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CIB1 and CaBP1 bind to the myo1c regulatory domain

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Abstract

Myo1c is a member of the myosin-I family that binds phosphoinositides and links the actin cytoskeleton to cellular membranes. Recent investigations suggest that targeting of myo1c to some subcellular regions requires the binding of an unknown protein to the IQ motifs in the myo1c regulatory domain. We identify two myristoylated proteins that bind the myo1c regulatory domain: calcium-binding protein 1 (CaBP1) and calcium- and integrin-binding-protein-1 (CIB1). CIB1 and CaBP1 interact with myo1c *in vivo* as determined by pull-down experiments and fluorescence microscopy where the endogenously expressed proteins show extensive cellular colocalization with myo1c. CIB1 and CaBP1 bind to the myo1c IQ motifs in the regulatory domain where they compete with calmodulin for binding. CaBP1 has a higher apparent affinity for myo1c than CIB1, and both proteins better compete with calmodulin in the presence of calcium. We propose that these proteins may play a role in specifying subcellular localization of myo1c.

Keywords

Myosin-I; Myo1c; IQ motif; CaBP1; CIB1; Calcium; Cell motility; Myristoylation; EF hand

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Introduction

Myosin-Is are membrane-associated members of the myosin superfamily that are found in most eukaryotic cells. They play roles in membrane dynamics, cytoskeletal structure, mechanical signal-transduction, and membrane trafficking (e.g., Bose et al. 2002; Holt et al. 2002; Ruppert et al. 1995; Tang and Ostap 2001). Morphological and biochemical studies have established the association of myosin-I isoforms with the plasma membrane, the actin-rich cell cortex, specialized membrane projections, and cytoplasmic organelles. There is overlap in the cellular distribution of myosin-I isoforms expressed in a single cell type, yet isoforms also have distinct localizations (Balish et al. 1999; Ruppert et al. 1995; Tyska et al. 2005), suggesting that myosin-Is contain information for targeting to specific compartments.

The motor and tail domains of myosin-I are required for proper subcellular localization (Ruppert et al. 1995; Tang and Ostap 2001). The motor binds to tropomyosin-free actin filaments (Tang and Ostap 2001), and the tail domain of at least one isoform binds directly to phosphoinositides (Hokanson et al. 2006; Hokanson and Ostap 2006). Recently, several proteins that bind to vertebrate myosin-I isoforms have been discovered, including sucrase isomaltase (Tyska and Mooseker 2004), PHR1 (Etournay et al. 2005), cadherin 23 (Siemens et al. 2004), and NEMO (Nakamori et al. 2006). It is possible that these proteins play roles in specifying myosin-I localization, but it is also possible that myosin-I serves as an anchor to concentrate to transport these proteins to the actin-membrane interface (Jung et al. 2001; Novak and Titus 1998).

It has been proposed that the regulatory domain of myo1c, a widely expressed vertebrate myosin-I isoform, plays a role in directing myo1c subcellular localization in the presence of calcium (Cyr et al. 2002; Phillips et al. 2006). This regulatory domain contains three IQ motifs, which are consensus sequences of ~25 amino acids that bind calmodulin with high affinity (Bahler and Rhoads 2002; Houdusse et al. 1996; Manceva et al. 2007). Non-calmodulin proteins have been found to bind the IQ motifs of myosin-I of lower eukaryotes (Wang et al. 1997), myosin-V (Watanabe et al. 2005), and vertebrate myosin-X (Rogers and Strehler 2001). In this study, we identify two myristoylated calmodulin-like proteins that bind to the regulatory domain of myo1c, a widely expressed vertebrate myosin-I isoform, and we propose that these proteins play roles in specifying the subcellular localization of this myosin.

Materials and methods

Antibodies and expression vectors

A mouse monoclonal antibody that binds CIB1 (UN2) was provided by L.V. Parise (University of North Carolina; Naik et al. 1997), and a mouse monoclonal antibody (M2) that binds the tail domain of myo1c was provided by P G. Gillespie (Wagner et al. 1992). A goat polyclonal antibody to CaBP1 was purchased from Abcam, Inc. (Boston, MA).

