CARMIL Is a Potent Capping Protein Antagonist

IDENTIFICATION OF A CONSERVED CARMIL DOMAIN THAT INHIBITS THE ACTIVITY OF CAPPING PROTEIN AND UNCAPS CAPPED ACTIN FILAMENTS

Takehito Uruno, Kirsten Remmert, and John A. Hammer III

From the Laboratory of Cell Biology, Section on Molecular Cell Biology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-8017

Acanthamoeba CARMIL was previously shown to co-purify with capping protein (CP) and to bind pure CP. Here we show that this interaction inhibits the barbed end-capping activity of CP. Even more strikingly, this interaction drives the uncapping of actin filaments previously capped with CP. These activities are CP-specific; CARMIL does not inhibit the capping activities of either gelsolin or CapG and does not uncap gelsolin-capped filaments. Although full-length (FL) CARMIL (residues 1–1121) possesses both anti-CP activities, C-terminal fragments like glutathione S-transferase (GST)-P (940–1121) that contain the CARMIL CP binding site are at least 10 times more active. We localized the full activities of GST-P to its C-terminal 51 residues (1071–1121). This sequence contains a stretch of 25 residues that is highly conserved in CARMIL proteins from protozoa, flies, worms, and vertebrates (CARMIL Homology domain 3; CAH3). Point mutations showed that the majority of the most highly conserved residues within CAH3 are critical for the anti-CP activity of GST-AP (862–1121). Finally, we found that GST-AP binds CP ~20-fold more tightly than does FL-CARMIL. This observation together with the elevated activities of C-terminal fragments relative to FL-CARMIL suggests that FL-CARMIL might exist primarily in an autoinhibited state. Consistent with this idea, proteolytic cleavage of FL-CARMIL with thrombin generated an ~14-kDa C-terminal fragment that expresses full anti-CP activities. We propose that, after some type of physiological activation event, FL-CARMIL could function in vivo as a potent CP antagonist. Given the pivotal role that CP plays in determining the global actin phenotype of cells, our results suggest that CARMIL may play an important role in the physiological regulation of actin assembly.

The spatial and temporal control of actin polymerization is essential for a host of fundamental cellular processes such as cell migration, endocytosis, and cytokinesis (1–3). In vivo, actin polymerization occurs primarily at the barbed (fast-growing) end of the actin filament. Mechanisms that control the number of barbed ends are crucial, therefore, in the regulation of actin assembly within cells. Cells appear to use at least three mechanisms to generate barbed ends (1, 4). First, barbed ends are created de novo by the action of the Arp2/3 complex and the formins, two actin-nucleating machines that drive the formation of branched (Arp2/3) and unbranched (formin) actin structures (5, 6). Second, proteins like coflin generate barbed ends by severing pre-existing actin filaments (4, 7). Third, the active removal of capping protein or gelsolin from the barbed end of pre-existing, capped actin filaments by factors like phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is thought to generate free barbed ends (8–10).

Capping protein (CP) is a highly conserved, ubiquitously expressed, heterodimeric actin-binding protein that is essential for normal actin dynamics in vivo (11–13). CP binds the barbed end of the actin filament tightly (K$_d$ = 0.1–1.0 nM), and its presence there blocks both the association and dissociation of actin monomers (14). The association of CP with the barbed end (capping) terminates filament elongation, whereas the dissociation of CP from a pre-capped actin filament (uncapping) creates a free barbed end, allowing the resumption of filament elongation. The extent to which CP can influence actin assembly and organization in vivo was revealed in dramatic fashion by a recent study that used RNA-mediated interference to reduce the cellular level of CP (15). Specifically, B16 melanoma cells depleted of the majority of CP exhibited a striking diminution in the size of their lamellipodia and a dramatic increase in the number of filopodia on their surface. In addition to demonstrating a pivotal role for CP in determining the global organization of the actin cytoskeleton, this (15) and previous studies (11–13) of CP function in vivo highlight the necessity of identifying physiological regulators of CP.

One potential regulator of CP is CARMIL. CARMIL proteins comprise a novel family of molecules whose two most conspicuous features are a central, leucine-rich repeat domain and a C-terminal proline-rich domain (16, 17). Genes encoding CARMIL proteins have been identified so far in Acanthamoeba, Dictyostelium, Caenorhabditis elegans, Drosophila, mouse, and man (16–18). The first CARMIL protein to be identified was Acan125 (19). This 125-kDa Acanthamoeba protein was discovered based on its ability to bind to the isolated SH3 domain of Acanthamoeba myosin-IC, a monomeric unconventional myosin. Mapping studies showed that two PXXP motifs present within the Acan125 C-terminal proline-rich domain are responsible for its interaction with the myosin I SH3 domain (16). Using a similar approach, Jung et al. (17) subsequently identified p116, the Dictyostelium homologue of Acan125. Importantly, the eluates of their myosin I SH3 domain affinity columns contained on a consistent basis not only p116 but also CP and the Arp2/3 complex. Immunoprecipitation reactions and other experiments provided evidence that p116 forms a complex with CP, Arp2/3, and myosin I in vivo and that p116 serves as the scaffold for assembly of this complex by binding CP, Arp2/3 and myosin I at independent sites. Given its central role in forming the complex, Jung et al. (17) gave p116 the name CARMIL for capping protein, Arp2/3, myosin I linker.

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To whom correspondence should be addressed: Laboratory of Cell Biology, Bldg. 50, Rm. 2523, 9000 Rockville Pike, Bethesda, MD 20892-8017. Tel.: 301-496-8960; Fax: 301-402-1519; E-mail: hammerj@nhlbi.nih.gov.

2 The abbreviations used are: PIP$_2$, phosphatidylinositol 4,5-bisphosphate; CP, capping protein; SAS, spectrin-actin seeds; SH3, Src homology domain 3; FL, full-length; CAH3, CARMIL homology domain 3; GST, glutathione S-transferase; DTT, dithiothreitol; a.a., arbitrary units; GA, gelsolin-actin complex; Mops, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin.
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Recent efforts to purify *Acanthamoeba* CARMIL have provided evidence that CARMIL interacts tightly with CP (20). First, CARMIL and CP were found to co-purify through a lengthy purification procedure that included ammonium sulfate fractionation, hydrophobic interaction chromatography, affinity chromatography, and anion exchange chromatography. Second, the mixture of CARMIL and CP that was obtained from these steps was only partially resolved by size exclusion chromatography despite the fact that these two proteins differ enormously in Stokes radius. Complete resolution of CARMIL and CP by gel filtration required the use of a running buffer with a pH of 5.4. Third, measurements of bound and free fractions in CARMIL-CP mixtures using analytical ultracentrifugation and SH3 domain pull-down assays indicated that CARMIL binds CP with an affinity of ~0.4 μM or tighter. Given this affinity and the reported cellular concentrations of both proteins (~1 μM for CP (21) and ~2 μM for CARMIL (22)), Remmert et al. (20) suggested that a significant amount of CP might be complexed with CARMIL in vivo. In terms of the possible functional consequences of forming this complex, Remmert et al. (20) raised two possibilities. First, if CP can no longer cap the barbed end when bound to CARMIL, then the combination of CARMIL-CP complex would reduce the level of free, active CP, favoring actin polymerization. If on the other hand CP still functions when bound to CARMIL, then the CARMIL-CP complex could function as a novel barbed end cap that might further recruit myosin I and the Arp2/3 complex to the filament end.

In this study we used CARMIL and CARMIL fragments to characterize the effects of the protein on CP function. Our data show that the binding of CARMIL to CP inhibits the ability of CP to cap the barbed end. We also found to our surprise that CARMIL promotes the rapid dissociation of CP from the ends of actin filaments previously capped with CP, i.e. it promotes uncapping. Although FL-CARMIL is able to perform both of these anti-CP functions, C-terminal fragments of CARMIL that contain its CP binding site perform both tasks much more effectively. These and other observations suggest that, in the absence of some uncharacterized activating event, FL-CARMIL probably folds into a low activity state. Consistent with this, thrombin digestion of FL-CARMIL generates a C-terminal fragment that expresses full anti-CP activity. Together, our results suggest that CARMIL might play a significant role in controlling actin assembly in vivo by antagonizing in a regulated fashion the function of CP in two important ways.

