CARMIL Is a Bona Fide Capping Protein Interactant*

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CARMIL, also known as Acan 125, is a multidomain protein that was originally identified on the basis of its interaction with the Src homology 3 (SH3) domain of type I myosins from Acanthamoeba. In a subsequent study of CARMIL from Dictyostelium, pull-down assays indicated that the protein also bound capping protein and the Arp2/3 complex. Here we present biochemical evidence that Acanthamoeba CARMIL interacts tightly with capping protein. In biochemical preparations, CARMIL copurified extensively with two polypeptides that were shown by microsequencing to be the α- and β-subunits of Acanthamoeba capping protein. The complex between CARMIL and capping protein, which is readily demonstrable by chemical cross-linking, can be completely dissociated by size exclusion chromatography at pH 5.4. Analytical ultracentrifugation, surface plasmon resonance and SH3 domain pull-down assays indicate that the dissociation constant of capping protein for CARMIL is ~0.4 μM or lower. Using CARMIL fusion proteins, the binding site for capping protein was shown to reside within the carboxyl-terminal, ~200 residue, proline-rich domain of CARMIL. Finally, chemical cross-linking, analytical ultracentrifugation, and rotary shadowed electron microscopy revealed that CARMIL is asymmetric and that it exists in a monomer ↔ dimer equilibrium with an association constant of 1.0 × 10^6 M⁻¹. Together, these results indicate that CARMIL self-associates and interacts with capping protein with affinities that, given the cellular concentrations of the proteins (~1 and 2 μM for capping protein and CARMIL, respectively), indicate that both activities should be physiologically relevant.

In 1995, Zot (1) and colleagues identified a ~125-kDa protein from Acanthamoeba on the basis of its ability to bind to the isolated Src homology 3 (SH3) domain of Acanthamoeba myosin IC (1). This protein, which they called Acan 125, commu-
noprecipitated with myosin IC and appeared to colocalize with the myosin in cellular surface projections involved in pinocytosis. The subsequent cloning of the Acanthamoeba gene for Acan 125 (2) revealed a multidomain protein dominated by a central, ~460-residue leucine-rich repeat (LRR) domain. LRRs are ~29-residue sequences that contain a loose consensus dominated by leucines, form amphipathic α-β-structural units and mediate protein-protein interactions, either by serving as the ligand binding sites themselves or by increasing the affinity and/or specificity of binding at a separate site (3). The second most striking structural feature of Acan 125 is its ~200-residue, proline-rich COOH-terminal domain. Consistent with the fact that SH3 domains mediate protein-protein interactions by binding to proline-rich target sequences containing the core element PXXP (4), Xu et al. (2) showed that a fusion protein containing the COOH-terminal 344 residues of Acan 125 bound to the isolated SH3 domain of myosin IC and that this interaction was abrogated by an 18-residue deletion spanning two PXXP motifs fitting the consensus for SH3 domain target sequences (2, 5). Subsequent studies have estimated an affinity of 20–150 nM for the interaction between Acan 125 and the SH3 domains of Acanthamoeba myosins IA (6) and IC (5). These values, together with the cellular concentrations of myosin I and Acan 125 (~1 and ~2 μM, respectively), suggest that type I myosins and Acan 125 may be largely associated in vivo, barring some type of regulation (6).

Parallel efforts in our laboratory to purify and characterize proteins that bind to the SH3 domains of type I myosins from Dictyostelium led to the identification of p116, the Dictyostelium homolog of Acan 125 (7). Importantly, however, the eluates of the Dictyostelium myosin I SH3 domain affinity columns contained on a consistent basis not only p116, but also the seven-member Arp2/3 complex, the central player in the de novo nucleation of actin filament assembly and in the formation of branched filament networks, and capping protein, the central player in the termination of actin filament assembly. Immunoprecipitation reactions and other experiments provided evidence that Dictyostelium myosins IB (myoB) and IC (myoC) form a complex with p116, Arp2/3, and capping protein in vivo and that p116 serves as the scaffold for assembly of the complex, binding myosin I, capping protein, and Arp2/3 at independent sites. Given its central role in complex formation, we proposed the name CARMIL for p116, which stands for Capping protein, ARp2/3, Myosin I Linker. In further work by Jung et al. (7), CARMIL was shown to localize along with the Arp2/3 complex, myoB, and myoC in dynamic actin-rich cellular extensions, including the leading edge of cells undergoing chemotactic migration and dorsal, cup-like macroinvasive extensions. Moreover, cells in which the CARMIL gene was rendered nonfunctional by homologous recombination exhibited striking defects in the formation of these macroinvasive structures, a concomitant reduction in the rate of fluid phase pino-
cytosis, and a significant decrease in the efficiency of chemotactic aggregation. Together, these results identified a complex that links key players in the nucleation (Arp2/3) and termination (capping protein) of actin filament assembly with a ubiquitous barbed end-directed motor (myosin I), indicated that the protein responsible for the formation of this complex (CARMIL) is physiologically important, and suggested that previously reported myosin II mutant phenotypes in Dictyostelium might be, in part, due to defects in the assembly state of actin.