GFP-tagged mouse myo1c-tail constructs were created as described (Hokanson et al. 2006) in pEGFP-C1 (Clontech, Mountain View, CA). Expression constructs contain a GFP, the myo1c-tail domain, and the first three IQ motifs, the second two IQ motifs, the third IQ motif, and no IQ motifs.

Vectors for the expression of GST-calcium- and integrin-binding protein (GST-CIB1), GST-calcium-binding protein-1 (GST-CaBP1), GST-neuronal-calcium-sensor-1 (GST-NCS-1), GST-calsenilin, and GST-sorcin were obtained as described (White et al. 2006). Plasmids for the expression of untagged CIB1 and CaBP1 were constructed in pMW172 (Way et al. 1990).

Protein purification

GST fusion proteins were expressed and purified as described (White et al. 2006). Untagged CIB1 and CaBP1 were expressed in BL21(DE3)pLysS cells. Cell pellets from 1 l cultures were suspended in 30 ml lysis buffer (50 mM Tris pH 7.5, 5 mM DTT, 1 mM $CaCl_2$, 100 mM NaCl, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin and 1 mM PMSF), lysed by sonication, and centrifuged at 27,000*g* for 30 min. The supernatant was loaded on a 10 ml phenyl-sepharose CL-4B column equilibrated with lysis buffer. The column was washed sequentially with 30 ml of Ca^{2+} buffer (50 mM Tris, pH 7.5, 5 mM DTT, 1 mM $CaCl_2$), 30 ml Ca^{2+} buffer plus 100 mM NaCl, and again in 30 ml Ca^{2+} buffer. CIB1 and CaBP1 were eluted with 90 ml of EDTA buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 5 mM DTT) and concentrated. Concentrated protein was loaded onto a MonoQ column (Amersham Biosciences) equilibrated in 20 mM Tris pH 7.5, 1 mM EGTA 1 mM DTT and eluted with a linear 0-1M NaCl gradient. Fractions containing CIB1 or CaBP1 were combined, $CaCl_2$ was added to 7 mM, and the fractions were loaded on a 2 ml phenyl-sepharose CL-4B column equilibrated in 20 mM Tris pH 7.5, 1 mM EGTA 1 mM DTT, and 300 mM NaCl. The column was washed with 60 ml Ca^{2+} buffer. Pure CIB1 or CaBP1 was eluted with 20 ml of EDTA buffer. CIB1 and CaBP1 were dialyzed into appropriate assay buffers.

A mouse myo1c-tail construct (NM_008659) containing residues 690–1028 (myo1c-tail^{IQ1-3}), which consists of an N-terminal his-tag for purification, three calmodulin-binding IQ motifs, and the tail domain, was co-expressed with calmodulin as described (Tang et al. 2002). The purified protein contains three bound calmodulins.

Cell culture, transfection, and immunofluorescence

COS-7 cells were grown in DMEM with 10% FBS and transfected with GFP constructs using Lipofectamine 2000 (Invitrogen). Transfected cells were harvested for pull-down and immunoprecipitation assays after incubation at 37°C for 34–36 h. PC12 cells were grown in RPMI 1640 with 10% heat-inactivated horse serum, and 5% FBS (Invitrogen). Cells were differentiated in RPMI 1640 with 2% heated-inactivated horse serum, 1% FBS and 100 ng/ml NGF for 5–7 days. PC12 cells for microscopy were plated on coverslips coated with 0.01% poly-L-Lysine (Sigma).

COS-7 cells were fixed for immunofluorescence in freshly prepared 4% formaldehyde for 15 min at room temperature, and permeabilized in PBS with 0.1% saponin, and 0.5% BSA at room temperature for 30 min. Fixed cells were sequentially labeled with UN2 and a FITC-labeled anti-mouse secondary antibody. The cells were then fixed again in 4% PFA at room temperature for 5 min to prevent dissociation of UN2 and the secondary antibody. UN2-labeled cells were then labeled with M2 antibody conjugated directly to Cy3 monofunctional dye (GE Life Sciences). PC12 cells were double labeled with antibodies M2 and goat anti-CaBP1. Cells were then incubated with Cy3 goat anti-mouse and Alexa 488 rabbit anti-goat IgG. Fluorescence was visualized with a Leica epifluorescence microscope.