MATERIALS AND METHODS

**Proteins**—Full-length (FL) *Acanthamoeba* CARMIL and CARMIL lacking its P domain (∆P-CARMIL) were expressed in Sf9 cells with a FLAG tag at their N terminus. For FL-CARMIL, the FLAG tag (MDYKDDDDK) was filled with a small double-stranded fragment made by annealing the following two oligonucleotides: 5ʹ-CCCTAGAGCGCCCTGTTGAGGTGGGCTGCCGGC-3ʹ and 5ʹ-AGCTTGGCAGCGGACGACCATCTGCTTACATGCGGTT-3ʹ (this fragment contains, in order, an Sphl site cohesive end, a stop codon, a short stretch of 3ʹ-untranslated region, a NotI site, and a Hind III site cohesive end). The insert in the resulting construct was released with EcoRI and NotI and cloned into pVL1939. After sequence confirmation, both pVL1939 constructs were transfected into Sf9 cells, and amplified virus stocks were prepared by standard procedures. We assume that the addition of the FLAG tag to CARMIL does not alter in any significant way its biochemical properties.

For protein expression, 4 × 10⁶ Sf9 cells were suspended in 250 ml of serum-free SF900-II medium (Invitrogen), mixed with 12 ml of amplified viral stock, distributed to ten 150-mm dishes, and incubated at 27 °C in the dark for 72 h. Cells were collected by centrifugation, subjected to one freeze/thaw cycle, and resuspended in 10 ml of lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 40 μg/ml 4-(2-aminoethyl) benzene-sulfonyl fluoride). Cell lysate was stabilized by homogenization using a glass Dounce homogenizer (30 strokes on ice) and incubation on a rotating wheel at 4 °C for 30 min. The lysate was then clarified by centrifugation at 27,000 × g for 45 min at 4 °C, mixed with 1 ml of anti-FLAG M2 affinity resin (Sigma, A2220), and incubated on a rotating wheel at 4 °C for 2 h. The resin was washed 5 times (15 ml per wash) using 1× TBS (50 mM Tris–HCl (pH 7.5), 150 mM NaCl) and FLAG-tagged CARMIL eluted using 3 ml of 1× TBS containing 0.1 mg/ml synthetic FLAG peptide (DYKDDDDK). The eluted fractions were examined by SDS-PAGE followed by staining with Coomassie Blue, and the fractions containing FL-CARMIL or ∆P-CARMIL were pooled and dialyzed against Mono Q buffer A (25 mM Mops (pH 7.2), 50 mM KCl, 1 mM EDTA, 1 mM DTT). CARMIL proteins were further purified by chromatography on a Mono Q column (Amersham Biosciences) connected to a fast protein liquid chromatography system (Amersham Biosciences) using a 50–500 mM KCl gradient made with Mono Q buffer A and Mono Q buffer B (25 mM Mops (pH 7.2), 500 mM KCl, 1 mM EDTA, 1 mM DTT). Peak fractions were pooled and dialyzed against storage buffer (10 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM MgCl₂, 0.5 mM DTT, 1 mM EGTA). Aliquots were snap-frozen in liquid nitrogen and stored at ~80 °C until use.

The preparation of the GST fusion proteins GST-VAP, GST-VA, GST-AP, and GST-P was reported previously (20). To prepare GST-51aa, a DNA fragment containing CARMIL residues 1071–1121 with flanking BamHI and EcoRI sites was created by PCR using *Phuturbo* DNA polymerase (Stratagene), pBS-FLAG-Acan125 as the template, and the primers 5ʹ-CGGGGATCCAGAAGCCCGAACCGCTCTGCAGGGA-3ʹ and 5ʹ-AAAACTGGATCTAGTGGCGGTCTCATGTCATGGGCCTGC-3ʹ. The resulting fragment was digested with EcoRI and BglII and ligated into pBluescript SK digested with EcoRI and BamHI. An internal MluI/SphI fragment spanning ~2.7 kb of the PCR product was replaced with the corresponding fragment from the parental Acan125 cDNA clone. The insert in the resulting construct (pBS-FLAG-Acan125) was released with EcoRI and NotI and cloned into pVL1939 (Invitrogen). To create AP-CARMIL, which lacks the C-terminal 167 residues of CARMIL, a portion of the FL-CARMIL insert in pBS-FLAG-Acan125 was removed by digestion with SphI and HindIII, and the gap was filled with a small double-stranded fragment made by annealing the following two oligonucleotides: 5ʹ-CCCTAGAGCGCCCTGTTGAGGTGGGCTGCCGGC-3ʹ and 5ʹ-AGCTTGGCAGCGGACGACCATCTGCTTACATGCGGTT-3ʹ (this fragment contains, in order, an Sphl site cohesive end, a stop codon, a short stretch of 3ʹ-untranslated region, a NotI site, and a Hind III site cohesive end). The insert in the resulting construct was released with EcoRI and NotI and cloned into pVL1939. After sequence confirmation, both pVL1939 constructs were transfected into Sf9 cells, and amplified virus stocks were prepared by standard procedures. We assume that the addition of the FLAG tag to CARMIL does not alter in any significant way its biochemical properties.
addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. The cells were harvested by centrifugation, subjected to one freeze/thaw cycle, resuspended in lysis buffer (PBS containing 1 mM DTT, 1 mM EDTA, and protease inhibitors), and broken by 2 passages through a French press (20,000 p.s.i.). The lysate was then made 0.4% with Triton X-100, incubated on ice for 20 min, and clarified by centrifugation for 1 h at 15,000 × g at 4 °C. The supernatant was mixed with 0.8 ml of glutathione-Sepharose 4B beads (Amersham Biosciences) and incubated for 4 h at 4 °C with rotation. The resin was washed 5 times (15 ml per wash) with PBS at 4 °C, and the fusion protein was eluted with elution buffer (200 mM Tris-HCl (pH 8.0), 20 mM reduced glutathione (Sigma, G4251)). Eluted proteins were further purified using a Mono Q column as described above. Proteins were dialyzed into storage buffer (10 mM Tris-HCl (pH 7.3), 50 mM KCl, 1 mM MgCl$_2$, 0.5 mM DTT, 1 mM EGTA) and stored at −80 °C.

To prepare the GST-free, 51-residue peptide (51aa), 1 ml of GST-51aa beads was resuspended in 0.9 ml of PBS containing 1 mM CaCl$_2$ and 50 units of thrombin protease (Amersham Biosciences). After incubation at 24 °C for 16 h, the supernatant containing cleaved 51aa was passed three times through a column containing 1 ml of p-aminobenzamidine-agarose beads (Sigma, A-7155) and 0.1 ml of glutathione-Sepharose 4B beads to remove thrombin and GST proteins, respectively. The eluate containing 51aa was dialyzed against storage buffer (Tris-HCl (pH 7.5), 50 mM KCl, 1 mM MgCl$_2$, 0.5 mM DTT, 1 mM EGTA) and concentrated using a Centricon 3 membrane (Amicon). Mass spectrometric analysis confirmed that the cleaved peptide contained the last 51 residues of CARMIL and an additional 8 amino acids (GSRRASVG) derived from pGEX-2TK.

Acanthamoeba capping protein was purified using a novel method$^3$ in which purified GST-AP was covalently coupled to CNBr-activated Sepharose 4B beads (Amersham Biosciences) and used as an affinity matrix for purifying CP from crude cell extracts. Briefly, 50 g of frozen Acanthamoeba cell pellet was resuspended in 2 volumes of 1× TBS (Tris-HCl (pH 7.5), 150 mM NaCl) containing protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 20 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride), 1 mM DTT, and 1 mM EDTA, and lysed by thawing on ice. After centrifugation at 100,000 × g for 3 h at 4 °C, the supernatant was supplemented with Triton X-100 to a final concentration of 0.1% and incubated with 5 ml of GST-AP affinity matrix for 2 h at 4 °C with gentle rotation. The matrix was washed extensively with 1× TBS at 4 °C, and CP was eluted with low pH elution buffer (100 mM MES (pH 5.4), 500 mM KCl, 1 mM DTT, 1 mM EDTA). Peak fractions were identified by SDS-PAGE/Coomassie Blue staining, pooled, dialyzed against Mono Q buffer A, and purified by chromatography on a Mono Q column as described above. Purified CP was dialyzed against CP storage buffer (50% glycerol, 10 mM imidazole (pH 7.3), 50 mM KCl, 1 mM MgCl$_2$, 0.5 mM DTT, 1 mM EGTA) and stored in aliquots at −80 °C.

Protein concentrations were determined by Bio-Rad assay using BSA as standard and verified by SDS-PAGE/Coomassie Blue staining using a dilution series of the sample and the BSA standard. For 51aa, the Bio-Rad assay underestimated its concentration by a factor of 8–10, so the concentration of 51aa was estimated using Coomassie Blue-stained gels loaded with known amounts of GST-51aa cleaved with thrombin.