In the present study we sought to purify CARMIL to homogeneity and to begin to characterize its biochemical properties. We chose to purify the protein from Acanthamoeba given our laboratory’s previous success in using this organism for large scale preparations of cytoskeletal proteins. We show by a variety of methods that Acanthamoeba CARMIL is a bona fide capping protein (CP) interactant that could bind a significant fraction of cellular CP in vivo.

EXPERIMENTAL PROCEDURES

CARML Purification—The purification of CARMIL was based on a method described by Xu et al. (2) with several modifications. Acanthamoeba castellanii were grown in suspension culture to late-log phase, harvested, and resuspended in 1:2 (v/v) Tris-buffered saline (TBS; 1 mM EDTA, 150 mM NaCl, 6 mM KCl, 50 mM Tris-Cl, pH 8.0) supplemented with protease inhibitors (5 μg/ml leupeptin, 20 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 20 μg/ml benzamidine, 20 μg/ml TLCK, 50 μg/ml AEBSF), and broken in a Parr bomb at 375 psi for 5 min. After clarification of the lysate by centrifugation at 100,000 × g for 3 h (“high speed supernatant”), proteins were fractionated by ammonium sulfate precipitation. The fraction between 25 and 55% (NH₄)₂SO₄ was collected, resuspended in TBS supplemented with 1 mM ammonium sulfate and protease inhibitors, and applied to a phenyl-Sepharose 6B column (Amersham Biosciences). Proteins were eluted with a linear gradient from 1.0 to 9 mM ammonium sulfate in TBS. Fractions were tested for the presence of CARMIL by Western blotting, and positive fractions were pooled and dialyzed against TBS. CARMIL was bound to a SH3 affinity column and eluted with 5 × TBS. The eluate was dialyzed against buffer A (50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 25 mM MES, pH 6.0) supplemented with protease inhibitors (5 μg/ml leupeptin, 20 μg/ml TLCK, 50 μg/ml AEBSF), loaded on a Mono Q column (Amersham Biosciences), bound to glutathione-Sepharose 4B beads (Amersham Biosciences) for 2–3 h at 4 °C overnight and incubated with polyclonal antibodies against CARMIL or CP (1:10,000 and 1:1,500, respectively) in 2% bovine serum albumin and TBST for 3 h at room temperature. Specific binding was detected with horseradish peroxidase-conjugated secondary antibodies using an enhanced chemiluminescence system (ECL; Amersham Biosciences).

Antibodies—A polyclonal antibody against Acanthamoeba CP was raised using a compound peptide containing two α-subunit sequences that were obtained by microsequencing (see above; DESILNSAPFTF and FGEVGNQYLDPI) and three glycine residues as linker. This peptide was conjugated to keyhole limpet hemocyanin and injected into rabbits (Zymed Laboratories Inc.), and the resulting serum was affinity purified using the immunogenic peptide immobilized on nitrocellulose. Polyclonal antibodies against the α- and β-subunits of Dictyostelium CP were a kind gift from John A. Cooper (Washington University, St. Louis). Generation of the polyclonal antibody against a portion of the LRR domain of Acanthamoeba CARMIL was described previously (13) using a PerkinElmer Life Sciences Lambda 18 spectrophotometer and the model XL-1 equipped with interference optics. A capillary synthetic model XL-A and XL-1 analytical ultracentrifuges (Beckman Inc.) were equipped with a four-place An-Ti rotor. Sedimentation velocity and sedimentation equilibrium experiments were performed at 20 and 4 °C, respectively. The density (ρ) of the dialysate, buffer A (25 mM MOPS, 250 mM KCl, 1 mM EDTA, and 2 μM mercaptoethanol, pH 7.0) was determined to be 1.0101 g/ml at 20.00 ± 0.01 °C with the Anton Paar model DMA 58 densitometer, and the relative viscosity was determined to be 1.0139 (11). A partial specific volume of 0.720 ml/g for CARMIL was calculated from the amino acid composition and the values of Zamyatin (12). Specific absorbance coefficients for CARMIL were determined previously (13) using a PerkinElmer Life Sciences Lambda 18 spectrophotometer and the model XL-1 equipped with interference optics. A capillary synthetic boundary cell centerpiece and sapphire windows were employed with 0.140 ml of dialyzed protein (1.144 mg/ml CARMIL or 0.767 mg/ml CP) loaded in the right side and 0.400 ml of buffer A on the left side. After formation of the boundary by slow acceleration to 15,000 rpm, the speed was decreased to 3,000 rpm for CARMIL and 5,000 rpm for CP, while maintaining the temperature at 20 °C. Repeated interference scans were taken 20 or 30 s apart for 15 min during which time solvent base line and the protein plateau remained flat. The calibration value of 3.191 ± 0.055 fringes (mg/ml)⁻¹ (13) was corroborated with a solution of 30 μg/ml bovine serum albumin in TBS containing 0.1% SDS. A calibration curve of 0.474 ± 0.01% (cm²/mg) or ε = 57,640 cm⁻¹·m²·g⁻¹ for CARMIL and 1.140 ± 0.02 or ε = 70,520 cm⁻¹·m²·g⁻¹ for CP was determined. After