GST pull down and immunoprecipitation

Rat lung, COS-7, and PC12 lysates were prepared in lysis buffer (10 mM Hepes, pH 7.0, 200 mM NaCl, 1 mM DTT, 1 mM ATP, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 1% Triton X100, 1 mM PMSF, 0.01 mg/ml aprotinin and 0.01 mg/ml leupeptin). Lysates were centrifuged at 27,000*g* for 30 min to remove insoluble material.

Pull-down assays were performed by incubating lysate at 4°C for 2 h with GST-fusion proteins bound to glutathione-beads. The beads were centrifuged to remove the supernatant, washed 3× in lysis buffer and eluted in gel sample buffer. Immunoprecipitations were performed by incubating COS-7 lysate and 1 μg UN2 antibody at 4°C for 4–12 h, followed by incubation

with protein-G-sepharose at 4°C for 1 h. The beads were washed, resuspended in SDS sample buffer. Normal mouse IgG was used as negative control.

Ca^{2+}-dependent binding assays

COS-7 and PC12 cells were collected and lysed in 20 mM Hepes, pH 7.0, 200 mM NaCl, 1 mM DTT, 2 mM K^+-ATP, 2 mM $MgCl_2$, 1% Triton X100, 1 mM PMSF, 0.01 mg/ml aprotinin and 0.01 mg/ml leupeptin. Aliquots (500 μl) of lysate were adjusted to appropriate free calcium concentrations by using EDTA, EGTA, and HEDTA buffers as necessary. Free calcium concentrations were confirmed using fluorescent indicator dyes (Foskett et al. 2007). GST-CIB1 or GST-CaBP1 (1 μM) bound to glutathione beads were incubated with lysates at 4°C for 1 h. The beads were centrifuged, washed, and eluted as above.

In-vitro competition binding assays

Myo1c-tail^{IQ1-3} (1 μM) with bound calmodulin was mixed with 1 μM free calmodulin, CIB1 or CaBP1, and 1% BSA in binding buffer (10 mM Hepes, pH 7.0, 100 mM NaCl, 1 mM EGTA and 1 mM BME) and incubated for 30 min at room temperature in the absence or presence of 1.5 μM free calcium. Ni-NTA (10 μl) beads were added to the protein mixture to bind the his-tagged myo1c-tail^{IQ1-3} and sedimented. The beads were washed 3 times in binding buffer, and the myo1c-tail^{IQ1-3} with bound calmodulin and CIB1 or CaBP1 was eluted in 250 mM Imidazole, pH 7.5, 100 mM NaCl, 25 mM Tris, 1 mM EGTA and 1 mM BME. Myo1c-tail^{IQ1-3}, calmodulin, and CIB1 were resolved by SDS-PAGE. The concentration of calmodulin and CIB1 were determined by monitoring the fluorescence intensity of protein bands on gels stained with Sypro-red (Molecular Probes) with a Typhoon 8600 variable mode imager. CaBP1 binding was determined by quantitative western blots. Concentrations were determined by comparing the intensities with CIB or CaBP1 standards resolved on the same gel.

Results and discussion

CIB1 and CaBP1 interact with cellular myo1c

Recent reports propose that subcellular targeting of myo1c depends, in part, on the binding of unknown proteins to the calmodulin-binding IQ motifs in the myo1c regulatory domain (Cyr et al. 2002; Phillips et al. 2006). Reasoning that these unknown proteins have properties similar to calmodulin, we surveyed five proteins from different EF-hand containing calcium-binding families for association with myo1c. Pull down experiments from whole-lung lysates were performed to probe for the association of endogenous myo1c with GST-chimeras of sorcin (Van der Bliek et al. 1986), CaBP1 (Haeseleer et al. 2000), CIB1; (Naik et al. 1997), calsenilin (Burgoyne and Weiss 2001), and NCS-1 (De Castro et al. 1995) (Fig. 1a) Lung lysates were used because of the high level of myo1c expression in this tissue (Ruppert et al. 1995). We found that GST-CIB1 and GST-CaBP1 pull down myo1c from cell extract, with GST-CaBP1 pulling down significantly more myo1c based on the intensity of the protein band.

