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Purification of Capping Protein Using the Capping Protein Binding Site of CARMIL as an Affinity Matrix

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Abstract

Capping Protein (CP) is a ubiquitously expressed, heterodimeric actin binding protein that is essential for normal actin dynamics in cells. The existing methods for purifying native CP from tissues and recombinant CP from bacteria are time-consuming processes that involve numerous conventional chromatographic steps and functional assays to achieve a homogeneous preparation of the protein. Here we report the rapid purification of *Acanthamoeba* CP from amoeba extracts and recombinant mouse CP from *E. coli* extracts using as an affinity matrix GST fusion proteins containing the CP binding site from *Acanthamoeba* CARMIL and mouse CARMIL-1, respectively. This improved method for CP purification should facilitate the *in vitro* analysis of CP structure, function and regulation.

Keywords

Capping protein; CARMIL; Affinity matrix; CAH3 domain

Introduction

The regulation of actin filament organization and dynamics is essential for numerous, fundamental cellular events such as cell migration, cytokinesis, and vesicle trafficking (for review, see [1]). This regulation requires the action of a large number of actin binding proteins. The actin binding protein Capping Protein (CP) is a ubiquitously expressed, α/β heterodimer that binds with very high affinity ($K_d \sim 0.1$ – 1 nM) to the fast-growing barbed end of the actin filament (for review, see [2]). This interaction blocks further growth at the barbed end, while dissociation of CP from the barbed end allows growth to resume. CP is one of five proteins required for the *in vitro* reconstitution of actin polymerization-driven motility [3], and it is an essential component of the dendritic nucleation model of actin assembly [1]. Moreover, a recent report by Akin and Mullins [4] suggests that blocking the barbed end of actin filaments by CP actually increases Arp2/3 complex-based nucleation and branching. Consistent with all of these observations, perturbation of CP function in living cells causes profound changes in the organization and dynamics of the actin cytoskeleton [5;6;7;8].

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To date, several proteins have been shown to interact with CP (reviewed in [2;9;10]). One of these is CARMIL, an ~125 kDa multi-domain protein that was initially identified based on its interaction with the SH3 domain of type I myosins from *Acanthamoeba* (at which time it was called Acan 125) [11;12]. Subsequent work showed that the *Dictyostelium* homolog of Acan 125 also interacts with CP and the Arp2/3 complex, creating a multi-protein complex that contains a barbed end-directed motor (myosin I), the major terminator of actin assembly (CP), and a major *de novo* nucleator of actin filament assembly (Arp2/3) [13]. Given its central role as a scaffold in forming this complex, the *Dictyostelium* protein was named CARMIL, an acronym for Capping protein ARp2/3 Myosin I Linker. Subsequent efforts to purify CARMIL from *Acanthamoeba* showed that it interacts strongly with CP [14]. Specifically, CARMIL and CP were found to co-purify through a lengthy purification procedure that included ammonium sulfate fractionation, hydrophobic interaction chromatography, SH3 domain affinity chromatography, and anion exchange chromatography. Moreover, 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 greatly 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. Finally, 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 μ M or tighter [14].

Initial domain mapping experiments localized the CP binding site in *Acanthamoeba* CARMIL to the proline-rich, C-terminal ~180 residues of the protein [14], and more recently to the last 51 residues of the protein [15]. This latter sequence contains a stretch of 25 residues that is highly conserved in CARMIL proteins from protozoans, *Drosophila*, *C. elegans*, and vertebrates, and which we refer to as CARMIL Homology domain 3 (CAH3). Consistent with these observations, a 123-residue fragment of mouse CARMIL-1 (mCARMIL-1) termed C1, which contains mCARMIL-1's CAH3, also contains mCARMIL-1's CP binding site [16]. Importantly, binding isotherms obtained using GST fusion proteins containing C1 from mCARMIL-1 and the C-terminal proline-rich domain of *Acanthamoeba* CARMIL yield affinities for CP in the low nanomolar range (~1 nM for C1 and ~20 nM for GST-AP, which contains the C-terminal 256 residues of *Acanthamoeba* CARMIL) [15;16].