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determination of the absorbance coefficient for CARMIL, the speed was increased to 44,000 rpm, and 20 scans at 1 min apart were taken to determine the sedimentation coefficient under conditions where >90% of CARMIL was dimeric. For time derivative analysis (14), the procedures of Zolkiewski et al. (15) were used. Observed sedimentation coefficients ($s_{obs}$) were corrected to the density and viscosity of water at 20 °C, where $s_{E. coli} = 1.0457$ (reported in Svedberg units, S).

For sedimentation equilibrium runs in the XL-A, liquid columns of 0.080 or 0.110 ml of CARMIL in buffer A (with 0.015 ml more dialysate buffer A in the reference channel) and a speed of 7,500 rpm were used. Scans at 280 nm were collected at 2-h intervals in step mode (0.001-cm steps) with 11 or 13 averages/scan. Equilibrium was attained in 28 h, as determined by overlaying scans at different times. Global, weighted fits of the sedimentation equilibrium data obtained at two concentrations of CARMIL, in different runs to a model of reversible monomer-dimer association (with fully competent species present) were made using software provided by Allen P. Minton (NIDDK, NIH). Higher oligomeric forms than dimer were not detected. The monomer molecular weight of CARMIL (calculated from the amino acid composition) and base lines at zero 280 nm absorbance were held constant. Residuals from fits of the data to a monomer ↔ dimer equilibrium were randomly distributed around zero with < 0.1 absorbance deviations. For conversion of the observed association constant ($K_{obs}$) to a true molar concentration-dependent association constant (expressed per mole subunit), $K_s$, Equation 1 was used,

$$\log K_s = \log K_{obs} + \log \gamma$$

where the constant was calculated from log 2/e, where e is the molar extinction coefficient of the monomer for a 1.2-cm path length and the dimer was assumed to equal 2e.

Purified CARMIL and CP were mixed together in 0.15 ml of buffer A to give final concentrations of 1.93 and 3.28 μM, respectively (corresponding to a molar ratio of 1.7:1, CP to CARMIL). The mixture was equilibrated versus the same volume of buffer A in a double sector centerpiece in a 12-mm cell with sapphire windows for 48–54 h at 10,000 rpm and 4 °C. Interference optics were used to collect data and later analyzed for the equilibrium components present, where the molecular weights for CP and the CARMIL dimer were 61,860 and 108.5, respectively (see "Results").

Far UV circular dichroism spectra measurements were performed with a Jasco J-710 spectrometer using a water-jacketed cylindrical cell with a path length of 0.01 cm. The temperature of the cell was controlled at 20 °C by an external programmable water bath (Neslab RTE-111). Spectra were corrected for the CD signal of solvent (buffer A without β-mercaptoethanol). For determining secondary structure, for UV spectra were the average of 30 accumulations taken at 200 nm/min. Mean residue molecular weights of 108.5 for CARMIL were used for calculations of $\gamma$. Secondary structural components were calculated using the SELCON analysis program in Softmac™ applications (Softwood Company).

Rotary Shadowing—Purified CARMIL, CP, and mixtures of both proteins at an initial concentration of ~0.1 mg/ml were diluted with 2 parts of glycerol, sprayed onto freshly cleaned mica chips (16), and rotary shadowed at room temperature and at an angle of 9° in a Balzers 301 freeze fracture apparatus, or an RMC RDF 9010 (Boeckeler Instruments Inc., Tucson, AZ).

Chemical Cross-linking—DSG, DSP, EDAC, and S-NHS were obtained from Pierce and used at final concentrations of 2.5 mM for DSG and DSP and 5 mM for EDAC and S-NHS. Stock solutions (10×) of DSG and DSP were prepared in dimethyl sulfoxide whereas EDC and S-NHS were dissolved in H2O. The final concentration of dimethyl sulfoxide to cross-linking reactions never exceeded 10%. Cross-linking reactions were incubated for a maximum of 30 min at 24 °C. Products were resolved on 3–8% NuPAGE gels and either stained with Coomassie Blue or processed for Western blotting.

Actin Polymerization Assay—The kinetics of actin polymerization were measured by monitoring the change in fluorescence intensity of pyrene-labeled actin upon incorporation into the growing filament (9, 17). In brief, G-actin and pyrene-actin were mixed at a ratio of 20:1 and polymerized at a final actin concentration of 4 μM by adding 10× actin polymerization buffer (500 mM KCl, 10 mM MgCl₂, 10 mM EGTA, 100 mM imidazole, pH 7.0). CP was diazylated in 1× actin polymerization buffer and added to the actin solution immediately before the polymerization reaction was started. Fluorescence intensities (ex, 365 nm; em, 407 nm) were measured at a rate of 0.2 point/sec in a Pti QuantaMaster spectrofluorometer (Photon Technology International, Santa Clara, CA) over 30 min.