CIB1, also called calmyrin, was originally discovered as a protein that binds to the cytoplasmic domain of the αIIb-integrin subunit (Naik et al. 1997). CIB1 contains two EF-hand domains and is myristoylated, which is important for its cellular localization (Stabler et al. 1999). CIB1 is expressed in many different tissues and cell types and has been shown to bind to eukaryotic DNA-dependent protein kinase (Wu and Lieber 1997), presenilin-2 (Stabler et al. 1999), Rac3 (Haataja et al. 2002), the $InsP_3$ receptor (White et al. 2006), and Pax3 (Hollenbach et al. 2002). CaBP1 is a calmodulin-like protein (~56% similarity) that is expressed in the brain and retina (Haeseleer et al. 2000). It contains three functional EF-hand domains and is myristoylated. Unlike calmodulin, CaBP1 acts as a high affinity agonist of the inositol-triphosphate-receptor-Ca^{2+}-release channel (Yang et al. 2002). CaBP1 also regulates Ca(v)2.1 channels in a manner that is different from calmodulin (Lee et al. 2002).

We were able to immunoprecipitate endogenous myo1c from COS-7 cell extracts using an anti-CIB1 antibody, thus demonstrating an *in vivo* association between CIB1 and myo1c (Fig. 1b). Control immunoprecipitations using normal mouse IgG did not pull down CIB1 or myo1c. We were also able to immunoprecipitate CaBP1 from whole brain extracts using an anti-myo1c antibody (not shown).

Immunofluorescence micrographs of COS-7 cells labeled with anti-CIB1 and anti-myo1c antibodies show significant colocalization of the proteins within the cell margin, membrane ruffles, and punctate regions on the cell membrane (Fig. 2, top). However, not all CIB1 colocalizes with myo1c. Most notably, CIB1 is concentrated on structures that may be stress fibers or actin cables, which are regions that are known to exclude myosin-I isoforms (Fig. 2, top, arrows). The lack of complete colocalization is not surprising given the large number of proteins in which CIB1 has been proposed to interact.

CaBP1 and myo1c are endogenously expressed in PC12 cells (Haynes et al. 2004; Wagner et al. 1992), and immunofluorescence micrographs of cells labeled with anti-CaBP1 and anti-myo1c antibodies show significant colocalization of the two antibodies within the cell margin and punctate regions on the cell membrane (Fig. 2, bottom). As with CIB1, not all CaBP1 colocalizes with myo1c, possibly reflecting the multiple functions of this protein.

The association of GST-CIB1 and GST-CaBP1 with myo1c is calcium dependent (Fig. 1d). Calcium and calcium-buffers were added to COS-7 cell lysates to attain 0.01–10 μM free calcium concentrations. Endogenous myo1c was pulled down by GST-CIB1 and GST-CaBP1 at the lowest free calcium concentration tested (0.01 μM), and these associations were increased significantly at free calcium concentrations >0.8 μM (Fig. 1d).

CIB1 and CaBP1 bind directly to the myo1c regulatory domain

GST-CIB1 and GST-CaBP1 bind directly to purified myo1c^{IQ1-3} in pull down experiments, indicating that the interactions are not mediated by adaptor proteins or cell membranes (Fig. 1c). Control experiments show that myo1c-tail^{IQ1-3} does not bind to GST alone.

To identify the region of myo1c that binds CIB1 and CaBP1, we performed pull down experiments with GFP-myo1c constructs serially truncated from the first IQ motif to the tail domain (Fig. 3a, b). Removal of the first two IQ motifs in the regulatory domain did not affect the ability of GST-CIB1 to pull down myo1c, but removal of the third IQ motif (IQ3) eliminated binding (Fig. 3b, center). Removal of the three IQ motifs also greatly reduced binding of GST-CaBP1 to myo1c (Fig. 3b, right). However, removal of an additional 23 amino acids was required to eliminate binding completely, indicating that a portion of the binding site is outside of the well defined IQ motifs in a region proposed to contain a poorly conserved IQ motif (Gillespie and Cyr 2002).