Existing protocols for the purification of native CP from skeletal muscle [17] and *Acanthamoeba* [18], as well as untagged, recombinant mouse CP from bacteria [19], are lengthy procedures involving a series of chromatographic steps and time-consuming activity measurements. Here we describe a novel, two-step procedure for the purification of CP from *Acanthamoeba* and recombinant mouse CP from bacteria that uses as an affinity matrix the CP binding site in *Acanthamoeba* CARMIL and mCARMIL-1, respectively, followed by chromatography on Mono Q. This rapid, high-yield method should facilitate the further characterization of CP structure, function and regulation.

Material and Methods

Generation of the Affinity Matrix for *Acanthamoeba* Capping Protein

A GST fusion called GST-AP, which contains the C-terminal acidic and proline-rich domains of *Acanthamoeba* CARMIL (residues 862–1121, which includes the protein's CAH3 domain (residues 1071–1121)), and which was used previously to map the CP binding site in *Acanthamoeba* CARMIL [14], was covalently coupled to Sepharose beads and used as an affinity matrix to purify *Acanthamoeba* CP. Briefly, an overnight culture of *E. coli* strain BL21 harboring the pGEX 2T plasmid containing the AP domain was diluted 1:10 into 200 ml of fresh LB medium containing 100 μ g/ml ampicillin and grown to an OD_{600nm} of 1.0. Protein expression was then induced by the addition of IPTG (isopropyl- β -thio-galactopyranoside) to a final concentration of 0.5 mM. Following incubation for 3 hr at 37°C, the cells were harvested,

resuspended in TBS supplemented with protease inhibitors (1 mM AEBSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 µg/ml pepstatin A), 1 mM EDTA and 1 mM DTT and lysed by two passes through a French press (Thermo Spectronic) at 20,000 psi. Triton X-100 was added to a final concentration of 0.4 %. Following 15-min incubation on ice, the lysate was centrifuged at 12,000×g for 10 min. The supernatant from this spin was then mixed in batch format with 1.0 ml (settled volume) of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 2 hr at 4°C. Following extensive washing of the resin with TBS, the GST-AP fusion protein was eluted with 10 volumes of 20 mM reduced glutathione (#G-4251, Sigma) dissolved in 200 mM Tris (pH 8.0). Following extensive dialysis against coupling buffer (500 mM NaCl, 100 mM NaHCO₃ (pH 8.3)), the sample of GST-AP was batch-incubated with HCl-washed CNBr-activated Sepharose beads (Amersham Pharmacia Biotech) for 2 hours at 20°C. After several washes of the beads with coupling buffer, remaining reactive CNBr-groups were inactivated by 1-h incubation with two column volumes of 100 mM Tris (pH 8.0) on ice. Unbound proteins were then removed by alternating washes with 500 mM NaCl, 100 mM Tris-HCl (pH 8.0) and 500 mM NaCl, 100 mM Na-acetate (pH 4.0). Following a final wash with 5 volumes of TBS, the GST-AP-charged beads were used as the affinity matrix for purifying *Acanthamoeba* CP. The resulting affinity matrix can be reused at least four times without loss in performance. However, it should be re-equilibrated in TBS right after elution of CP (see below).