Actin Assembly Assays and Estimation of Actin Critical Concentration—Actin was purified from Acanthamoeba castellanii according to the method of Gordon et al. (23) followed by size exclusion chromatography on a HiPrep Sephacryl S200 column (Amersham Biosciences). Purified, monomeric actin was stored at 4 °C with continuous dialysis in fresh G buffer-Ca (3 mM imidazole (pH 7.5), 0.1 mM CaCl$_2$, 0.5 mM ATP, 0.75 mM β-mercaptoethanol, and 0.02% NaN$_3$). Pyrene-labeled actin was prepared by labeling monomeric actin with N-(1-pyrenyl)iodoacetamide (Molecular Probes) according to the method of Koyama and Mihashi (24). Labeled actin was purified by size exclusion chromatography on a HiPrep Sephacryl S200 column and stored lyophilized at −80 °C. Before use, pyrene-labeled actin was resuspended in G buffer-Ca and dialyzed in the same buffer extensively.

Actin critical concentration measurements were made according to standard methods (25). Briefly, a stock solution of G-actin (20 μM, 5–10% pyrene-labeled) in G buffer-Ca was incubated with 0.1 volume of 10× exchange buffer (10 mM EGTA, 1 mM MgCl$_2$) for 3 min at 24 °C and polymerized for 1 h at 24 °C by the addition of 0.1 volume of 10× KMEI buffer (100 mM imidazole (pH 7.0), 500 mM KCl, 10 mM MgCl$_2$, and 10 mM EGTA). Polymerized actin was diluted into 1× polymerization buffer without additions or with tested proteins at fixed concentrations to make an actin dilution series. Pyrene fluorescence was measured after 24 h to determine the extent of polymerization, and the data were plotted as a function of actin concentration.

Pointed end elongation assays were performed as described (26) using the gelsolin-actin complex as seeds. Briefly, bovine plasma gelsolin (Sigma, G-8032) was resuspended in G buffer-Ca and dialyzed against a buffer containing 10 mM imidazole (pH 7.0), 0.2 mM EGTA, 0.5 mM DTT, and 3 mM NaN$_3$. Dialyzed gelsolin was mixed with a 2-fold molar excess of Ca-ATP-G-actin in G buffer-Ca supplemented with 0.5 mM CaCl$_2$ and incubated for 2 h at room temperature and overnight at 4 °C to prepare a stock of gelsolin-actin complex (5 μM). Acanthamoeba Arp2/3 complex was purified as described previously (27).

Barbed end elongation assays were performed using either phalloidin-stabilized actin filaments as seeds or spectrin-actin seeds (SAS). To prepare phalloidin-stabilized actin filament seeds, Mg-ATP-G-actin (10 μM, 10% pyrene-labeled) was polymerized at room temperature for 1 h, mixed with an equal volume of 1× polymerization buffer (G-buffer-Mg plus 1× KMEI) containing 10 μM phalloidin (Molecular Probes, P-3456), and incubated at 24 °C for 1 h. Profilin was purified from Acanthamoeba as described previously (27) and used at final concentration of 4 μM in assays employing phalloidin-stabilized actin filament seeds to ensure that actin filament elongation occurs primarily at the barbed end. Spectrin-actin seeds were prepared from human red blood cells as described (28) and were a generous gift of S. Zigmond. SAS were stored in 50% ethylene glycol at −20 °C. Recombinant CapG was a kind gift of F. Southwick.

For barbed end elongation assays, G-actin was added to the reaction as Mg-ATP-actin. A stock solution of monomeric Mg-ATP-actin (20 μM, 5% or 10% pyrenyl-actin) was generated by adding 0.1 volume of a solution containing 10 mM EGTA and 1 mM MgCl$_2$ to a stock of Ca-ATP-actin and incubating for 3 min at 24 °C. Assays were assembled by applying separate drops of F buffer (G-buffer-Mg supplemented with 0.1 volume of 10× KMEI, 10× KMEI buffer, and Mg-ATP-actin in G buffer-Mg (2 mM Tris-HCl (pH 8.0), 0.1 mM MgCl$_2$, 0.2 mM ATP, 0.5 mM DTT, and 0.01% NaN$_3$) to the sides of Eppendorf tubes and mixing by three gentle strokes of pipetting. Test proteins were added either to the F buffer or as separate drops. Actin polymerization was then initiated by the addition of seeds. Actin polymerization was monitored by measuring the change in fluorescence of pyrenyl actin (excitation at 365 nm and emission at 407 nm) using either a PTI fluorescence luminometer (Model QM-1 system) or an LS55 luminescence spectrophotometer (PerkinElmer Life Sciences). The concentration of filament ends was calculated using the equation $R(t) = k_0 \cdot [End(t)](G(t) - C(t))$, where $R(t)$ equals the elongation rate at a given time, $k_0$ equals the monomer association rate constant (10 μM$^{-1}$·s$^{-1}$) (29), $[End(t)]$ equals the concentrates

$^3$ K. Remmert, T. Uruno, and J. Hammer, manuscript in preparation.
tion of free barbed ends (μm) at time t, G(t) equals the concentration of actin monomer at time t (μm), and Cc equals the actin critical concentration (assumed to be 0.2 μm under all conditions). The elongation rate, R(t), is dF(t)/dt = -dG(t)/dt. F(t) is the mass of actin polymer at time t, derived from the fluorescence of pyrene-actin, and G(t) was calculated from G(t) = G(0) - F(t). G(0) equals the original actin concentration. The time-dependent change in the concentration of filament ends was calculated to obtain the concentration of barbed ends at steady state for each condition.

Determination of the Apparent Affinity of GST-AP for CP—An E. coli lysate containing GST-AP was mixed with a lysate expressing GST alone at a volume ratio of ~1.8 and incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) at 4 °C for 4 h. Control beads loaded with GST only were prepared in the same way. Beads were then washed extensively with PBS at 4 °C. To quantitate the amount of GST-AP on beads, 5 μl of washed beads were boiled in SDS-PAGE sample buffer (50 mM Tris-HCl (pH 6.8), 6% SDS, 6% 2-mercaptoethanol, 1.5 mM EDTA, 15% sucrose, 0.06% bromphenol blue) and resolved together with BSA standards (1.36, 2.72, 4.08, 5.44, 6.8, 8.5, 10.9 μg) on a 10–20% gradient SDS-PAGE gel (Invitrogen). The gel was then stained with Coomassie Blue and scanned using an Odyssey Infrared Imaging system (LI-COR Biosciences) at 700 nm. The intensities of the GST-AP and GST bands were converted to protein amounts using Scion Image for Windows (version Beta 4.0.2) (Scion Corp.) and a standard curve generated using the BSA standards. The GST-AP beads used in Fig. 9 contained GST-AP and GST at a molar ratio of 1:18. GST-AP beads were prepared in this way because an extremely high density of GST-AP on the surface of glutathione beads was found to underestimate the GST-AP apparent affinity for CP, presumably because of sterical hindrance due to overcrowding and because it was not practical, given GST-AP high affinity for CP, to add small enough amounts of GST-AP to binding assays when the beads were charged with GST-AP alone. To perform binding assays, purified CP at a final concentration of 9 nM was mixed with different amounts of GST-AP to binding assays when the beads were charged with GST-AP alone. To perform binding assays, purified CP at a final concentration of 9 nM was mixed with different amounts of GST-AP to binding assays when the beads were charged with GST-AP alone.
Amersham Biosciences), and Hyperfilm ECL film (Amersham Biosciences). The film was scanned with an Epson scanner, and the scanned image was analyzed using Scion Image for Windows. Serial dilutions of a control sample containing CP but no beads were used to create a standard curve for quantitating the amount of CP remaining in the supernatant in reactions containing GST-AP or GST beads. The amount of CP associated with beads charged with GST-only was considered nonspecific binding and was subtracted from the amount CP bound to GST-AP beads to calculate specific binding. Data were fitted with the equation for one site binding (hyperbola) using the GraphPad Prism software.