Competition binding experiments with purified proteins show that CIB1 does not bind to myo1c^{IQ1-3} that contains three bound calmodulin in the absence of calcium at total CIB1 concentrations below 20 μM (Fig. 4a). However, in the presence of 10 μM free calcium, CIB1 binds to myo1c^{IQ1-3} at a ratio of 1:1 and displaces one of the three bound calmodulins. Since myo1c^{IQ1-3} binds only one CIB1, and GST-CIB1 pulls down myo1c construct containing only IQ3 (Fig. 3), it is likely that CIB1 competes with calmodulin for binding to IQ3.

In the absence of calcium and in the presence of a high concentration of CaBP1 (20 μM), CaBP1 binds myo1ctail^{IQ1-3} at a ratio of 1:1 without displacing a calmodulin (Fig. 4b). This binding might be mediated by association with regions outside of the well defined IQ motifs as stated above (Gillespie and Cyr 2002). It is also possible that CaBP1 competes for IQ binding

with a single, loosely-bound lobe of calmodulin. Single-lobe light-chain binding has been proposed for Mlc1p binding to the IQ motifs of myosin-V (Terrak et al. 2005).

In the presence of 10 μM free calcium, CaBP1 binding appears to be much tighter. CaBP1 (2 μM) binds myo1ctail^{IQ1-3} at a ratio of 1:1 while displacing a calmodulin, indicating that CaBP1 binds to one of the IQ motifs with the same or tighter affinity than calmodulin. Increasing the CaBP1 concentration to 20 μM in the presence of calcium results in the binding of three CaBP1 molecules to myo1ctail^{IQ1-3} and displacement of the remaining bound calmodulins (Fig. 4b). Therefore, CaBP1 competes for calmodulin binding to the regulatory domain in the presence of calcium, and CaBP1 has a higher apparent affinity than CIB1 for myo1c-tail^{IQ1-3} in the presence of calmodulin. It remains to be determined if these increased affinities in the presence of calcium are due to weakening of the calmodulin affinity, or to calcium-dependent increases in the CIB1 and CaBP1 affinities.

Conclusions

We identify CIB1 and CaBP1 as two EF-hand containing proteins that bind the IQ motifs in myo1c and are able to effectively compete with calmodulin for binding in the presence of calcium. GST-CaBP1 and GST-CIB1 are able to pull myo1c out of lung tissue lysates, indicating that they can compete with other cellular factors for myo1c binding. Additionally, immunoprecipitations and immunofluorescence microscopy suggest *in vivo* associations of endogenous CIB1 and CaBP1 with myo1c.

We propose that CIB1 and CaBP1 play roles in regulating the localization and dynamics of cellular myosin-I. Myo1c binds directly to the membrane via a phosphoinositide binding site in its tail domain (Hokanson et al. 2006; Hokanson and Ostap 2006). Since CIB1 and CaBP1 are myristoylated, they may provide a further calcium-dependent membrane attachment for a subset of myo1c molecules. It is also possible that CIB1 and CaBP1 link myo1c to other membrane associated proteins that require association with a membrane protein. Further experiments are required to probe the motile and cellular consequences of the association of CIB1 and CaBP1 to myo1c and to determine if other calmodulin-like proteins interact with the myosin-I regulatory domain.