RESULTS

Capping Protein Copurifies with CARMIL—The procedure we used for the isolation of Acanthamoeba CARMIL was based in part on a protocol devised by Xu et al. (2). The key steps in that procedure were hydrophobic-interaction chromatography followed by affinity chromatography that takes advantage of the tight and highly specific association between PXXP motifs located in the proline-rich carboxyl-terminal region of CARMIL and the SH3 domain of Acanthamoeba myosin IC (2). Fig. 1 illustrates the enrichment of CARMIL we obtained with a modified procedure that made use of these two steps. In brief, a high speed supernatant of the crude lysate (lane 1) was fractionated with ammonium sulfate, and the 25–55% precipitate (lane 2) was subjected to a hydrophobic interaction chromatography step (lane 3). CARMIL-containing fractions were identified by Western blotting with an antibody raised previously against the Acanthamoeba protein (7). Pooled fractions were subjected to SH3 domain affinity chromatography (lane 4). Three predominant polypeptides elute from the GST-SH3 resin: a ~125-kDa band (filled arrowhead) corresponding to CARMIL and two unknown polypeptides of ~32 and ~30-kDa molecular mass (open arrowheads). After a final ion exchange chromatography step using Mono Q (lane 5), we consistently obtained peak fractions containing essentially the three aforementioned polypeptides. The fact that the smaller proteins copurified with CARMIL throughout the entire procedure and that their abundance appeared to be roughly equimolar to each other and to CARMIL led us to speculate that they might form a stable complex with CARMIL. In this regard it is interesting to note that these two smaller proteins were not described in the previous study by Xu et al. (2).

We reported previously that CP is present along with CARMIL in GST-SH3-domain pull-down experiments made using Dictostelium cell lysates (7). Given those findings, and our observation here that the ~32-kDa and ~30-kDa polypeptides are always approximately equimolar, we reasoned that they corresponded to the two chains of heterodimeric CP. Consistent
with this, polyclonal antibodies raised against the α- and the β-subunits of CP from Dictyostelium cross-react in a subunit-specific fashion with the ~32- and ~30-kDa Acanthamoeba polypeptides, respectively (Fig. 2A). Moreover, using a purified, CARMIL-free fraction of the ~32/30-kDa proteins (see below) and actin assembly assays, we observed a dose-dependent stimulation of actin nucleation in accordance with the nucleation activity of CPs (Fig. 2B (18)). To confirm absolutely that the ~32/30-kDa doublet is CP, both bands were excised from polyacrylamide gels, digested with trypsin, and the resulting fragments subjected to microsequencing by Edman degradation. As shown in Fig. 2C (top), the partial sequences of two peptides derived from the ~32-kDa band were found to be highly similar to sequences in the α-subunits of CP from Dictyostelium and various vertebrates. Likewise, three peptides obtained from the ~30-kDa band (Fig. 2C, bottom) exhibited extensive sequence similarity to the β-subunits of CP from various species. Taken together, these results establish unequivocally that the ~32/30-kDa polypeptides copurifying extensively with CARMIL are the α- and β-subunits of Acanthamoeba CP.

CARMIL and CP Can Be Separated by Gel Filtration at Low pH—Preparations of CARMIL which are free of CP are required to investigate the biochemical properties of the protein. Given the large difference in the molecular masses of CARMIL (~125 kDa) and CP (~60 kDa for the obligate heterodimer), we chose size exclusion chromatography as an appropriate method for their separation. Chromatography at physiological pH and elevated salt concentrations (500 mM) (Fig. 3A) resulted in a bimodal CP elution profile with a significant peak of CP comigrating precisely with CARMIL and a second peak exhibiting the mobility of free CP. We concluded, therefore, that these conditions were not effective in completely disrupting the CARMIL-CP complex. The use of even higher salt concentrations (800 mM) or the addition of the nonionic detergent CHAPS had no beneficial effect on the separation (data not shown). In contrast, decreasing the pH of the running buffer from 7.2 to 5.4 resulted in complete separation of CARMIL from CP (Fig. 3B).

When this low pH gel filtration step was incorporated into our large scale purification, and a second Mono Q run used to reconcentrate the proteins, we routinely obtained CARMIL that was 97% pure as determined by SDS-PAGE densitometry (Fig. 3C, left lane). The yield of was typically ~1 mg of CARMIL from 500 g of Acanthamoeba cells. As a byproduct of this purification we also obtained ~0.75 mg of pure CP (Fig. 3C, right lane). Separate experiments showed that CP purified in this way was equally active in terms of nucleating and capping activity as CP purified at physiological pH (data not shown). To assist in all subsequent experiments, the precise extinction coefficients of CARMIL and CP were determined by synthetic boundary centrifugation experiments performed on the purified proteins (A_{280}, 1 cm) of 0.4740 ± 001 (cm/mg) or ε_{280} = 57,640 M⁻¹ cm⁻¹ for CARMIL and 1.1400 ± 002 or ε_{280} = 70,520 M⁻¹ cm⁻¹ for CP).