**Assay for the CARMIL-catalyzed Physical Dissociation of CP from Actin Filaments**—F-actin cosedimentation assays were utilized to examine the CARMIL-catalyzed dissociation of CP from actin filaments. Mg-ATP-G-actin (20 μM) was polymerized in F buffer in the presence of phalloidin (20 μM) for 2 h at 24 °C and then diluted with an equal volume of F buffer to prepare a stock solution of phalloidin-stabilized actin filaments (10 μM; non-sheared). To prepare sheared actin filaments, the stock solution of phalloidin-stabilized actin filaments was passed 4 times through a 27–1/2-gauge needle connected to a 1-ml syringe. This material was mixed immediately with CP (final 30 nM) and incubated for 3 min at 24 °C to obtain CP-capped sheared filaments. Each reaction mixture (180 μl total) was prepared in a 7 × 20-mm polycarbonate centrifuge tube (Beckman, number 343775) by adding in order 100 μl of F buffer containing bovine serum albumin (0.15 mg/ml, which serves to block nonspecific binding), 53 μl of non-sheared or sheared actin filaments previously incubated with CP, and a mixture of F buffer and CARMIL fragments (51aa, GST-AP, and GST-APR1098E) totaling 27 μl. Samples were incubated for 5 min at 24 °C and then centrifuged at 200,000 × g for 20 min at 20 °C. 30 μl of supernatant fraction was recovered, mixed with an equal volume of SDS sample buffer, boiled for 5 min, and resolved by SDS-PAGE. The fraction of total CP present in the supernatant was determined by immunoblotting using anti-CP polyclonal antibody and quantitative densitometry as described above.

**Thrombin Digestion of FL-CARMIL**—A stock solution of FL-CARMIL (0.8 μg/μl) was mixed with an equal volume of PBS containing 2 mM CaCl₂ and thrombin protease (final concentration, 0.02 units/μl) (Amersham Biosciences). The mixture was incubated at 24 °C for 2 h. No further fragmentation of FL-CARMIL occurred over an additional 4 h of incubation, as determined by SDS-PAGE analysis. The digested sample was then incubated with 5-aminobenzamidine-agarose beads (Sigma, A-7155) for 1 h at 24 °C to absorb thrombin. The activity of protein samples prepared in this way and stored on ice was constant for at least 24 h. The sites of cleavage in FL-CARMIL were determined by reverse phase HPLC-mass spectrometry (Agilent 1100 series) using freshly digested samples.

**RESULTS**

**Proteins Used in This Study**—Fig. 1A shows the positions within the 1121-residue Acanthamoeba CARMIL polypeptide of the leucine-rich repeat domain, the verprolin-like sequence (V), the acidic domain (A), and the C-terminal proline-rich domain (P), shown previously to contain the CARMIL CP binding site (20) (see the legend to Fig. 1 for additional details). Fig. 1B shows in schematic form the CARMIL proteins and fragments used in this study. Full-length CARMIL (FL-CARMIL) tagged at its N terminus with FLAG was expressed in S9 cells. The GST fusion proteins GST-VAP, GST-VA, GST-AP, and GST-P, which were used previously to map the CP binding site to the CARMIL P domain (20), were expressed in E. coli. The GST fusion protein GST-

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![Image](221x26 to 248x38)

**FIGURE 2.** FL-CARMIL and C-terminal fragments containing its CP binding site inhibit barbed end capping by CP. A–C, shown are barbed end elongation assays in which 1.0 μM G-actin (10% pyrene-labeled) with or without 3 nM CP was mixed with the indicated concentrations of FL-CARMIL (A), GST-VAP (B), GST-VA (B), GST-P (B), GST-AP (C), or ΔP-CARMIL (C), and polymerization was initiated by the addition of 1.25 μM phalloidin-stabilized actin filament seeds supplemented with 4 μM profilin to suppress growth at the pointed end. Actin polymerization was monitored by pyrene fluorescence.

51aa, which corresponds to the C-terminal 51 residues of the P domain/CARMIL polypeptide, was also expressed in E. coli. CARMIL lacking its P domain (ΔP-CARMIL) was expressed as a FLAG-tagged fusion in S9 cells. Fig. 1C shows a Coomassie Blue-stained gel of these proteins after SDS-PAGE analysis. The indicated concentrations of FL-CARMIL (A), GST-VAP (B), GST-VA (B), GST-P (B), GST-AP (C), or ΔP-CARMIL (C), and polymerization was initiated by the addition of 1.25 μM phalloidin-stabilized actin filament seeds supplemented with 4 μM profilin to suppress growth at the pointed end. Actin polymerization was monitored by pyrene fluorescence.
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that of seeds alone (Fig. 2A). An even more striking inhibition of CP activity was obtained, however, using each of the three GST fusion proteins that include the P domain, which contains the CARMIL CP binding site. Specifically, GST-VAP, GST-AP, and GST-P all completely inhibited the activity of 3 nM CP when used at a concentration of 30 nM, restoring the rate of elongation to that of seeds alone (Figs. 2, B and C). In contrast, GST-VA and H9004P-CARMIL, both of which lack the P domain, had no effect on CP activity when used at a concentration of 100 nM (Fig. 2, B and C). Together, these results indicate that CARMIL inhibits the ability of CP to cap the barbed end, that the P domain is both required and sufficient for CARMIL to perform this task, and that the anti-CP activity of FL-CARMIL is modest relative to C-terminal fragments of the protein containing the P domain.

The Anti-CP Activity of CARMIL Resides within Its C-terminal 51 Residues—The P domain (residues 940–1121) can be subdivided into two approximately equal portions based on sequence characteristics. Specifically, the N-terminal portion spanning residues 940–1030 is very rich in prolines (25%) and somewhat hydrophobic (18% Leu, Val, Ile, and Phe; 14% Asp, Glu, Lys, and Arg), whereas the C-terminal portion spanning residues 1031–1121 contains fewer prolines (15%) and is much more hydrophilic (4% Leu, Val, Ile, and Phe; 45% Asp, Glu, Lys, and Arg). Given this, the fact that the two PXXP motifs responsible for the interaction between the P domain and the myosin I SH3 domain reside in the N-terminal portion (between residues 979 and 994) (16) and the fact that CARMIL can bind both the SH3 domain and CP simultaneously (17, 20), we decided to focus on the C-terminal portion of the P domain as the region most likely to inhibit CP function. Based on sequence alignments with other CARMIL P domains (see below), which narrowed our focus even further, we prepared GST-51aa, a GST fusion protein containing the C-terminal 51 residues of the P domain/CARMIL polypeptide (residues 1071–1121). In all of the following experiments, seed elongation assays were performed using actin seeds capped at their pointed end with spectrin (28) since these SAS allow for a more rigorous estimation of dynamics at the barbed end.

We initially retested the anti-CP activity of GST-P using SAS (Fig. 3A). As expected, CP strongly inhibited elongation from SAS, with 3 nM CP blocking ~95% of the barbed ends in the assay (Fig. 3A; see also Fig. 3F, which re-plots the data in Figs. 3, A–E, as the concentration of CARMIL or CARMIL fragment versus the percentage of free barbed ends in the assay relative to the seed-only control; the intercept of all of the curves with the y axis shows the percent of free barbed ends in the presence of 3 nM CP alone (~5%). The addition of increasing amounts of GST-P (20–160 nM) to parallel samples inhibited the activity of CP in a roughly dose-dependent manner up to ~50 nM, at which point ~70% of the barbed ends in the assay appeared to have been freed (Figs. 3, A

FIGURE 3. GST-AP, GST-P, and the C-terminal 51 residues of the P domain/CARMIL polypeptide potently inhibit CP function, whereas FL-CARMIL inhibits CP function only modestly. A–E, shown are barbed end elongation assays in which 2 μM G-actin (5% pyrene-labeled) with or without 3 nM CP was mixed with the indicated concentrations of GST-P (A), GST-AP (B), GST-51aa (C), 51aa (D), or FL-CARMIL (E), and polymerization was initiated by the addition of 0.6 nM SAS. Actin polymerization was monitored by measuring pyrene fluorescence. F, shown are the concentration dependencies and the extents of CP inhibition exhibited by GST-P (open triangles), GST-P after correction for its weak barbed end-capping activity (open squares), GST-51aa (filled squares), 51aa (filled triangles), and FL-CARMIL (filled circles). The concentration of free barbed ends, which reports the extent of CP inhibition, was calculated from the data in A–E (the slope of each polymerization curve is a function of the concentration of free barbed ends; see “Materials and Methods” for details). The concentration of free barbed ends provided by the SAS (0.6 nM) (indicated by the dotted line) was reduced by ~95% by the presence of 3 nM CP.

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The anti-CP activity of FL-CARMIL is modest compared with that of its C-terminal fragments. In agreement with the data in Fig. 2, the C-terminal fragment of FL-CARMIL, GST-AP, and GST-51aa (51aa) by itself had no effect on the rate of elongation at the barbed end (Fig. 5A). Similar to GST-51aa, 51aa by itself had no effect on the rate of elongation at the barbed end (Fig. 5A).