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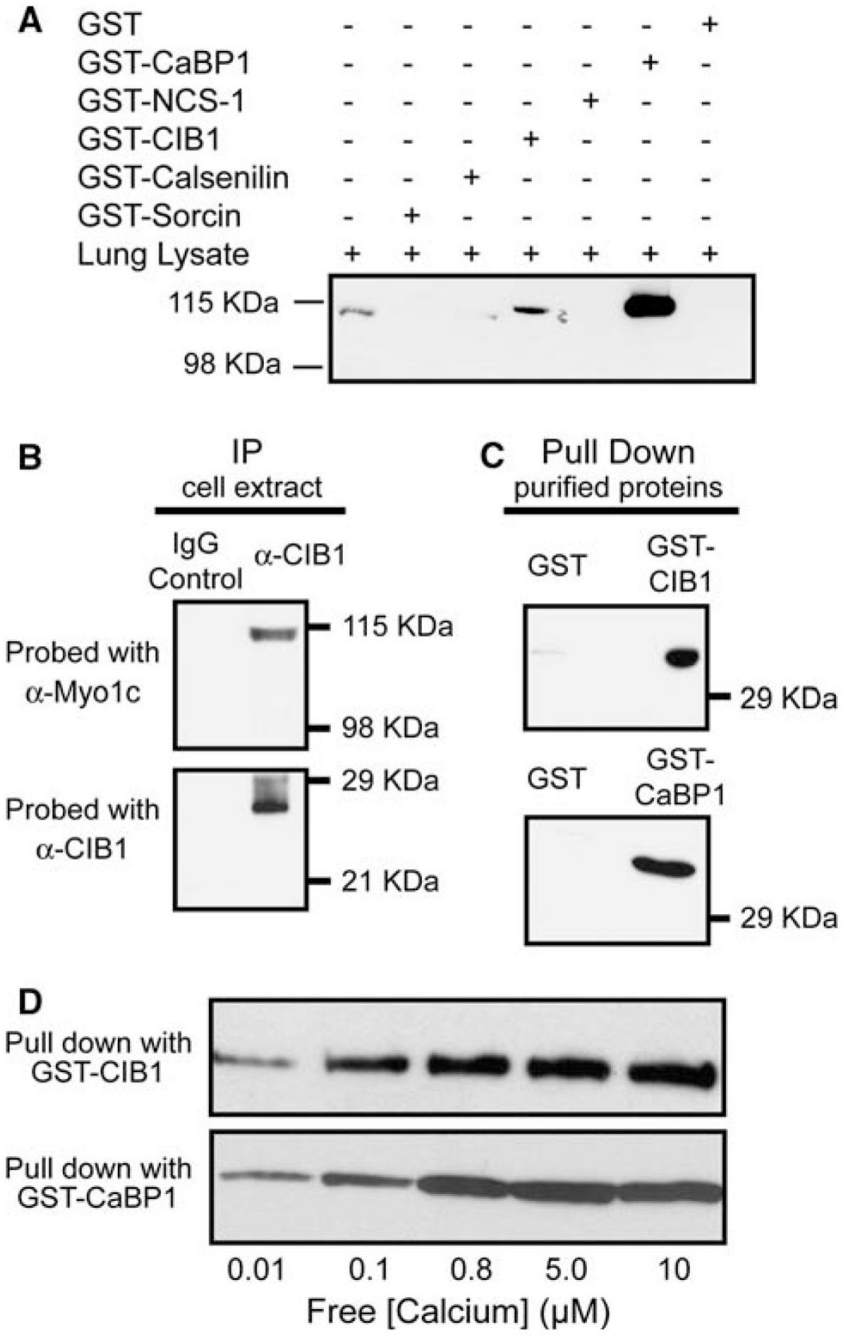

Fig. 1.

Myo1c interacts with CIB1 in vivo and in vitro. (**a**) Western blot probed with anti-myo1c antibody showing endogenously expressed myo1c is pulled out of lung extract by GST-CIB1 and GST-CaBP1. Equal concentrations of GST-fusion proteins were used in the pull-down experiments. (**b**) Co-immunoprecipitation of CIB1 and myo1c from COS-7 cell extracts. CIB1 and myo1c did not co-precipitate with normal mouse IgG in control experiments. (**c**) Pull down experiment showing purified myo1c-tail^{IQ1-3} interacts with GST-CIB1 and GST-CaBP1 bound to glutathione beads, but not with GST bound to beads. (**d**) Western blot probed with anti-myo1c antibody showing the calcium dependence of endogenously expressed myo1c pulled

out of COS-7 cell extract by (top) GST-CIB1 and (bottom) GST-CaBP1. Free calcium concentrations are reported