Purification of *Acanthamoeba* CP from *Acanthamoeba* Cell Lysates

Acanthamoeba cells were grown to late-logarithmic phase as described previously [14] and harvested by centrifugation at 2,000 × g for 20 min at 4°C. Cell pellets were washed twice with 10 volumes of TBS, resuspended in 4 volumes of TBS supplemented with 1 mM DTT and protease inhibitors (2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin, 20 µg/ml AEBSF, and 1 mM EDTA), and lysed using a Parr bomb (375 psi, 5 min). The lysed cell extract was then centrifuged at 100,000 × g for 3 hours at 4°C. The resulting high-speed supernatant from 100 g of wet cell pellet was either subjected to ammonium sulfate fractionation (see text and [14]) or, in the final version of our purification procedure, added directly in batch format to ~2 ml (settled volume) of the GST-AP affinity matrix after addition of Triton X-100 (Pierce) to a final concentration of 0.1 %. Following incubation for 2 h at 4°C with continual mixing, the resin was washed five times with 10 volumes of TBS, and CP was eluted by incubation for 20 min at 4°C with 5 ml of elution buffer (500 mM KCl, 1 mM DTT, 1 mM EDTA, 100 mM MES (pH 5.4)). The eluate was immediately supplemented with protease inhibitors and dialyzed against Mono Q Buffer A (50 mM KCl, 0.5 mM DTT, 1 mM EDTA, 25 mM MOPS (pH 7.3)) for 12 h at 4°C. The dialyzed material was then loaded on a 1 ml Mono Q column (APB) equilibrated with Mono Q Buffer A. After washing the column with five column volumes of Mono Q Buffer A, CP was eluted with a linear gradient of KCl (50 to 500 mM) in Mono Q Buffer A. Fractions enriched in CP, which eluted between 280 and 300 mM KCl (as determined by OD at 280 nm), were pooled and either dialyzed into TBS containing leupeptin (10 µg/ml) and AEBSF (10 µg/ml) for immediate use, or into TBS containing 50% glycerol, leupeptin and AEBSF for long term storage at -20°C.

Generation of the Affinity Matrix for Mouse CP

To generate an affinity matrix for mouse CP, we created a GST-fusion protein (GST-mCAH3) that contains the CAH3 domain of mCARMIL-1 (GenBank accession number AAR96060). The specific fragment of mCARMIL-1 that we used spans mCARMIL-1 residues 964 to 1038. The experiments that led to the choice of these specific residues, which span what we term the a and b sub domains of mCARMIL1's CAH3 domain, and which correspond essentially to the N-terminal 75 residues of the 123-residue CI fragment used by Yang et al. [16], will be described elsewhere (Urano et al. unpublished observations). To create (GST-mCAH3), a DNA fragment containing mCARMIL-1 residues 964 to 1038 followed by a stop codon was amplified from a full-length mCARMIL-1 cDNA using PfuTurbo polymerase (Stratagene) and

the following primers: 5'-CGGGGATCCGTCGGCAAGAGAAGCGAAGCTCGGGG-3' and 5'-AAGCGAATTCTACACCTTCTTGGTGAAAACTCATCTAC-3'. The amplified, 235-bp fragment was then purified using the QIAquick PCR Purification Kit (QIAGEN), digested with Bam HI and Eco RI, repurified, and cloned into the GST vector pGEX-2T vector (Amersham Pharmacia). The final plasmid was verified by DNA sequencing. GST-mCAH3 was expressed in BL21-CodonPlus (DE3)-RIPL cells (Stratagene). An overnight culture of transformed cells was diluted 1:40 in fresh LB medium containing 100 µg/ml ampicillin, incubated at 37°C for 1.5 hr, and protein expression was induced by the addition of IPTG (0.5 mM final concentration). Following incubation at 37°C for 4 h, cells were harvested by centrifugation at $2,500 \times g$ for 20 min at 4°C. To prepare the fusion protein, a cell pellet derived from 300 ml of expression culture was frozen at -80°C, thawed on ice, resuspended in 20 ml of PBS (150 mM NaCl, 5 mM Na₂HPO₄, 1.7 mM KH₂PO₄ (pH 7.4)) supplemented with 1 mM DTT, 1 mM EDTA, and protease inhibitors (Complete, EDTA-free, protease inhibitor cocktail tablet; Roche), and disrupted by three passes through a French press at 20,000 psi. Following addition of Triton-X 100 to a final concentration of 0.4 %, the lysate was incubated on ice for 20 min and centrifuged at $13,200 \times g$ for 60 min at 4°C. The ~20 ml supernatant was then mixed in batch format with 1 ml (settled volume) of Glutathione Sepharose 4B (GE Healthcare) and the slurry was incubated at 4°C for 3 hr with continual mixing. The beads were then washed five times with PBS and packed in a disposable 10 ml column (Bio-Rad) for protein elution. The GST-mCAH3 fusion protein was eluted by application of 1-ml aliquots of elution buffer (20 mM L-glutathione (#G-4251, Sigma) in 200 mM Tris-HCl (pH 8.0)). Eluted fractions were analyzed for protein content by Bio-Rad assay and purity by SDS-PAGE followed by staining with Coomassie Blue. The fractions with highest protein concentration were pooled and dialyzed extensively in Coupling Buffer (500 mM NaCl, 100 mM Tris-HCl (pH 8.0)) by exchanging the buffer 3 times over 2 days. Coupling of GST-mCAH3 to HCl-washed CNBr-activated Sepharose beads was performed as described above for the coupling of GST-AP. The final GST-mCAH3 affinity resin was equilibrated in PBS and stored at 4°C until use. The predicted capacity of this resin is ~1 mg CP per ml resin.