The potency of this 51-residue peptide as a CP antagonist could be further demonstrated by titrating its activity using increasing amounts of CP (Fig. 5). In the absence of the peptide, the addition of increasing amounts of CP (0.1–2 nM) to SAS inhibited assembly at the barbed end in a dose-dependent manner such that CP at a concentration of 2 nM inhibited the assembly rate by ~95% (Fig. 5A). In the presence of a 1200 nM concentration of the 51-residue peptide on the other hand, 2 nM CP had no effect on assembly at the barbed end, and the addition of as much as 100 nM CP only partially inhibited assembly (Fig. 5B). Plotting the data in Figs. 5, A and B, as a function of the concentration of CP (Fig. 5C) shows that the concentration of CP required to cap 50% of the total barbed ends in the assay was ~0.6 nM in the absence of 51aa and ~70 nM in the presence of 51aa. Thus, the presence of this active peptide lowered the effective concentration of CP by ~100-fold. Finally, 51aa itself did not induce spontaneous actin polymerization or affect the rate of elongation when used at a concentration as high as 1800 nM (Fig. 5D).

The anti-CP activity of FL-CARMIL is modest compared with that of its C-terminal fragments. In agreement with the data in Fig. 2, the anti-CP activity of FL-CARMIL estimated using SAS was much weaker than that exhibited by 51aa, GST-P, and GST-AP. Specifically, the addition of increasing amounts of FL-CARMIL (80–640 nM) to SAS in the and F). At higher concentrations of GST-P, however, the rate of actin polymerization began to decrease progressively (Figs. 3, A and F). Control reactions performed without CP (Fig. 4A) revealed that this latter effect was due to an inhibition of actin polymerization by GST-P. Additional experiments showed that this dose-dependent inhibition of barbed end elongation by GST-P was unaffected when the concentration of actin monomers in the assay was raised from 1.0 to 2.5 μM. When the concentration of seeds was tripled from 0.2 to 0.6 nM (Fig. 4B), these results argue that the inhibitory effect of GST-P on barbed end elongation is due to a weak barbed end-capping activity rather than to the sequestration of actin monomers. When the dose response curve for GST-P in the presence of CP was corrected for its inhibitory effect on actin polymerization, GST-P could be seen to potently antagonize CP function (Fig. 3F; see GST-P corrected). Approximately 30 nM GST-P was sufficient to cause an ~50% inhibition of the activity of 3 nM CP, and ~80 nM GST-P was able to free ~95% of the barbed ends in the assay.

Estimation of the anti-CP activity of GST-AP was less complicated because this C-terminal fragment did not inhibit actin polymerization (Fig. 4A). Fig. 3B shows that the addition of increasing amounts of GST-AP (8–300 nM) to assays containing 3 nM CP restored the rate of elongation in a dose-dependent manner. Re-plotting the data in Fig. 3F shows that ~20 nM GST-AP was sufficient to cause an ~50% inhibition of the activity of 3 nM CP, whereas 300 nM GST-AP freed ~90% of the barbed ends in the assay. Consistent with these results, the large shift in critical concentration for actin that occurred upon the addition of 6 nM CP (from 0.3 μM without CP to 0.6 μM with CP) was completely abolished when the assay also contained 120 nM GST-AP (supplemental Fig. S2). To test if the A domain of GST-AP serves to suppress the weak barbed end-capping activity of the adjacent P domain, we prepared a GST-A fusion protein. GST-A by itself had no effect in seed elongation assays (supplemental Fig. S3, inset). More importantly, the inhibitory effect of GST-P on barbed end elongation was not diminished by the addition of 4 μM GST-A (supplemental Fig. S3), indicating that the A domain does not serve to suppress the weak capping activity of the P domain, at least not in trans. We are at present unclear as to the physiological significance of the weak barbed end-capping activity exhibited by GST-P. Importantly, Fig. 4A shows that FL-CARMIL, GST-AP, and GST-51aa/51aa do not inhibit to any appreciable extent the rate of elongation at the barbed end, i.e., they do not exhibit barbed end-capping activity. These results suggest that the weak barbed end-capping activity exhibited by GST-P might represent some kind of spurious gain-of-function resulting from the incorrect or incomplete folding of this isolated, proline-rich domain.

Having confirmed the anti-CP activities of GST-P and GST-AP using SAS, we then tested the ability of GST-51aa to inhibit CP activity. Fig. 3C shows that the addition of increasing amounts of GST-51aa (3.6–280 nM) to assays containing 3 nM CP restored the rate of elongation in a dose-dependent manner. Plotting the data as a function of the concentration of GST-51aa (Fig. 3F) showed that ~20 nM GST-51aa was sufficient to cause an ~50% inhibition of the activity of 3 nM CP, and ~150 nM GST-51aa was able to free ~90% of the barbed ends in the assay. To extend these observations, we repeated these assays using the 51-residue peptide (51aa) obtained after cleavage of GST-51aa with thrombin and removal of the GST moiety. The addition of increasing amounts of this peptide (10–100 nM) to assays containing 3 nM CP caused an even stronger, dose-dependent inhibition of CP function (Fig. 3D), with ~20 nM 51aa sufficient to inhibit the activity of 3 nM CP by ~50% and with just ~100 nM 51aa sufficient to free ~100% of the barbed ends in the assay (Fig. 3F). Similar to GST-51aa, 51aa by itself had no effect on the rate of elongation at the barbed end (Fig. 4A).

The potency of this 51-residue peptide as a CP antagonist could be further demonstrated by titrating its activity using increasing amounts of CP (Fig. 5). In the absence of the peptide, the addition of increasing amounts of CP (0.1–2 nM) to SAS inhibited assembly at the barbed end in a dose-dependent manner such that CP at a concentration of 2 nM inhibited the assembly rate by ~95% (Fig. 5A). In the presence of a 1200 nM concentration of the 51-residue peptide on the other hand, 2 nM CP had no effect on assembly at the barbed end, and the addition of as much as 100 nM CP only partially inhibited assembly (Fig. 5B). Plotting the data in Figs. 5, A and B, as a function of the concentration of CP (Fig. 5C) shows that the concentration of CP required to cap 50% of the total barbed ends in the assay was ~0.6 nM in the absence of 51aa and ~70 nM in the presence of 51aa. Thus, the presence of this active peptide lowered the effective concentration of CP by ~100-fold. Finally, 51aa itself did not induce spontaneous actin polymerization or affect the rate of elongation when used at a concentration as high as 1800 nM (Fig. 5D).
presence of 3 nM CP resulted in only a modest dose-dependent inhibition of CP function (Fig. 3E). Plotting the data as a function of the concentration of FL-CARMIL (Fig. 3F) further emphasized the large difference between FL-CARMIL and the 51-residue peptide in terms of their abilities to antagonize CP function. Specifically, FL-CARMIL required a concentration of \(-350 \text{ nM}\) or about 15 times higher than that of the peptide to inhibit the activity of 3 nM CP by \(-50\%\), and \(640 \text{ nM}\) FL-CARMIL (the highest concentration tested) was able to free only \(-60\%\) of the barbed ends in the assay. Stated another way, 51aa freed \(-90\%\) of the total barbed ends in the assay at the molar ratio to CP.
CARMIL Does Not Antagonize the Activity of Other Barbed End Capping Proteins Such as Gelsolin and CapG—We tested the effect of FL-CARMIL and various CARMIL fragments on the barbed end-capping activities of two additional capping proteins, gelsolin (32) and CapG (33). As expected, control assays showed that gelsolin (Fig. 6A) and CapG (Fig. 6C) both efficiently capped the barbed end. Specifically, 10 nM gelsolin and 50 nM CapG capped >90% of the barbed ends in standard SAS assembly assays. In striking contrast to the results with CP, the addition of FL-CARMIL, GST-AP, GST-51aa, or 51aa over a wide range of concentrations had no effect on the barbed end-capping activities of either gelsolin (present at 10 nM) (Fig. 6B) or CapG (present at 50 nM) (Fig. 6D). These results indicate that the ability of CARMIL to antagonize capping of the barbed end is CP-specific and argue along with other data that the inhibition of CP activity by CARMIL involves its interaction with CP, not with the barbed end.