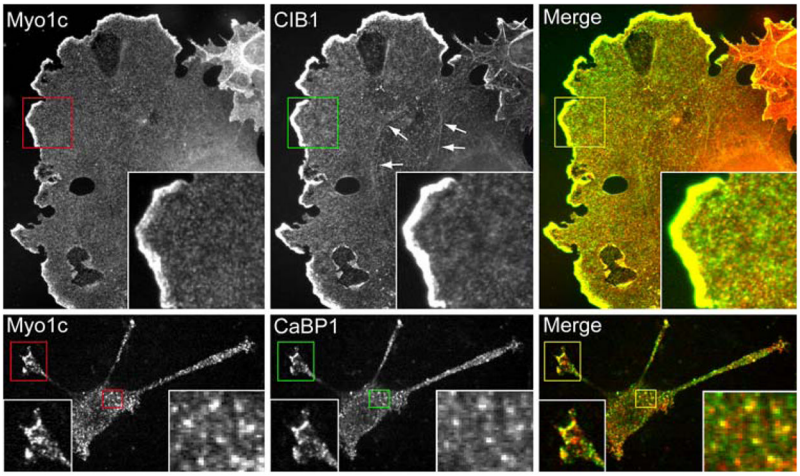

Fig. 2.

Endogenously expressed myo1c partially co-localizes in CIB1 and CaBP1 cells. (*Top*) Fluorescence micrographs of a fixed and permeabilized COS-7 cell labeled with (*left*) anti-myo1c and (*center*) anti-CIB1 antibodies. Arrows show unique localization of CIB1 to cytoplasmic fibers (*Bottom*). Fluorescence micrographs of PC12 cells labeled with (*left*) anti-myo1c and (*center*) anti-CaBP1 antibodies. Merged images show colocalization of (*red*) myo1c and (*green*) CaBP1 or CIB. Insets show expanded regions to highlight regions of colocalization

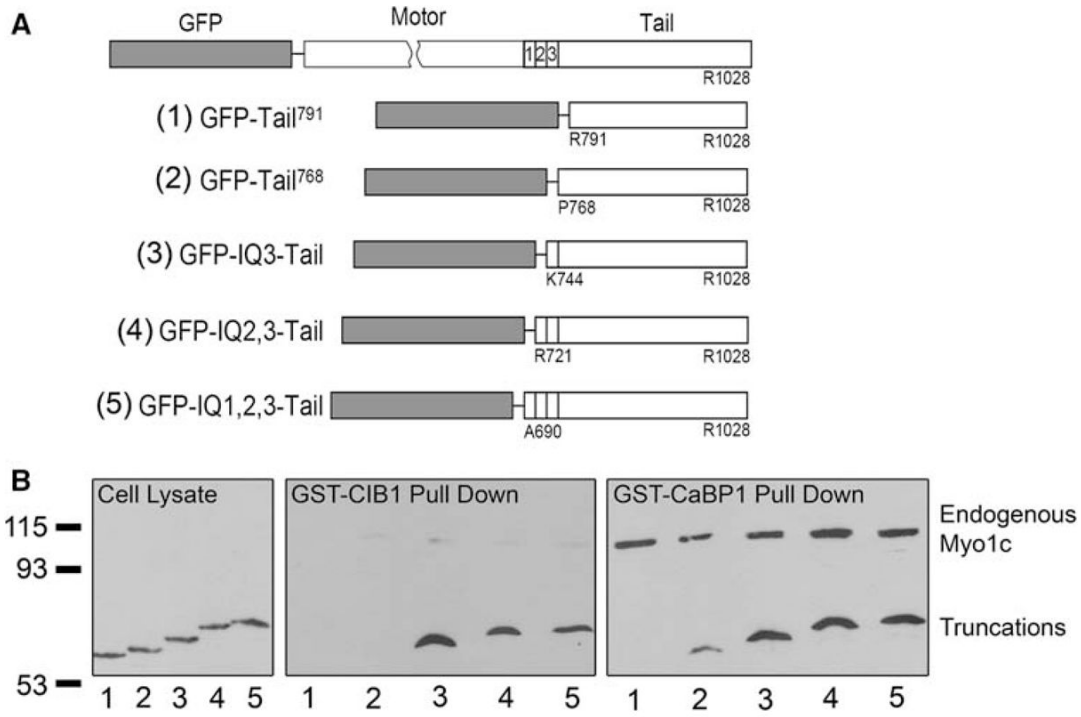

Fig. 3.