Purification of Recombinant Mouse CP from Bacterial Lysates

Mouse CP (mCP) was expressed as a recombinant, untagged protein using an expression vector carrying the genes for mCP α 1 and β 2 subunits in tandem (a kind gift from Pekka Lappalainen [19]). BL21-CodonPlus (DE3)-RIPL cells transformed with this mCP expression vector were cultured overnight in LB medium containing ampicillin at 200 µg/ml, diluted 1:30 into a fresh LB medium containing ampicillin at 100 µg/ml, and incubated for 2 hr at 37°C before the addition of IPTG (0.5 mM final concentration) to induce protein expression. Following incubation at 34°C for 4 h, the cells were harvested by centrifugation at $2400 \times g$ for 30 min at 4°C and the cell pellet was frozen at -80°C. The lysate from frozen pellets was prepared exactly as described above for GST-mCAH3. The lysate from 200 ml of expression culture was then loaded directly onto 1 ml (settled volume) of GST-mCAH3 affinity resin prepacked in a 10 ml disposable column (Bio-Rad). In early versions of the purification procedure, the lysate was subjected to ammonium sulfate fractionation prior to loading on the affinity matrix (see text). After loading the lysate, the resin was washed with 20 column volumes of wash buffer (50 mM Tris-HCl (pH 7.5), 135 mM NaCl, 0.02% Tween-20, 1 mM EDTA, and 0.5 mM DTT), and mCP was eluted with 10 ml of elution buffer (100 mM sodium acetate (pH 5.2), 500 mM NaCl, 1 mM β -mercaptoethanol, 1 mM EDTA). The eluate was then dialyzed against Mono Q Buffer A and applied to a 1 ml Mono Q column (APB) equilibrated with Mono Q Buffer A. Mouse CP was eluted with using a linear gradient of 50 to 500 mM KCl in Mono Q Buffer A. Mouse CP eluted at ~230 mM KCl. Peak fractions were pooled, dialyzed against the mCP storage buffer (50 mM KCl, 1 mM MgCl₂, 1 mM DTT, 1 mM EGTA, 10 mM imidazole (pH 7.3) [9;15] supplemented with glycerol at a final concentration of 50% and stored at -80°C.

SDS-PAGE and Western Blotting

SDS-PAGE was performed using 4–20 % or 12% Tris/Glycine- buffered polyacrylamide gels (Invitrogen). Samples were denatured in SDS-PAGE sample buffer (2.5% β -mercaptoethanol, 5% SDS, 0.002% bromophenol blue, 0.75% sucrose, 1 mM EDTA and 30 mM Tris/HCl (pH 6.8)) by boiling for 5 min. After electrophoresis, proteins were either stained with Coomassie blue or transferred onto nitrocellulose by semi-dry electroblotting (BioRad). After incubation at 4°C for 16 h in Blocking Buffer (TBS containing 0.1% Tween 20 (TBST) and supplemented with 2% (w/v) BSA or 4% (w/v) nonfat dry milk), blots were probed for 3 hr at room temperature with either a rabbit polyclonal antibody against the alpha subunit of *Acanthamoeba* CP [14] diluted 1:1000 in Blocking Buffer, or a mouse monoclonal antibody against the beta subunit of mCP [20] diluted 1:2000 in Blocking Buffer. Blots were developed with appropriate HRP-conjugated secondary antibodies using an enhanced chemiluminescence system (ECL; APB). For densitometric measurements, films were analyzed using an Alpha Innotech gel documentation system and ChemImager analysis software. The concentration of CP at individual steps in the purification procedure for *Acanthamoeba* CP was determined by quantitative Western blotting of preparation samples along with a CP standard of known concentration (determined by analytical ultracentrifugation) [14]. The concentration of mCP was determined by absorbance at 280 nm using its extinction coefficient ($78.31 \mu\text{M}^{-1}\text{cm}^{-1}$).