C-terminal Fragments of CARMIL Potently Uncap CP-capped Actin Filaments, Whereas the Uncapping Activity of FL-CARMIL Is Modest—In all of the experiments with CP described above, CARMIL fragments were mixed together with CP before the addition of seeds. It is unclear from those experiments, therefore, whether these fragments antagonize CP function solely by binding to free CP and inhibiting its association with the barbed end (sequestering) or whether they can also bind to CP already present on the barbed end and promote its dissociation (uncapping). To test for such uncapping activity we incubated a mixture of actin monomers, SAS, and CP for 100 s (after which time the elongation rate remained constant) to prepare CP-capped seeds and then added CARMIL proteins. The addition of increasing amounts of either GST-AP (15–300 nM; Fig. 7A) or 51aa (6–30 nM; Fig. 7B) caused an immediate and dose-dependent stimulation of actin polymerization, indicating the rapid generation of free barbed ends. Moreover, concentrations of just 100 nM GST-AP and 30 nM 51aa were sufficient to induce rates of actin polymerization that were ~80 and ~95%, respectively, that of the seed-only control. Importantly, we did not observe any appreciable delay in the onset of actin polymerization after the addition of either GST-AP or 51aa, indicating that the dissociation of CP from the capped end of filaments occurred within ~25 s (the time between the addition of GST-AP or 51aa and re-starting the recording). This extremely rapid effect argues strongly that these CARMIL fragments forcibly uncapped previously capped filaments rather than simply sequestering CP molecules that dissociated on their own from the barbed end, since the off-rate for this latter reaction (2–10 × 10⁻⁴ s⁻¹) (9, 34) predicts a half-time for such dissociation in our assays of 10–60 min. Moreover, results obtained from F-actin/CP cosedimentation assays (supplemental Fig. S4) provided evidence that the addition of GST-AP or 51aa to CP-capped actin filaments causes the physical dissociation of CP from the filaments.

Fig. 7C shows that whereas FL-CARMIL can also uncap CP-capped actin filaments, it does so much less effectively than GST-AP or 51aa. Specifically, a concentration of 300 nM FL-CARMIL restored the rate of actin assembly to only ~60% that of the seed-only control. By comparison, 30 nM GST-AP and 12 nM 51aa were sufficient to restore the rate of actin assembly to ~60% that of the seed-only control. Together, these data indicate that GST-AP and the active, 51-residue peptide contained within it not only inhibit the barbed end-capping activity of CP, they also actively promote the dissociation of CP from the barbed end of capped filaments. The data also reveal that whereas FL-CARMIL can also uncaps, its uncapping activity is at least 10-fold weaker than that of its active, C-terminal fragments.

The Uncapping Activity of CARMIL Is CP-specific—We next sought to determine whether the uncapping activity of CARMIL is specific for barbed ends capped by CP. To test this, we first prepared gelsolin-capped seeds by incubating a mixture of actin monomers, SAS, and CP for 100 s (after which time the elongation rate remained constant) to prepare CP-capped seeds. The near complete block in barbed end elongation caused by the presence of gelsolin was not relieved by the subsequent addition of 200 nM 51aa, a concentration that robustly restored the rate of elongation from CP-capped seeds (Fig. 8B). Moreover, Fig. 8B shows that the restoration of barbed end elongation caused by the addition of 51aa to CP-capped filaments is subject to rapid inhibition by the subsequent addition of 10 nM gelsolin. Together, these results indicate that CARMIL uncapping activity, like its ability to antagonize barbed end capping, is CP-specific, and they argue that uncapping of CP-capped filaments by CARMIL leaves a free barbed end.

Identification of CAH3, a Stretch of 25 Residues within the Active, 51-Residue C-terminal Fragment of Acanthamoeba CARMIL That Is Highly Conserved in All CARMIL Proteins and That Contains Numer-
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ous Residues Required for Anti-CP Activity—Having demonstrated the importance of the C-terminal 51 residues of Acanthamoeba CARMIL for its anti-CP activities, we searched the sequences of Dictyostelium CARMIL, C. elegans CARMIL, Drosophila CARMIL, mouse CARMIL-1 and CARMIL-3, rat CARMIL-1 and CARMIL-2, and human CARMIL-1 and CARMIL-2 for the presence of similar sequences. The alignments revealed a stretch of 25 residues falling within the 51-residue Acanthamoeba CARMIL sequence that is highly conserved in all ten CARMIL proteins (Fig. 9). We named this sequence CAH3 for CARMIL homology domain 3, since it is the most C-terminal of three regions of sequence that are highly conserved in all CARMIL proteins (the other two being CAH1 and CAH2). Within this 25-residue stretch lies the core sequence LHXGTKXR, which is remarkably conserved. Also highly conserved are residues ELP, found at the N-terminal end, a proline residue at the C-terminal end, and 2–4 basic residues (K/R) that precede this proline (see consensus sequence).

We used site-directed mutagenesis of GST-AP (Fig. 10A) to determine which of the conserved residues marked with an asterisk in the CAH3 domain of Acanthamoeba CARMIL (see Fig. 9) are critical for its ability to inhibit the barbed end-capping activity of CP. As shown in Figs. 10B–D, all 10 mutations affected the anti-CP activity of GST-AP, as judged by changes in both concentration dependence and maximal effect on CP function, although the magnitude and direction of the effects varied. The most dramatic effect was produced by R1098E and R1104E/R1105E, both of which completely abolished the anti-CP activity of GST-AP (Fig. 10B). Almost as detrimental were mutations L1091E (Fig. 10B) and H1093D and H1093L (Fig. 10C). Mutations P1099G and P1107G (Fig. 10D) also significantly impaired the activity of GST-AP, suggesting that these prolines may be important for the local conformation of CAH3 assumed upon binding to CP. In contrast, T1095L weakened activity only slightly (Fig. 10C). Moreover, mutations E1083K and E1088K potentiated the activity of GST-AP (Fig. 10B). Specifically, the concentrations required to inhibit the activity of 3 nM CP by 50% were ~10, ~20, and ~50 nM for E1083K, E1088K, and wild type GST-AP, respectively. Collectively, these results identify a conserved sequence within CARMIL proteins (CAH3) that is crucial for anti-CP activity, estimate the relative contribution to anti-CP activity of individual conserved residues within the CAH3 domain of Acanthamoeba CARMIL, and highlight the extreme importance in this regard of the core portion of CAH3 and the C-terminal basic residues.

GST-AP Binds CP Much More Tightly Than Does FL-CARMIL—The fact that C-terminal fragments of CARMIL such as GST-AP inhibit...
CP much more effectively than FL-CARMIL suggests that the affinity of these C-terminal fragments for CP should be significantly higher than the affinity of FL-CARMIL for CP, which has been reported to be 0.4 μM or tighter (20). To test this idea, we estimated the affinity of GST-AP for CP by pull-down assay using beads coated with either GST-AP or GST alone (Fig. 11). Increasing amounts of these beads were incubated with 9 nM CP and sedimented, and the amount of CP remaining in the supernatant was determined by quantitative immunoblotting (Fig. 11A). The left half of the upper panel in Fig. 11A shows a dilution series (center to left, 1.0 to 0.0) for the supernatant from a control sample that lacked beads. This was used as a standard curve to quantify the depletion of CP from the supernatant after the addition of increasing amounts of GST-coated beads (Fig. 11A, upper panel, center to right) or GST-AP-coated beads (Fig. 11A, lower panel). The inset in Fig. 11B shows these data plotted as the percent of total CP bound to the beads versus the volume of beads used, whereas the remainder of Fig. 11B shows the specific binding of CP by GST-AP (calculated as described in the legend to Fig. 11). The apparent $K_d$ obtained for the interaction between GST-AP and CP in this experiment was 10.1 nM. A second experiment, which contained more data points at low concentrations of GST-AP, yielded an apparent $K_d$ of 19.9 nM (Fig. 11C). We conclude, therefore, that the C-terminal portion of CARMIL binds CP with an affinity of ~20 nM, or about 20-fold more tightly than full-length CARMIL. This large difference in affinity is consistent with the large difference between FL-CARMIL and GST-AP (as well as other C-terminal fragments) in terms of their ability to inhibit CP function.