GST-CIB1 and GST-CaBP1 binds to the IQ motifs of myo1c. (**a**) Diagram showing constructs for the expression of truncated GFP-myo1c proteins in COS-7 cells. (**b**, *left*) Western blot probed with anti-myo1c antibody showing expression of truncations in COS-7 cells. Lanes are numbered to correspond to the constructs in (**a**). Western blots probed with anti-myo1c antibody showing GFP-myo1c constructs that are pulled down by (**b**, *center*) GST-CIB1 and (**b**, *right*) GST-CaBP1. Note that GST-CIB1 and GST-CaBP1 constructs also pull down endogenous myo1c from COS-7 cell extracts

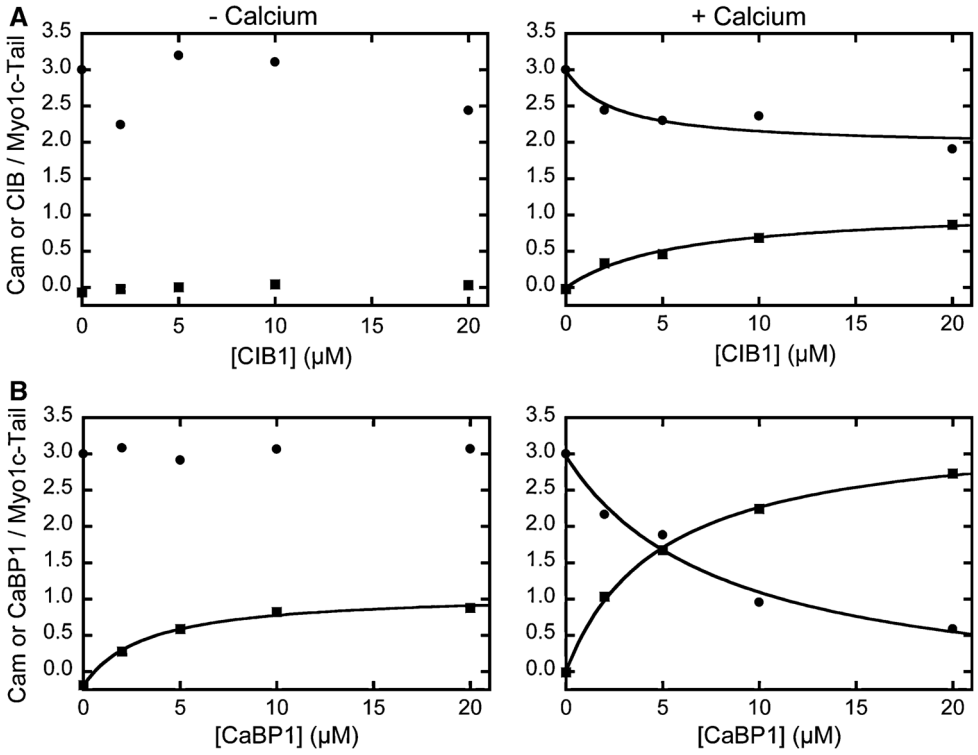

Fig. 4.

Stoichiometry of CIB1 and CaBP1 binding to myo1c-tail^{IQ1-3}. Binding of 0–20 μM untagged (*top*) CIB1 and (*bottom*) CaBP1 to 1 μM myo1c-tail^{IQ1-3} in the (*left*) absence and (*right*) presence of 1.5 μM free calcium. Three calmodulins are bound to each myo1c-tail^{IQ1-3} and solutions contained 1 μM free calmodulin, so the total calmodulin concentration before sedimentation was 4 μM. The molar ratios of (●) calmodulin and (■) CIB1 or CaBP1 to myo1c^{IQ1-3} are plotted. Each point is the average of two experiments