Results

Affinity purification of *Acanthamoeba* CP

The generation of the affinity matrix for the purification of CP from *Acanthamoeba* extracts was based on previous mapping experiments, which showed that the C-terminal, ~180-residue proline-rich domain of the 1112-residue *Acanthamoeba* CARMIL polypeptide contains the protein's binding site for CP [14]. Those mapping experiments made use of three GST fusion proteins that span increasingly larger portions of CARMIL's C-terminus: GST-P, GST-AP, GST-VAP ('P' stands for proline-rich domain (residues 940–1121), 'A' stands for acidic domain (residues 862–939), 'V' stands for Verprolin homology domain (residues 684–861)). Comparison of these three fusion proteins (all of which bind CP efficiently; [14]) with regard to their solubility, purity and yield revealed that GST-AP would be the most suitable for generation of the affinity matrix (data not shown). Figure 1 shows a Coomassie-stained SDS-PAGE gel that illustrates the principle steps in the purification of GST-AP from *E. coli* and its subsequent coupling to a solid support. Briefly, expression for 3 h at 37°C resulted in the appearance in *E. coli* extracts of a prominent band corresponding in size (~70 kDa) to the 256-residue AP-domain of CARMIL fused to GST (compare lane 2 to lane 1). Following removal of insoluble material by centrifugation, the high speed supernatant (HSS) (lane 3) was incubated in batch format with Glutathione Sepharose beads, the beads were washed extensively, bound material was eluted with excess free glutathione, and the eluate was subjected to extensive dialysis against coupling buffer. As can be seen in Figure 1, lane 4, the dialyzed eluate contained predominantly the ~70 kDa GST-AP fusion protein, although some bands of smaller molecular mass were present. These bands probably represent truncated fragments of the full-length fusion protein, as is typical for high-level expression in *E. coli*. Finally, the subsequent covalent attachment of the dialyzed GST-AP fusion protein to CNBr-activated Sepharose beads (see Methods) is essentially quantitative since the fusion protein is not typically detected in the flow through from the coupling reaction (lane 5) or in subsequent wash fractions (data not shown).

To prepare the *Acanthamoeba* cell lysate for incubation with the GST-AP affinity matrix, we initially used as the loading material a 25–55% ammonium sulfate cut of the high speed supernatant (HSS) of lysed *Acanthamoeba*, which corresponds to the first step in our standard purification procedure for CARMIL from *Acanthamoeba* [14]. While this ammonium sulfate

fraction contained most of the CARMIL in the HSS ([14]; data not shown), semi-quantitative western blotting showed that a significant portion (~40%) of the total CP in the HSS was present in the pellet of the 0–25% ammonium sulfate cut (Figure 2A; compare lane 2 to lane 1). Conversely, essentially all of the CP present in the HSS was found in the pellet of a 0–55% ammonium sulfate cut (Figure 2B; compare lane 3 to lane 1). Subsequent efforts showed, however, that the final yield and purity of *Acanthamoeba* CP from the GST-AP matrix was not significantly different whether we started with the unfractionated HSS or the 0–55% ammonium sulfate cut of the HSS (data not shown). Given this, and the fact that omission of the ammonium sulfate fractionation step reduced the time between cell lysis and affinity chromatography from 24 h to 8 h, we settled on the direct addition of the unfractionated HSS to the GST-AP affinity matrix for the large scale purification of CP. That said, we wish to highlight the fact that in these analytical experiments ~40% of the total CP loaded on to the GST-AP matrix did not bind. This was routinely the case whether the 0–55% ammonium sulfate cut was loaded on to the matrix (Figure 2B; compare lane 5 to lane 4), or the unfractionated HSS was loaded on to the matrix (Figure 2C; compare lane 3 to lane 2). In both cases, this unbound fraction of CP may correspond to the fraction of total CP that is inaccessible to the affinity matrix because it is in a complex with endogenous *Acanthamoeba* CARMIL.