To confirm that the copurification of CP with CARMIL was the result of its interaction with CARMIL and not fortuitous coelutions, we performed an SH3-GST pull-down assay with purified CP with and without purified CARMIL. As shown in Fig. 3D, CP was pulled down in the presence of CARMIL, but not in its absence (the ~31-kDa band present in the bound...
fraction from the CP-only sample is GST-SH3 domain fusion protein that leeched from the resin; see Fig. 3 legend). This result with purified proteins demonstrates unequivocally that the proteins are truly in a complex and is in agreement with our previous study demonstrating that the CARMIL homolog from Dictyostelium is able to recruit CP from cell extracts (7). The fact that CARMIL and CP copurify extensively over four purification steps and that a pH of 5.4 is required to separate them, suggests that the affinity of CP for CARMIL must be fairly high.

Finally, we note that the apparent molecular mass of the CARMIL-CP complex that we obtained from these gel filtration experiments using standard proteins (data not shown) is ≈500 kDa, which is much larger than the predicted molecular mass for a 1:1 complex between CARMIL and CP (~185 kDa). Furthermore, pure CARMIL elutes at a similar volume, indicating that it is largely responsible for the large apparent molecular mass of the complex (data not shown). These findings suggest that CARMIL is either asymmetric, forms higher order structures, or both.

**Fig. 3.** CP and CARMIL can be separated quantitatively by gel filtration at pH 5.4. Analytical gel filtration chromatography at physiological pH (A) and at pH 5.4 (B) was quantitated for all three polypeptides by densitometry of Coomassie-stained protein gels (bottom) and plotted against elution volume (top). With regard to the origin of the free CP in the elution profile in A, separate experiments showed that the elution of pure CP, pure CARMIL, and CARMIL-CP complex from Mono Q are overlapping. Therefore at least some of the free CP in A may have been present in the material loaded on the column. Moreover, the majority of CP in elutions at physiological pH was either coeluting precisely with CARMIL or precisely at the position of free CP, i.e. there is relatively little CP trailing in between the two peaks. We conclude, therefore, that CARMIL-CP complexes undergo relatively little dissociation during their passage through the column at physiological pH. C. SDS-PAGE of pooled peak fractions of CARMIL and CP. Samples were re concentr ated on Mono Q before electrophoresis. D. CP binds to CARMIL and not to the SH3 domain of myosin IC. GST-SH3 domain pull-down assays are shown using purified CARMIL and CP together (left) or CP without CARMIL (right). I, input; UB, unbound fraction; B, bound fraction. The ~31-kDa band present in the bound fraction from the CP-only pull-down (and comigrating with the α-subunit of CP in the bound fraction from the CARMIL + CP pull-down) is a portion of the GST-SH3 domain fusion protein that leeched from the beads during elution with 5 × TBS.
show that CP and CARMIL are both present in complex 3, whereas only CARMIL is found in complex 2. We conclude from these experiments that CARMIL forms homodimers and that a CARMIL homodimer associates with a CP heterodimer into what appears to be a heterotetrameric complex (although we cannot determine the exact stoichiometry of CP to CARMIL from these experiments).

**Sedimentation Equilibrium Analyses Indicate that the CARMIL Protein Is in a Monomer ↔ Dimer Equilibrium**—We performed sedimentation analyses in the analytical ultracentrifuge to gain further insight into the apparent ability of CARMIL to self-associate. The purified CARMIL used in these studies was homogeneous as evidenced by sedimentation velocity experiments, which showed a single symmetrical boundary (data not shown). Sedimentation equilibrium results obtained with two preparations of CARMIL are shown in Fig. 5A (bottom), together with the fit of the data to a monomer ↔ dimer equilibrium. One preparation of CARMIL was devoid of CP (circles and diamonds), whereas the other contained 3.5% CP (squares). Irrespective of the presence of a small amount of CP, global fitting of data sets for the two CARMIL preparations gave excellent fits to a monomer ↔ dimer equilibrium model with an association constant of $1.0 \times 10^6 \text{ M}^{-1}$ at pH 7.0 and 4°C. No oligomeric species larger than the dimer could be detected, in agreement with the cross-linking data above. We also note that the dimerization constant for CARMIL is probably considerably tighter than $1.0 \times 10^6 \text{ M}^{-1}$ at 20°C because only dimer was detected in sedimentation velocity derivative profiles for 4–7 μM CARMIL run at this higher temperature (data not shown). Finally, CD spectra of pure CARMIL revealed 59% α-helix, 10% β-sheet, and 31% turns.