5 We note that our previous estimate of the affinity of FL-CARMIL for CP based on pull-down assays (100 nM or tighter) is considerably tighter than our previous estimate based on hydrodynamic analyses of CARMIL-CP mixtures (~400 nM) (20). The higher affinity yielded by the pull-down assays is not only more consistent with the extensive co-purification of CARMIL and CP (20) but also with estimates of the affinity of FL-CARMIL for CP based on its dose-dependent inhibition of CP activity (whereas these estimates are complicated by the sigmoidal shape of the FL-CARMIL dose dependency curve (see Fig. 3F), the concentration of FL-CARMIL required for 50% inhibition of CP activity suggests an apparent affinity of <100 nM). Together, these observations suggest that the affinity of FL-CARMIL for CP is significantly tighter than the value of 400 nM highlighted in our previous study, i.e. that the difference relative to the measured affinity of GST-AP for CP (~20 nM) might be less than 20-fold. Having said this, a rough estimate of the affinity of GST-AP for CP based on its dose-dependent inhibition of CP activity suggests that its affinity for CP may also be significantly tighter (~5 nM) than the value of ~20 nM obtained from the direct binding data in Fig. 11. We conclude, therefore, that whatever the absolute affinities are for the interaction of FL-CARMIL and its active fragments with CP, the affinity of FL-CARMIL for CP is probably at least 10-fold weaker than the affinity of its more active, C-terminal fragments for CP.
Cleavage of FL-CARMIL with Thrombin Unmasks Its Anti-CP Activities

We hypothesized that the large difference between FL-CARMIL and its C-terminal fragments with regard to CP affinity and anti-CP activities might be due to the limited accessibility of the C-terminal CP binding site in FL-CARMIL resulting from some form of autoinhibition. As a first test of this idea, we sought a way to relieve this putative structural constraint in FL-CARMIL. We found that incubation of FL-CARMIL with thrombin resulted in the generation of two relatively stable fragments of 96 kDa and 17 kDa (Fig. 12A). Immunoblotting with an anti-FLAG antibody showed that the larger fragment corresponds to the N terminus of CARMIL (data not shown). High performance liquid chromatography coupled with mass spectroscopy confirmed that the larger fragment corresponds to the N-terminal, 95.7 kDa of CARMIL and showed that the smaller 17-kDa fragment was actually a mixture of three fragments of 11.8, 12.7, and 14.6 kDa generated by quantitative cleavage at arginines 867 and 994 and partial cleavage at arginine 985 (Fig. 12B, see the legend for additional details). Importantly, mass spectrometry showed that the most C-terminal fragment, which begins at arginine 994, was intact to the C terminus, i.e. this 14.6-kDa fragment contained the complete, 51-residue active sequence. To test the effect of thrombin digestion on the anti-CP activity of CARMIL, 80 nM concentrations of FL-CARMIL, mock-treated FL-CARMIL, and thrombin-treated FL-CARMIL were added to seeds precapped with 3 nM CP (Fig. 12C). Fig. 12C shows that whereas the two controls antagonized CP function only modestly, the thrombin-digested protein completely blocked CP function. To test the effect of thrombin digestion on the uncapping activity of CARMIL, 40 nM concentrations of FL-CARMIL, mock-treated FL-CARMIL, and thrombin-treated FL-CARMIL were added to seeds precapped with 3 nM CP. Fig. 12D shows that whereas the two controls exhibited essentially no uncapping activity, thrombin-digested CARMIL rapidly induced a rate of actin polymerization that was indistinguishable from that of the seed-only control. Indeed, the uncapping activity of 40 nM thrombin-treated FL-CARMIL was indistinguishable from that of 40 nM GST-51aa in terms of both magnitude and time course (Fig. 12D). Together, these results show that the anti-CP activities of thrombin-digested FL-CARMIL not only exceed to a large extent that of FL-CARMIL, they also approach relatively closely that of the isolated P domain and its active, 51-residue fragment.

FIGURE 11. GST-AP binds CP with an affinity of ~20 nM. CP (9 nM) was incubated for 45 min at 24 °C with increasing amounts of GST or GST-AP immobilized on beads (see “Materials and Methods” for details). After pelleting the beads (bound fraction), the supernatant (unbound fraction) was resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to quantitative, ECL-based Western blotting using an antibody against CPs. Depletion of CP from the supernatant was used to estimate the size of the bound fraction (which equals the difference between the CP input and the CP remaining in the supernatant). The left half of the upper panel in A shows the Western blot for a serial dilution of the supernatant from a “control” sample that lacked beads (shown from center to left are 1.0, 0.8, 0.4, 0.2, and 0.1 times the input CP present in the supernatant of this control sample). This Western blot was subjected to densitometry to generate a standard curve that was then used to estimate the depletion of CP from the supernatant by GST- and GST-AP-coated beads. The right half of the upper panel in A and the lower panel in A show the Western blots of the supernatants from a series of binding reactions containing increasing amounts of GST-coated beads or GST-AP-coated beads, respectively. B, shown in the inset is the raw data from A converted to percent CP bound as a function of the volume of GST- or GST-AP-coated beads, whereas the remainder of the plot shows the data for GST-AP (solid circles) corrected for the background binding by GST-coated beads and converted to the concentration of CP bound as a function of the concentration of GST-AP. The apparent equilibrium dissociation constant (Kd) for these data, which used GST-AP over a concentration range of 0–240 nM, was determined by simple regression analysis of the hyperbolic binding data. C, shown are the results of a second experiment, presented in the same fashion as in B, in which GST-AP was used over a concentration range of 0–60 nM.
conclude, therefore, that the C-terminal ~14 kDa of FL-CARMIL possesses high intrinsic anti-CP activity that can be artificially unmasked by proteolytic cleavage, suggesting that the modest activity of FL-CARMIL is due to some form of autoinhibition.

**DISCUSSION**

Here we have shown that CARMIL antagonizes CP function in two ways. First, by binding to free CP, CARMIL inhibits the association of CP with the barbed end. Second, CARMIL actively removes CP already present on the barbed end. Importantly, these activities are modest for FL-CARMIL and robust for C-terminal fragments containing the CARMIL CP binding site (i.e. CAH3). These differences in activity are consistent with differences in their affinities for CP and likely arise from some form of autoinhibition of the CAH3 domain in the full-length molecule. Our data on thrombin digestion of FL-CARMIL provide support for this idea. We propose that CARMIL reaches its full potential as a CP antagonist *in vivo* after some type of activation event (e.g. protein-protein interaction and/or post-translational modification) that is triggered in concert with the activation of other pathways that generate free barbed ends.

**CARMIL as a CP Antagonist**—We show that CARMIL does not antagonize the barbed end-capping activities of either gelsolin or CapG. This important observation distinguishes CARMIL from VASP, whose anti-capping activity does not discriminate between CP, gelsolin, or CapG (35, 36). VASP is thought to accomplish such a nonspecific effect by binding at or near the barbed end, thereby protecting the end from all types of barbed end-capping proteins (36, 37). The fact that CARMIL does not behave in this fashion, that it does not inhibit the rate of elongation at the barbed end, and that its ability to antagonize the activity of free CP and to uncap CP-capped actin filaments, both, require its direct interaction with CP all argue that CARMIL inhibits CP function by binding to CP rather than to the barbed end.

Uncapping activity requires that the complex of CARMIL and CP associate with the barbed end however transiently *i.e.* that this complex can cap to some finite extent. For this reason it is more precise to say that the binding of CARMIL to CP reduces its affinity for the barbed end.
end. Having said that, the fact that several active C-terminal fragments of CARMIL (51aa, GST-P, and the ~14-kDa fragment liberated from FL-CARMIL by thrombin digestion) appear to completely reverse the effects of CP on barbed end growth argues that the barbed end-capping activity of the CARMIL/CP complex must be quite weak. The weaker the capping activity of the complex is, the more the interaction of CARMIL with free CP should approach true sequestering activity. The situation for FL-CARMIL is more difficult to gauge, however, because the curve describing its dose-dependent inhibition of CP function is sigmoidal and appears to plateau well short of complete inhibition. Whether this latter behavior is a consequence of the complex of CP and FL-CARMIL having more significant barbed end-capping activity will require further work.

CAH3 as a Novel CP Binding Site—Our data indicate that CAH3 is the central element of a novel CP binding site. This highly conserved sequence is predicted to form a short helix followed by a solvent-exposed random coil. The core motif in CAH3 (LXHATKXKR) probably serves as the principal site of contact with CP, since the conserved residues in this motif are all required for normal anti-CP activity, and the spacing between them is maintained in all CARMIL proteins. Basic residues C-terminal of the core motif also appear to be crucial and most likely provide additional contacts with CP, although their precise position relative to the core motif is somewhat variable. The two conserved prolines in CAH3 are probably important for confining the structure of the CAH3 domain and/or for influencing its fit with CP, which may be significantly induced upon contact.

