Identification of Acan125 as a Myosin-I-binding Protein Present with Myosin-I on Cellular Organelles of Acanthamoeba

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We have discovered the first protein to bind to a non-filamentous myosin, aside from actin. This protein, Acan125, is a 125-kDa protein from Acanthamoeba that associates with the SH3 domain of Acanthamoeba myosin-I and not the SH3 domain of human fodrin. Antibodies raised against Acan125 recognize a single protein of 125 kDa from a whole cell lysate of Acanthamoeba; antibodies to myosin-I (M1.7 and M1.8) do not recognize Acan125 on the same blot. Double labeling of Acanthamoeba shows Acan125 and myosin-I to be present on the same intracellular organelle, most likely amoebastomes. Immunoprecipitation with either anti-myosin-I or anti-Acan125 antibodies coprecipitates both Acan125 and myosin-I from a lysate of Acanthamoeba, demonstrating that Acan125 interacts with native myosin-I.

Binding through the Src homology domain, SH3, is a recognized means of linking signal transduction proteins (1–3), but a function has not been ascribed to the SH3 domain of the cytoskeletal protein myosin-I. The isoforms of myosin-I that contain an SH3 domain include myosin-Is from Acanthamoeba (4), Dictyostelium (5), Saccharomyces (6), rat (7), and human (8). In the known myosin-I sequences, the SH3 domain invariably resides in tandem with one or two proline-rich domains (5, 9); a proline-rich sequence of 3BP1 has been identified as a motif that binds to the SH3 domain of Abl (10). An interaction has not been demonstrated between SH3 and proline-rich domains of myosin-I, but proline-rich domains of myosin-I have been shown to interact with actin (11–13). These results suggested to us that the SH3 domain of myosin-I might be available for interaction with another protein.

The proposal of proteins that interact with myosin-I is rooted in efforts to reconcile reconstitution results with cellular localization studies. In vitro binding (11, 14, 15) and motility (16) assays are consistent with myosin-I acting mechanically on the surface of any membrane containing the ubiquitous phospholipid phosphatidyserine. But immunostaining shows myosin-I to be excluded from most cell membranes and to be concentrated at the leading edges of migrating cells (17, 18) and on selected organelles (19). The contractile vacuole of Acanthamoeba has been demonstrated to selectively bind the myosin-IC isoform (20). Myosin-IA and myosin-IB were found, using immunogold, to be associated along one side of fractionated membranes (21), as though bound to proteins.

Myosin-I could associate with other proteins on membrane surfaces via interactions with SH3. SH3 domains have been shown to mediate specific associations between SH3-containing proteins and various binding partners, including phosphatidylinositol 3-kinase (22–25), p21 protein (26–28), and dynamin (29–31). In each of these studies, bacterially expressed fusion proteins of SH3 domains were used as affinity ligands to selectively extract the binding partner from a cell lysate. Selectivity may be dictated by the structures of both the SH3 domain and its binding partner (32, 33). Thus, binding partners for myosin-I might be identified by their association with the SH3 domain of myosin-I.

We report here one protein from Acanthamoeba, Acan125, which binds to the SH3 domain of myosin-I and colocalizes with myosin-I on cellular organelles.

EXPERIMENTAL PROCEDURES

Preparation of GST Fusion Proteins—GST (glutathione S-transferase) fusion protein constructs were prepared from polymerase chain reaction products of SH3 domains of Acanthamoeba myosin-I and human nonerythroid spectrin (fodrin). The DNA corresponding to amino acids 981-1031 of Acanthamoeba myosin-I was amplified from the plasmid p45L (gift from T. D. Pollard, Johns Hopkins University). The polymerase chain reaction primers were 5'-CCCGATCCCGCGCT-GCCGTGTA (sense) and 5'-GAAGATTCTACCAGTAGA (antisense), which included BamHI and EcoRI sites in the sense and antisense primers, respectively. We subcloned the product into Bluescript (Stratagene, La Jolla, CA) and verified the nucleotide sequence. The bona fide primers were used to create the expected restriction enzyme cloning sites of the bacterial expression vector pGEX-2TK (Pharmacia, Piscataway, NJ). We received the sequence verified DNA for the SH3 domain of human nonerythroid spectrin (fodrin) corresponding to residues 974-1030 in the pGEX vector (gift from J. P. Albanesi, University of Texas Southwestern Medical Center). Both constructs and the empty vector were transformed into Escherichia coli DH5α cells for expression.