**The CARMIL Dimer Is Highly Asymmetric**—We next addressed the issue of the shape of the CARMIL molecule because dimerization of CARMIL alone cannot account for its large apparent molecular mass (≥500 kDa) observed in gel filtration experiments. To this end we performed sedimentation velocity analyses in the analytical ultracentrifuge. When run under conditions at which the protein is >90% dimer (9.4 μM), CARMIL has a sedimentation coefficient ($s_{20,w}$) of 7.1 S. This indicates that the CARMIL dimer is quite asymmetric with a frictional coefficient of $f/f_0 = 2.0$, or about 2-fold greater than that of a spherical particle (19). For comparison, the α-helical, coiled-coil, rod-like tail of *Acanthamoeba* myosin II (149 kDa) has a frictional coefficient of 2.7, which yields an effective length of 82 nm assuming a 2-nm diameter and a prolate ellipsoid (15).

To extend these observations, we performed rotary shadowed electron microscopy (RSEM) on purified CARMIL. Highly concentrated CARMIL was diluted in glycerol, sprayed on mica, and rotary shadowed (Fig. 5B). Panels labeled 1–8 show representative examples of the most common objects seen. We think the “lollipop”-like structures in 1–3 are extended CARMIL, whereas the objects in 4–8 represent instances in which the linear portion is crumpled, folded, or entirely wrapped around the ball. We also think that the ball corresponds to the LRR domain (see below). Moreover, we think that the typical lollipop structure is the CARMIL dimer because the ball portion sometimes appears bilobed (9 and 10). Additionally, the size of the ball in CARMIL molecules that have been covalently cross-linked (11 and 12), where ≥98% of the protein appears as a dimer on SDS-PAGE, is about the same size as the ball for objects like the ones in 1–3. Like the hydrodynamic data above, these results indicate that the CARMIL dimer is asymmetric. Finally, RSEM of the CARMIL-CP complex (13–17) (and pure CP at higher magnification; 18) shows one CP/putative CARMIL dimer positioned at the middle of the linear portion of
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Fig. 5. Sedimentation equilibrium analyses and RSEM. A, sedimentation equilibrium ultracentrifugation of two different preparations of Acanthamoeba CARMIL run at 7,500 rpm and 4 °C for 34 and 36 h. The lower panel shows the absorbance scans at 280 nm of CARMIL preparation 1 (void of CP and at a concentration of 4.51 μM CARMIL) after 34 h (circles) and 36 h (diamonds). Also shown is a scan of CARMIL preparation 2 (containing 3.5% CP and at a concentration of 3.47 μM CARMIL) after 36 h (squares). The solid lines indicate the global fits of the three sets of data to a reversible, monomer-dimer association model with weighted standard squares of the differences from the fit (WSSQ) = 0.026 (0.008 mg/ml). Alternate models were considered and discarded. One of these fitted the data to a mixture of excess CP, CARMIL dimer, and CARMIL dimer complexed to two equivalents of CP. In this case, the CARMIL dimer is 60–65%, (CARMIL)2(CP) is 35–40%, and the affinity constant for CP binding to CARMIL becomes 10^11 M^-1, in which case no free CARMIL dimer should be present. A second model that was considered is one in which monomeric CARMIL is complexed to 1 eq of CP, which can then dimerize to form (CARMIL)2(CP). This model was rejected because it does not take into account the fact that the interaction of CP with CARMIL stabilizes the dimer at 4°C.

| Hours (at speed) | Free CP K_D | μM | % | μM |
|----------------|-------------|----|---|----|
| 50             | 12.2 [0.127]| 86.8 [0.838]| 0.97 | 2.42 | 2.7 × 10^6 |
| 52             | 14.9 [0.144]| 85.1 [0.821]| 0.98 | 2.48 | 2.3 × 10^6 |
| 54             | 12.8 [0.124]| 87.2 [0.841]| 0.97 | 2.46 | 2.8 × 10^6 |

Because monomeric CARMIL was not detected, the presence of CP promotes dimerization of CARMIL.

From the amount of noncomplexed CARMIL dimer, ([CARMIL]_2(CP)) and free CP ([CP]_free) present, the association constant for capping protein binding to CARMIL dimer can be calculated:

\[ K_D = \frac{([\text{CARMIL}]_2(CP))/([\text{CARMIL}]_2 \times [CP]_\text{free})}{([\text{CARMIL}]_2-CP)/((\text{CARMIL})^2-CP)} \]

CARMIL. Based on the mapping data below, which places the CP binding site in CARMIL within the carboxyl-terminal portion of the molecule, we conclude that the ball indeed corresponds to the LRR domain, whereas the linear structure corresponds to a carboxyl-terminal tail.

Capping Protein Binds to CARMIL with Submicromolar Affinity—The data described above suggest a fairly tight association between CP and CARMIL. We used three independent methods to obtain an estimate of their affinity for each other. First we employed surface plasmon resonance with CARMIL immobilized on the chip and CP in solution at concentrations ranging from 83 to 1,000 nM. CP was bound to CARMIL for 6 min and then dissociated by switching to buffer without CP. Fig. 6A shows that the plasmon resonance response increased in a dose-dependent fashion with increasing CP concentration. Based on the association and dissociation slopes, the dissociation constant was calculated to be 1 μM.

