution of 35 K, maximum injection time of 54 ms and AGC of 5e4.

Database search criteria were as follows: taxonomy Mus musculus (55029 sequences, July 15, 2019), carboxyamidomethylated (+57 Da) at cysteine residues for fixed modifications, oxidized at methionine (+16 Da) residues, Phosphorylation (+79.9 Da) at Serine, Threonine, and Tyrosine residues for variable modifications, two maximum allowed missed cleavage, 10 ppm MS tolerance, a 0.02-Da MS/MS tolerance. Only peptides resulting from trypsin digestion were considered. The target-decoy approach was used to filter the search results, in which the false discovery rate was less than 1% at the peptide and protein level.

**Pull-down assays**

Pull-down assays of biotinylated Myo1c-3IQ constructs in the presence of 14-3-3β were performed as follows: 100-200 µL of 0.25-0.8 µM biotinylated Myo1c-3IQ and 14-3-3β in KMg25 buffer containing 0.5 mg/ml BSA and 2 µM CaM, was mixed with 10 µL streptavidin agarose beads and rotated at 4 °C for 1 hour. Unbound proteins were washed out with 100-200 µL KMg25 buffer, 5 times. Beads were boiled at 100 °C for 5 min after adding 35 µL 1x SDS loading buffer, and then centrifuged. 15 µL supernatant was subjected to SDS–PAGE, visualized by Coomassie Brilliant Blue staining and blot with anti-Flag antibody.

**Steady-state fluorescence binding assay**

Steady-state fluorescence measurements were taken with a Photon Technology International fluorometer at room temperature. Two IQ1 peptides (IQ1-WT and IQ1-pS701) were synthesized by Peptide 2.0 Inc (Chantilly, VA), and were dissolved in KMg25 buffer. IQ1 (5 µM) peptide and 0-10 µM 14-3-3β were mixed, and binding was determined by monitoring changes in the steady-state fluorescence of the intrinsic tryptophans of IQ peptides and 14-3-3β. Tryptophan residues were excited at 295 nm and fluorescence emission spectra were collected in the range 310 - 400 nm, with 2nm excitation and emission monochromator slits. Because two intrinsic tryptophans (Trp61 and Trp230) exist within 14-3-3β, the final steady-state fluorescence spectra were corrected by subtracting the contribution of the fluorescence spectra of samples containing only 14-3-3β (0-10 µM ) from the corresponding total fluorescence spectra.

**ATPase activity measurement and Motility assay**

Steady-state ATPase activity was measured in KMg25 buffer (containing 50 µM free calcium) at 37°C using the NADH-coupled assay as described (42). The final protein concentrations after mixing were 100 nM Myo1c-3IQ, 10 µM CaM, 50 µM phalloidin-actin and 0-7.5 µM 14-3-3β.

In vitro motility assays were performed in standard motility chambers which were assembled with double stick tape and silicon vacuum grease.
Coverslips were coated with 1% collodion (Electron Microscopy Sciences). Streptavidin (1 mg/mL, 20 µL) was flowed into the chamber and incubated for 2 min. The surface was then blocked with 100 µL 1 mg/mL BSA for 2 min. Biotinylated myo1c-3IQ (600 nM, 50 µL) was added to the chamber and allowed to bind to the immobilized streptavidin for 2 min. Activation solution (100 µL; 10 mM Mops, pH 7.0, 25 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 5 mM Mg²⁺-ATP, 20 mM DTT, 2 µM CaM), 1 nM Rhodamine phalloidin-F-actin, 0-15 µM 14-3-3β, 1 mg/ml BSA, 5mg/ml glucose, and 1x GOC mixture (0.2 mg/ml Glucose oxidase, 0.04 mg/ml Catalase) were flowed into the chamber. The chamber was sealed with silicon grease and mounted on the microscope with a heated objective (37°C). The chamber was allowed to equilibrate to temperature for 8 min before image acquisition. The velocity of actin filaments gliding was measured using manual tracking plugin in ImageJ software.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the content of this article.

Data Availability

The raw mass spectrometry data can be found in MassIVE database (ID: MassIVE MSV000084445).
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FIGURE LEGENDS

**Figure 1:** Effects of calcium on the binding of 14-3-3β to Myo1c. (A; Upper panel) Cartoon representation of the crystal structure (PDB ID: 4BYF) of the Myo1c motor domain with 1-IQ (light grey) with bound CaM (green), Proposed phosphorylation site, S710, is highlighted in red. (Lower panel) Schematic of the primary structure of full length Myo1c and Myo1c-3IQ constructs. (B) Coomassie-stained gel showing results of streptavidin-bead mediated pull-down of biotinylated 0.8 μM Myo1c-3IQ WT and 2.0 μM CaM in the presence or absence of 2.5 μM 14-3-3β, 40 μM free calcium, and 1 mM Mg ATP.