GST fusion proteins were expressed in bacteria and purified by affinity chromatography on glutathione-agarose (Pharmacia Biotech Inc.). The protein was eluted with glutathione, dialyzed, and stored at 4°C with NaN3 added. Proteins were stable as determined by SDS-PAGE during the time of the experiments.

Affinity Chromatography of Acanthamoeba Lysate—Acanthamoeba (–2 g) were harvested, resuspended in 2 ml of 0.5 × TBS (1 × TBS: 25 mM Tris-HCl, pH 7.4, 3.7 mM KCl, 138 mM NaCl) supplemented with 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 10 μg/ml pepstatin A, 100 units/ml aprotinin, 1 mg/ml disopropyl fluorophosphate, and 1 μg dithiothreitol, and lysed with 10 strokes in a Dounce homogenizer. The lysate was cleared by centrifugation at 400,000 × g for 10 min and the supernatant passed through a 0.45-μm filter. A 0.5-ml volume of the filtrate was mixed with 0.2 ml of glutathione beads coupled with 250 μg of fusion protein for 15 min at 4°C. The beads were washed five times with 1.5 ml of 0.5 × TBS and then eluted with 0.2 ml of 5 × TBS. The proteins were separated on 10% SDS-PAGE and stained with Coomassie Blue.

Antibodies—Polyclonal antibodies were raised against Acan125 in rabbits. Rabbits were injected with 10–30 μg of Acan125 cut from an SDS-PAGE gel (8 × 1 × 0.1 cm) and mixed 1:1 with TiterMax adjuvant (Vaccol, Norcross, GA). The rabbits were boosted twice with the same antigen and once with the excised Acan125 gel band in phosphate-buffered saline (12 ml 0.9% NaCl, pH 7.4, 138 mM NaCl, 2.7 mM KCl).
Control serum was collected before immunizing the rabbit. Antibodies to myosin were obtained from other laboratories. From T. D. Pollard (J ohn Hopkins University), we received mouse monoclonal antibodies, M1.7 and M1.8, to Acanthamoeba myosin-I, and M2.42, to Acanthamoeba myosin-II. From I. C. Baines (National Institutes of Health), we received rabbit polyclonal antibodies to Acanthamoeba myosin-I. These antibodies have been characterized elsewhere (20). Specificity of the anti-Acan125 antibodies was determined by Western blot. A whole cell lysate of Acanthamoeba was separated on SDS-PAGE and a strip of the gel was cut and stained with Coomassie Blue. The remainder of the gel was transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and used for a Surf Blot (Idea Scientific, Minneapolis, MN) in which sealed wells of solution containing the antibodies overlay the filter. This creates lanes for antibody reactivity on a continuous blotting surface and eliminates the problems associated with aligning strips cut from a blot. Peroxidase coupled secondary antibodies to mouse or rabbit IgG (American Qualex, La Mirada, CA) were added to the wells, and the peroxidase reaction was developed on the whole blotting surface with chemiluminescent reagent (ECL, Amersham Corp.).

Immunoprecipitations of Acan125 and Myosin-I—Lysate (0.7 ml) of Acanthamoeba (described above) was mixed with 0.7 ml of 0.5 M TBS and with 20 µl of protein A beads alone (Pharmacia Biotech Inc. and Sigma) or an equal volume of protein A beads coupled with ~7 µg of IgG. The suspensions were incubated 60 min at 4°C with constant gentle mixing. The beads were recovered by gentle centrifugation (820 × g for 1 min) and washed five times with 1 ml of 0.5 M TBS. Proteins were eluted from the beads in 100 µl of 5 M guanidine hydrochloride, separated on SDS-PAGE, and transferred to a PVDF membrane. The strips were incubated with the primary antibodies indicated and developed using peroxidase-coupled secondary antibodies and chemiluminescent reagent.

Immunostaining of Acanthamoeba—M1.7 and anti-Acan125 immune serum in phosphate-buffered saline were directly labeled with fluorescein isothiocyanate (Molecular Probes, Eugene, OR) and purified on G-25 (Pharmacia Biotech Inc. and Sigma) or an equal volume of protein A beads coupled with 7 µg of IgG. The suspensions were incubated 60 min at 4°C with constant gentle mixing. The beads were recovered by gentle centrifugation (820 × g for 1 min) and washed five times with 1 ml of 0.5 M TBS. Proteins were eluted from the beads in 100 µl of 5 M guanidine hydrochloride, separated on SDS-PAGE, and transferred to a PVDF membrane. The strips were incubated with the primary antibodies indicated and developed using peroxidase-coupled secondary antibodies and chemiluminescent reagent.