Second we performed analytical ultracentrifugation. Purified CARMIL and CP were mixed together to achieve final concentrations of 1.93 and 3.28 μM, respectively (corresponding to a molar ratio of 1.7:1, CP to CARMIL), and subjected to sedimentation equilibrium ultracentrifugation. After reaching equilibrium, the data were analyzed for the different components present (Table I). The data fit best with a model in which there is 2–3% free CP, 13–15% CARMIL dimer, and 85–87% CARMIL dimer with one CP bound (see legend to Table I for consideration and elimination of alternate models). These values yield an association constant for CP binding to the CARMIL-CP complexes (the distance between the ball of CARMIL and the CP molecule measured from center to center of the two globular structures ranged from 13 to 34 nm, but most of them were ~18 nm; the linear portion of the tail carboxyl-terminal of CP was rarely visible, possibly because of a change in its conformation upon CP binding). 15 is pure CP. The magnification bar in 16B applies to 1–17 and represents 50 nm, whereas the magnification bar in 18 represents 25 nm. Images are shown in inverted contrast.
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MIL dimer of $2.6 \pm 0.2 \times 10^6$ M$^{-1}$ (i.e. a dissociation constant of $\sim 0.4 \mu M$). Interestingly, CARMIL monomers were not observed in the equilibrium mixture. This observation, together with the sedimentation equilibrium data for pure CARMIL in Fig. 5A, which would argue that CP-free CARMIL at 1.93 $\mu M$ would be only $\sim 50\%$ dimer, suggest strongly that the presence of CP promotes dimerization of CARMIL.

Finally we performed a GST pull-down assay in which purified CARMIL and CP were mixed together at final concentrations for both proteins of 1.0, 0.5, 0.2, or 0.1 $\mu M$, incubated overnight at 4°C, and CARMIL, along with any bound CP, was quantitatively removed with GST-SH3 domain beads. The free CP in the resulting supernatant was quantitated densitometrically using SDS-polyacrylamide gels that included a CP standard of known concentration (see “Experimental Procedures”). The bar graph shows the percentages of total CP that were free at the different molar concentrations of input proteins.

Based on these three measurements and the fact that surface plasmon resonance is frequently known to underestimate affinities (20), we conclude that the affinity of CP for CARMIL is in the submicromolar range. If we assume a dissociation constant of $\sim 0.4 \mu M$ and use cellular concentrations for CP and CARMIL of $1 \mu M$ (21) and $2 \mu M$ (6), respectively, we calculate that about 75% of the cellular CP could be complexed with CARMIL, barring some form of regulation or compartmentalization, and excluding other proteins that also bind CP (e.g. actin filament ends).

CP Binds to the Carboxyl-terminal, Proline-rich Domain of CARMIL—As a first step to delineate the region within CARMIL which binds CP, we performed a CP overlay assay with trypsin-digested CARMIL immobilized on nitrocellulose membrane (data not shown). In this initial experiment, CP bound to an $\sim 80$-kDa fragment of CARMIL, identified as a carboxyl-terminal fragment by its ability to bind the SH3 domain of myosin I. This portion of CARMIL contains previously described domains that reside between the end of the LRR domain and the carboxyl terminus (see Fig. 7A, top): the verprolin homology domain (V), the acidic domain (A), and the proline-rich domain (P) (7). To test the role of these domains in binding CP, we generated four GST fusion proteins: GST-VAP, GST-AP, GST-P, and GST-VA (Fig. 7A). Beads coated with these fusion proteins were then assayed for their ability to pull CP out of Acanthamoeba cell extracts. Fig. 7B (upper panel) shows that GST-P, as well as the two other GST fusions that include the proline-rich domain (GST-VAP and GST-AP), captured CP, whereas GST-VA, which lacks the proline-rich domain, did not. Moreover, a previously described GST fusion protein containing the corresponding proline-rich domain from Dictyostelium CARMIL (GST-PRO (7)) pulls down CP from Acanthamoeba extracts (Fig. 7B, bottom). Under identical assay conditions and Western blot exposure times, a pull-down using the Dictyostelium CARMIL fusion proteins GST-NT (7) and GST-LRR, which contain the amino-terminal 179 residues of CARMIL and its complete LRR domain (residues 180-643), respectively, did not yield a CP signal (Fig. 7B, lower panel; for comparison, the Acanthamoeba CARMIL GST-P fusion protein was included in this experiment). From these results we conclude that the binding site for CP resides within the carboxyl-terminal, $\sim 200$-residue, proline-rich domain of CARMIL.