**Figure 2:** Association of 14-3-3β with Myo1c-3IQ. (A, Top panels) SYPRO-stained gels showing results of streptavidin-bead mediated pull-down of biotinylated Myo1c-3IQ with various concentrations of 14-3-3β in the absence (1 mM EGTA) and presence of 100 μM free calcium. (A, Bottom panels)The stoichiometry of 14-3-3β and CaM to pulled-down Myo1c-3IQ as determined from quantification of gel bands as described in Methods. Plotted values are mean ± S.D. from 3-4 independent pull-down assays. (B) Coomassie stained gel showing pull-down of biotinylated Myo1c-3IQ with 1.25 μM 14-3-3β in EGTA and Ca²⁺ conditions. Myo1c-3IQ was treated with or without 0.5 mM TFP.

**Figure 3:** Effects of phosphorylation of S701 on the binding of 14-3-3β to Myo1c. (A) Coomassie-stained gel of pull-down of 0.8 μM biotinylated Myo1c-3IQ S701A and S701E in the presence of 2.5 μM 14-3-3β in the absence (EGTA) and presence of 40 μM free calcium. (B) Glutathione-agarose bead mediated pull-down of GST-14-3-3β in the presence of 1 μM Myo1c-3IQ-S701A or S701E. The “# of washes” indicates number of times the agarose beads were washed post pull-down. (Top panel) The pull-downed Myo1c-3IQ was detected by western blot using anti-Flag antibody. (Bottom panel) The GST-14-3-3β was detected by Coomassie staining. (C) Streptavidin-bead mediated pull-down of 0.5 μM Myo1c-3IQ S701A and S701E treated with TFP in the presence of 1.25 μM 14-3-3β.

**Figure 4:** Determination of the binding stoichiometry of IQ peptides and 14-3-3β. (A) Steady-state fluorescence emission spectra of 14-3-3β with IQ1 peptide (λex = 295 nm). The fluorescence emission spectra of 3.5 μM 14-3-3β dimer with 5 μM IQ1 peptide is shown in panel left. The corrected fluorescence spectra of IQ1 peptide was from subtracting the contribution of the spectra of samples contains only 3.5 μM 14-3-3β (A-B) as shown in panel right. (B) 5 μM IQ peptides (IQ1-WT and IQ1-pS701) were titrated with 0-10 μM 14-3-3β. Values (350 nm) are mean ± S.D. from 3 independent assays. Linear regression of the non-saturated and the saturated parts of the data points reveals saturation of IQ peptides at ~2.5 μM 14-3-3β dimer.

**Figure 5:** 14-3-3β does not affect Myo1c motor function. (A) ATPase activity of Myo1c-3IQ WT in the presence of 14-3-3β. Steady-state ATPase activity (37 °C) was measured in KMg25 containing 50 μM free Ca²⁺ using the NADH-coupled assay. The values are mean ± S.D. from 3 independent assays. (B) Determination of in vitro actin gliding velocity of Myo1c-3IQ with 0-15 μM of 14-3-3β in the presence of EGTA. The inhibited motility was not rescued for Myo1c-3IQ WT with 10 μM of 14-3-3β in the presence of 20 μM free Ca²⁺.
**Figure 1**

![Diagram of Myo1c-1IQ with CaM](image)

---

**A**

Myo1c-1IQ with CaM

MD-1IQ

S701

CaM

---

**B**

|        | EGTA | 40 μM Ca²⁺ |
|--------|------|------------|
| Myo1c-3IQ | ++   | +          |
| 14-3-3β  | -    | +          |
| MgATP    | -    | +          |

---

Myo1c-3IQ

14-3-3β

CaM

---

**Figure 1**

![Diagram of Myo1c-FL](image)

Motor Domain

R695

R766

R1027

Motor Domain

C767

AVI-Flag tag

M1

M1

Myo1c-1IQ with CaM

S701

CaM

---

Myo1c-3IQ
Figure 2
Figure 3
Figure 4

Fluorescence Intensity (x10^3)

A: IQ1-WT + 14-3-3β
B: 14-3-3β

Normalized Fluorescence Intensity

IQ1-WT: RRQSLATKIAWRGFHRQKFL
IQ1-pS: RRQXLATKIAWRGFHRQKFL
where X = phosphoserine.
Figure 5

A

![Graph A](image)

ATPase activity (s\(^{-1}\))

14-3-3β (μM)

0 0.25 1.25 3.75 7.5

0 1 2 3 4 5

B

![Graph B](image)

Gliding Speed (nm/s)

14-3-3β (μM)

0 0.5 2.5 5 10 15 20 50 100

0 0.5 2.5 5 10 15

WT S701E S701A

+2 μM Ca\(^{2+}\)
The regulatory protein 14-3-3β binds to the IQ motifs of myosin-IC independently of phosphorylation
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