RESULTS AND DISCUSSION

To isolate SH3-binding proteins, affinity beads was prepared with the ligand being the SH3 domain of Acanthamoeba myosin-IC (SH3AmyoIC) expressed as a fusion protein of GST. The fusion proteins GST, GST-SH3AmyoIC, or GST-SH3MyoIC (human fodrin SH3-negative control) were immobilized on glutathione beads and then mixed with a lysate of Acanthamoeba; best results were obtained using the lysate clarified by centrifugation at 400,000 × g. High salt was used to elute amoeba proteins without disrupting the association between fusion proteins and the beads. The same proteins that eluted from all beads containing fusion proteins were shown to be nonspecifically bound to the beads alone (Fig. 1). Specifically bound proteins were detected exclusively in the high salt wash from the beads containing GST-SH3AmyoIC (Fig. 1, a–d). Subsequent release of all proteins from the beads with SDS revealed that the same amount of fusion protein was bound to all beads and that no other proteins were specifically associated with SH3AmyoIC (data not shown). Thus, four proteins, Acan125,2 Acan62, Acan55, and Acan47 (Fig. 1), are reversibly associated with SH3AmyoIC.

Acan125 was selected for further study because it was the least likely to be a proteolytic fragment, the most abundant, and the most well separated from other proteins on the gel. A large preparation yielded ~400 µg of the Acan125, which was sufficient for the production of the antibodies and the attainment of microsequence data.

Given that the SH3 of Src binds a proline-rich motif (10) and that a proline-rich region is present in the sequence of SH3-containing isoforms of myosin-I (5), the SH3 domain of myosin-I could interact with the proline-rich region of another, possibly uncharacterized, myosin-I. To determine if Acan125 is a myosin-I, we transferred Acan125 to a PVDF membrane for microsequence determination. Attempts to obtain sequence directly failed, indicating that the N terminus is blocked. The bound Acan125 was digested with endoproteinase lysC and the peptides separated by high performance liquid chromatography. Several peptide sequences were obtained (data not shown) and searches of protein data bases (PIR 42 and Swiss-Prot 30) did not reveal a match to a known myosin-I.

Polyclonal antibodies were raised in rabbits immunized with Acan125 that was excised from SDS-PAGE. A single protein of 125 kDa was recognized on a Western blot of Acanthamoeba whole cell lysate using anti-Acan125 antibodies (Fig. 2); immune serum, protein A purified immune serum, and blot purified antibody gave identical results. On the same blot, mouse monoclonal antibodies to myosin-I (M1.7 and M1.8) recognized multiple bands of proteins (Fig. 2). Previously, M1.7 was shown to react with myosin-I, myosin-IC, and myosin-II; M1.8 was shown to react with the same proteins plus myosin-IB (34). The M1.7- and M1.8-reactive proteins on the blot of Acanthamoeba lysate (Fig. 2) correlate with the sizes of myosins, 130–190 kDa. The fact that the broad spectrum myosin antibodies M1.7 and M1.8 recognize proteins larger than the anti-Acan125-reactive protein is consistent with the assertion that Acan125 is not a myosin-I.

To demonstrate that Acan125 interacts with myosin-I, we immunoprecipitated the complex from a lysate of Acanthamoeba using M1.7 and M1.8. The myosin-I antibody M2.42 was used as a control. Immunoprecipitations with M1.7 and M1.8, but not with M2.42, showed a single band of reactivity to anti-Acan125 antibodies on a Western blot (Fig. 3A). Thus, Acan125 is precipitated specifically by myosin-I antibodies, indicating that a direct association between the
two proteins is likely.

To verify Acan125 association with myosin-I, myosin-I was coprecipitated with Acan125 antibodies from a lysate of Acanthamoeba. Anti-Acan125 and preimmune sera were used to form immunoprecipitates, but only the anti-Acan125 antibodies coprecipitated proteins that reacted with M1.7. The blot shows at least two bands of M1.7 reactivity in the immunoprecipitation (Fig. 3B), indicating that Acan125 interacts with more than one isoform of myosin-I. We identified one isoform, myosin-IC, in the lower band in Fig. 3B using antibodies specific for myosin-IC (data not shown); we have not yet identified a specific isoform of myosin-I in the upper band. The ability of anti-Acan125 antibodies to precipitate myosin-I isoforms from a soluble lysate suggests that complexes of Acan125 and myosin-I exist in Acanthamoeba.