**DISCUSSION**

Our data indicate that CARMIL and CP form a fairly tight complex that persists throughout a lengthy purification procedure and which completely dissociates during gel filtration only under relatively harsh conditions. Consistent with these empirical observations, the affinity of CP for CARMIL, as determined by three independent methods, is in the submicromolar range. Using a $K_d$ of $\sim 0.4 \mu M$ (the value obtained from the hydrodynamic measurements, which fell in between the values obtained by surface plasmon resonance and the pull-down assays, and which we take as the most accurate because it measures the equilibrium interaction between both components in solution), and average cellular concentrations of 1 and 2 $\mu M$ for CP and CARMIL, respectively, one would predict that a significant fraction of cellular CP can be bound to CARMIL in vivo, barring some form of regulation. Moreover, an affinity of $\sim 0.4 \mu M$ seems reasonable from a physiological standpoint because it would allow readily reversible association between CP and CARMIL, consistent with the critical role of CP in terminating actin filament assembly. Interestingly, myosin I, which has been reported to interact with CARMIL via its SH3 domain with an affinity approaching 20 nM (5, 6), and the Arp2/3 complex that persists throughout a lengthy purification procedure.
complex, which is present in variable amounts in pull-down assays of CARMIL from Dictyostelium (7), were not obvious contaminants in the latter stages of our Acanthamoeba CARMIL preparations. These observations suggest that CARMIL binds myosin I and the Arp2/3 complex more weakly than CP, and/or that the interaction of CARMIL with myosin I and Arp2/3 is minimized in biochemical preparations by negative regulation of their interactions.

We present mapping data that place the CP interaction site in CARMIL within the N-200-residue, proline-rich, COOH-terminal domain of the protein. This result conflicts with our previous work (7), which placed the CP binding site in CARMIL within the NH2-terminal -180 residues of the protein. Although we cannot fully explain this discrepancy, we note that the previous study lacked any significant controls. Moreover, when tested under identical conditions against COOH-terminal CARMIL fusion proteins that clearly recruit CP from cell extracts, the NH2-terminal fusion protein used in that previous study lacked any significant controls. Furthermore, we note that recent mapping data on the vertebrate form of CARMIL place the CP binding site within the CARMIL dimer (23), and V-1 (24) have been shown to bind CP. The possibility is not formally ruled out by the hydrodynamic data.

The placement of the CP binding site within the CARMIL proline-rich domain is significant because the myosin I SH3 domain binds to two PXXP motifs present within this same proline-rich region. These results raise the question of whether the binding of CP and myosin I to CARMIL is mutually exclusive. We conclude that their binding is not mutually exclusive because CARMIL that is bound to the myosin I SH3 domain typically binds CP in amounts that appear to be near stoichiometric (Ref. 7 and this paper). Moreover, myosin I is present along with CARMIL in immunoprecipitates made using anti-CP antibodies, and CP is present along with CARMIL in immunoprecipitates made using anti-myosin I antibodies (7). Having said this, we cannot rule out the possibility that the binding of myosin I to CARMIL influences to some extent the affinity of CARMIL for CP and vice versa.

In addition to demonstrating a tight association between CARMIL and CP, we show by chemical cross-linking and analytical ultracentrifugation that CARMIL alone self-associates into a dimer. Given the association constant we obtained (~1.0 × 10^6 M^-1 or stronger), and the estimate of the CARMIL concentration in the cell (~2 μM), we predict that a significant fraction of CARMIL is present as a dimer in vivo. This fraction may in fact be quite high given that CARMIL is not evenly distributed throughout the cell, but rather is highly concentrated in regions of active actin assembly. We also show by gel filtration on calibrated columns, sedimentation velocity ultracentrifugation, and RSEM that the CARMIL dimer is quite asymmetric.

With regard to the association of CP with CARMIL monomer versus dimer, we conclude based on the best fit of the hydrodynamic data that CP binds preferentially to the CARMIL dimer. Consistent with this, CP promotes CARMIL dimerization. Overall, then, the preferential binding of CP to dimeric CARMIL couples the monomer ↔ dimer equilibrium of CARMIL to the equilibrium between free CP and CP bound to the CARMIL dimer. With regard to the stoichiometry of CP-CARMIL interaction, the sedimentation equilibrium data indicate that one CP molecule binds/CARMIL dimer. An alternate possibility is that a second CP molecule can bind to a CARMIL dimer but that its binding exhibits strong negative cooperativity. This possibility is not formally ruled out by the hydrodynamic data.

To date, only the barbed end of the actin filament, S100 (22), Twinfilin (23), and V-1 (24) have been shown to bind CP. The biochemical data presented here argue strongly that CARMIL should be added to this short list of bona fide CP interactants.
This conclusion is all the more important given the widespread distribution of CARMIL in metazoans. The next major question to address is whether CP is still able to cap the barbed end of actin filaments when bound to CARMIL. This question is particularly important because the answer will drive the investigation of CARMIL function in one of two very different directions. If CP can no longer cap while bound to CARMIL, then the principal role of CARMIL as regards CP would be to buffer the cellular concentration of free, active CP. Conversely, if CP can still actively cap actin filaments when bound to CARMIL (or CARMIL can bind to capped filaments), then the cell can create barbed ends with a novel cap that is capable of recruiting both a barbed end-directed motor (myosin I) and a complex capable of nucleating de novo actin filament assembly (Arp2/3 complex).

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