The fact that the CAH3 domain can promote the removal of CP from the barbed end suggests that the contact site(s) on CP for CARMIL is solvent-exposed when CP is bound to the barbed end. Current thinking regarding the mechanism by which CP binds to the barbed end has been shaped largely by the recent determination of the three-dimensional structure of CP (38). This structure shows that the C-terminal ~30 residues of both CP subunits form a flexible, mobile extension called the "tentacle." In the intact CP heterodimer the two tentacles, which are far apart, are thought to make separate contacts with one or more actin molecules exposed at the barbed end (38). Consistent with this "tentacle model," removal of both tentacles abolishes the ability of CP to cap the barbed end (34). Interestingly, CP mutants containing just one tentacle are still able to cap, albeit very weakly (34). These and other observations suggest that the extremely high affinity of CP for the barbed end requires the synergistic action of both tentacles. Given the recent results showing that the affinity of mammalian CARMIL for CP is unaffected by the removal of both tentacles from CP (39), we suggest that conserved basic residues in the CAH3 domain bind to conserved acidic residues in areas of CP that are immediately adjacent to one of the two tentacles (e.g. in helix 5, helix 4, and/or the β-turn regions) and that this and subsequent interactions promote uncapping by inducing a large decrease in the affinity of the adjacent tentacle for the barbed end. Consistent with this idea, the measured off rate for CP mutants missing either one of the two tentacles is ~0.2 s⁻¹ (34). This off rate, which predicts that ~90% of capped ends will have dissociated in ~10 s, is quite compatible with the rate of CARMIL-induced uncapping we measured here, which is vastly more rapid than the dissociation rate for wild type CP by itself (2–10 × 10⁻¹ s⁻¹ (9, 34), which predicts 90% dissociation in 35–180 min). Identification of the site on CP where CAH3 binds represents the first step toward validating this model. On a broader note, we suggest that CAH3, which also appears in several proteins other than CARMIL, is a signature sequence for proteins that interact with CP and antagonize its function.

Autoinhibition of CARMIL—Our data are consistent with the idea that FL-CARMIL exists in a low activity, autoinhibited state where its abilities to inhibit the activity of free CP and uncap CP-capped actin filaments are suppressed. Our working hypothesis, supported by our thrombin cleavage data, is that this low activity state results from some form of intramolecular folding of FL-CARMIL that serves to shield its intrinsically active CAH3 domain from CP. Thrombin cleavage relieves this structural constraint, unmasking the full anti-CP potential of FL-CARMIL. At present, we do not know what physiological event triggers this putative unfolding and activation of FL-CARMIL. One interesting possibility is that the interaction of CARMIL with a Rho-related GTPase is the activating signal. Although a Rho GTPase binding site is not obvious in the primary sequences of the protozoan CARMILs, the three vertebrate CARMILs all contain a consensus CRIB domain-like sequence located near their N terminus, suggesting that they interact with one or more Rho-related GTPases. One distinct possibility, therefore, is that CARMIL proteins are regulated in a fashion similar to proteins like PAK kinase (40), WASp (41, 42), and the formins (43, 44). In these proteins an intrinsically active C-terminal domain is suppressed by an intramolecular folding event that brings the N and C termini of the proteins into close apposition. Binding of a Rho GTPase to the CRIB/ RBD in the N-terminal portion of these proteins triggers their unfolding, resulting in the derepression/activation of their C-terminal domain. For Acanthamoeba CARMIL, we think that thrombin cleavage artificially activates the protein by separating its active C terminus from an autoinhibitory domain in the N terminus (we assume that this putative autoinhibitory domain, which is still present with the active C-terminal fragment in our thrombin-digested material, does not inhibit the active fragment in trans to any significant extent because for such a bi-molecular reaction to have a significant inhibitory effect requires a much higher ratio of the inhibitory domain to the active fragment than the 1:1 molar ratio that exists in the mixture; see, for example, the data for WASp in Rohatgi et al. (45)). In terms of the location of this putative, N-terminal, autoinhibitory domain, we suggest that it resides somewhere between residues 1 and 683, since GST-VAP (684–1121) appears to be fully active. Future studies must identify this autoinhibitory domain in Acanthamoeba CARMIL (and, presumably, in vertebrate CARMILs, although see below), identify the physiological signal that binds to it, and define the molecular mechanism by which this signal induces unfolding and activation.

Control of CP Function by CARMIL—To what extent might CARMIL affect CP function in vivo? The estimated cellular concentrations of Acanthamoeba CARMIL and CP are ~2 and ~1 μM, respectively (21, 22). To us, these concentrations argue strongly that FL-CARMIL must be autoinhibited, since CP function would be dramatically and unnecessarily antagonized by 2 μM CARMIL if it were constitutively active, i.e. if it exhibited an affinity for CP of ~20 nM. Indeed, the vast majority of barbed ends in vivo appear to be capped (46). Given this, the relevant value is more likely the cellular

6 We attempted to apply some quantitative analysis to the data presented in Fig. 5C. If we assume that the complex of 51aa and CP is unable to cap the barbed end, i.e. that only free CP can cap the barbed end with an affinity of 0.6 μM, then the best fit of the data is obtained when the Kₐ of 51aa for CP is assumed to be ~10 μM. This value is much weaker, however, than the Kₐ estimated from 51aa concentration-dependent inhibition of CP activity (~1 μM, Fig. 3F). If on the other hand we assume that the complex of 51aa and CP can cap the barbed end with an affinity of ~120 μM, then the data fit nicely with the Kₐ for 51aa-CP interaction of ~1 μM. We conclude based on these calculations that the complex of 51aa and CP has a finite affinity for the barbed end and that this affinity is ~200-fold weaker than the affinity of CP for the barbed end. Stated in another way, 51aa reduces the affinity of CP for the barbed end by ~200-fold.

7 K. Remmert, T. Uruno, G. Jung, and J. Hammer, unpublished observations.
concentration of activated CARMIL. Indeed, the most pertinent value is probably the local concentration of activated CARMIL, since Dictyostelium CARMIL is not evenly distributed but rather concentrated in regions of active actin assembly such as in leading edge pseudopods and in cup-like cortical projections involved in macropinocytosis (crowns) (17). The maximum possible cellular consequence of activating the anti-CP activities of CARMIL would be the generation of a phenotype that resembles that of cells lacking CP, such as yeast CP-knock-out cells (11), which are devoid of actin cables, and B16 melanoma cells treated with small interfering RNA for CP (15), which exhibit global changes in actin organization. It is almost certainly the case that the physiological activation of CARMIL never results in such a large reduction in CP function (although ectopic overexpression of constitutively active CARMIL fragments might). What seems more likely is that the local activation of CARMIL anti-CP activities facilitates focused actin assembly. These activities could “kick start” local actin assembly by rapidly generating free barbed ends from pre-existing, CP-capped actin filaments and by extending the interval of time between the initiation of growth from these freed ends and the termination of their growth by CP. The ability of CARMIL to inhibit the barbed end-capping activity of CP could also influence the Arp2/3-dependent assembly of branched actin networks and the formin-dependent assembly of unbranched actin structures by increasing the time interval between nucleation and capping, leading to increased filament length. Finally, we note that the functional significance of the modest anti-CP activities exhibited by non-activated CARMIL should perhaps not be dismissed completely given the relatively high cellular concentration of CARMIL.

Direct insight into the function of CARMIL in vivo has come from the characterization of Dictyostelium cells that lack p116/CARMIL (17). These cells exhibit significant deficits in two cellular processes that depend on the actin-rich cortical structures where CARMIL concentrates most dramatically; that is, macropinocytic crowns and pseudopods at the leading edge of chemotaxing cells. Specifically, CARMIL null cells contain 25% less F-actin than wild type cells (17). To the extent that CARMIL functions solely to antagonize CP, Dictyostelium cells that overexpress or underexpress CP might mirror the properties of Dictyostelium cells lacking or overexpressing CARMIL, respectively. Such CP mutants have been characterized (12), so the phenotypes of CP overexpressers and CARMIL null cells can be compared (CARMIL overexpressing cells have not been examined, so they cannot be compared with CP underexpressers). Unfortunately, the parameters measured for CP overexpressers and CARMIL null cells are largely non-overlapping, so they are difficult to compare. One apparent discrepancy is that CP overexpressers move slightly faster than control cells (12), whereas CARMIL null cells take much longer to complete chemotactic aggregation (17). Future studies in Dictyostelium should test the effects on the actin cytoskeleton of overexpressing CARMIL with and without function blocking mutations in its CAH3 domain.

Further insight into the function of CARMIL in vivo has come from a recent study of CARMIL-1 from mouse (mCARMIL-1) (39), which appears to be quite similar to Acanthamoeba CARMIL in terms of its inhibitory effects on CP function. Yang et al. (39) show that green fluo-
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this domain, we suggest that CARMIL may be an important regulator of CP function in vivo.

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