To assess potential interactions in vivo, we stained Acanthamoeba cells for both myosin-I and Acan125. Myosin-I was detected using M1.7 labeled with rhodamine, and Acan125 was detected using protein A-purified anti-Acan125 antibodies labeled with fluorescein. Rhodamine-labeled M1.7 staining of Acanthamoeba was characterized by diffuse fluorescence throughout the cytoplasm excluding the interior of vacuoles, by intense fluorescence in the nuclear region, and by occasional intense fluorescence circumscribing a single round structure (Fig. 4A). The same round structure was stained by fluorescein-labeled anti-Acan125 antibodies in double-labeled cells (Fig. 4B).

Staining in the nuclear region by rhodamine-labeled M1.7 was observed in all cells (one cell is marked and two cells are unmarked in Fig. 4A). This fluorescence arises from intense staining of the nuclei that surrounds a single large unstained nucleolus (visible in the unmarked cells in Fig. 4A). The nucleoplasmic staining is absent from identically prepared cells stained with unlabeled M1.7 and a labeled secondary antibody. Although the nucleoplasmic staining appears to be an artifact of the rhodamine-labeled M1.7, this provides a convenient internal control for the present experiment. To verify Acan125 association with myosin-I, we stained Acanthamoeba cells for both myosin-I and Acan125. Myosin-I was detected using M1.7 labeled with rhodamine, and Acan125 was detected using protein A-purified anti-Acan125 antibodies labeled with fluorescein. Rhodamine-labeled M1.7 staining of Acanthamoeba was characterized by diffuse fluorescence throughout the cytoplasm excluding the interior of vacuoles, by intense fluorescence in the nuclear region, and by occasional intense fluorescence circumscribing a single round structure (Fig. 4A). The same round structure was stained by fluorescein-labeled anti-Acan125 antibodies in double-labeled cells (Fig. 4B).

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These round structures appear to be similar to structures described earlier as vacuoles in Acanthamoeba stained with M1.7 (19). But a more recent exhaustive study indicates that M1.7 stains myosin-I on tubular structures known as amoebastomes, which appear similar to vacuoles in cross-section. All amoebastomes that stained with M1.7 also stained with anti-Acan125 antibodies in our experiments. No structures were observed to be stained exclusively by anti-Acan125 antibodies, and we found no more than a single organelle per cell to be stained by both antibodies. Colocalization of Acan125- and myosin-I-reactive proteins on M1.7-stained organelles, which are probably amoebastomes, further indicates the formation of a complex of myosin-I and Acan125 in the cell.

Acan125 is presently the only protein aside from actin known to interact with myosin-I. However, we think it is likely that others will be identified including Acan62, Acan55, and Acan47, which bind myosin-I SH3 and do not react with anti-Acan125 antibodies on a blot. The SH3 domain of myosin-I may regulate or target myosin-I’s interactions with these proteins in response to cellular signals. The presence of Acan125 and myosin-I in the high speed supernatant suggest that they are both present in the cytoplasm, and the immunoprecipitation results demonstrate that Acan125 and myosin-I are competent to form a complex. The complex may be recruited to organelle surfaces where attachment could be mediated by either Acan125 or myosin-I. A role for the complex will become clearer when a function can be ascribed to Acan125.

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FIG. 3. Immunoprecipitations showing that Acan125 and myosin-I form a complex. Western blot A, separate immunoprecipitations were performed using M1.8 (lane 2), M1.7 (lane 3), M2.42 (lane 4), or no antibody (lane 5). A standard of Acan125 protein was included on the same blot (lane 1). The entire blot was probed with anti-Acan125 antibodies. Western blot B, separate immunoprecipitations were performed using no antibody (lane a), preimmune serum (lane b), anti-Acan125 antibodies (lane c), and M1.7 (lane d). The entire blot was probed with M1.7.

FIG. 4. Double-stained Acanthamoeba show the colocalization of myosin-I- and Acan125-reactive proteins. The same field shows M1.7 staining in the rhodamine channel (panel A) and anti-Acan125 staining in the fluorescein channel (panel B). Marked are amoebastomes (arrow), defined by M1.7 staining, and one example of nucleoplasmic staining (n).
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