Large-scale purifications of CP from *Acanthamoeba* HSS were performed as shown in Figure 3, which illustrates the enrichment of CP at each step in the short procedure. Briefly, a high-speed supernatant (HSS) of the crude lysate (lane 1) was supplemented with Triton X-100 (see Methods) and incubated with the GST-AP affinity matrix. Figure 3, lane 2, shows the flow through from the affinity matrix. Following extensive washing, bound CP was eluted by shifting the pH from 7.2 to 5.4 (lane 3). This treatment was shown previously to destabilize the interaction between CP and native CARMIL [14]. The eluate contains predominantly two bands of similar intensities at ~29 and 32 kDa, in agreement with the expected mobility of the two subunits of CP. A minor contamination at ~75 kDa was also present. CP was further purified and concentrated using a Mono Q column, from which CP eluted as a single peak between 280 and 300 mM KCl. As shown in lane 4, the resulting eluate represents an essentially homogenous preparation of CP. Table 1 summarizes the enrichment of CP from the HSS through the Mono Q pool. The yield was typically ~0.5 mg of CP from 50 g of *Acanthamoeba* cells. Using the Bradford colorimetric assay for all determinations of protein concentration, and quantitative Western blotting with a known CP standard [14] to estimate the amount of CP in samples, preps start with an initial apparent CP purity of 0.00073 mg CP/mg total protein in the HSS, and end with a final apparent CP purity of 0.56 mg CP/mg total protein in the Mono Q pool, representing an enrichment of ~770 fold (Table 1). The final apparent CP purity of 0.56 mg CP/mg total protein must represent an approximately two-fold underestimate in purity since the protein is essentially homogeneous by SDS-PAGE analysis (Figure 3). Consistent with this conclusion, we found previously [14] that the Bradford assay underestimates by a factor of ~2 the actual amount of CP, as determined by analytical ultracentrifugation. Of the total CP present in the HSS (2.3 mg), about 30% (0.68 mg) was recovered in the Mono Q pool, indicating that this rapid CP purification procedure is quite robust in terms of yield. Finally, the function of *Acanthamoeba* CP purified by this method was rigorously confirmed by its use throughout an extensive characterization of the anti-CP activities of *Acanthamoeba* CARMIL [15].

Affinity Purification of Mouse CP

Having demonstrated that protozoan CP can be purified to near homogeneity in a single step using as an affinity matrix the CP binding site of a protozoan CARMIL isoform, we next sought to apply this approach to the purification of vertebrate CP. While the GST-AP fusion protein used above does bind CP present in mouse brain extracts and rabbit skeletal muscle (data not shown), we chose to develop an affinity matrix for mouse CP (mCP) that uses the CP binding

site/CAH3 domain of mouse CARMIL-1. This decision was based in large part on the fact that while the core motif in the CAH3 domain (LXHXTKXRPXXXXXP) is highly conserved across species [15], protozoan and metazoan CARMILs differ significantly in the sequence that immediately follows the core motif and that is required for the CAH3 domain to antagonize CP function effectively in a species-specific fashion (Urano et. al., unpublished). Specifically, the required sequence C-terminal of the core motif is rich in basic residues in protozoan CARMILs and acidic residues in vertebrate CARMILs. Moreover, vertebrate CARMILs have a metazoan-specific insert immediately following the core motif (Urano et. al., unpublished observations). Furthermore, the affinity of mCAH3 to mCP is ~1nM, even higher than that of the last 51 residues of *Acanthamoeba* CARMIL that mediates binding to CP [15;16]. Given this, we defined (in experiments to be published elsewhere) a minimal, 75-residue fragment of mCARMIL-1 (residues 964 to 1038) that contains the essential elements of its CAH3 domain/CP binding site and that is efficiently expressed in bacteria as a GST-fusion protein (which we refer to as GST-mCAH3).

To purify mCP, we used GST-mCAH3 covalently coupled to CNBr-activated Sepharose as the affinity matrix and, as a source of mCP, *E. coli* expressing unfused mCP (the $\alpha 1$, $\beta 2$ isoform). Starting with a total lysate of bacteria expressing mCP (Figure 4, lane 1), the GST-mCAH3 affinity matrix removed essentially all of the CP from the lysate (Figure 4, lane 2) and yielded essentially homogeneous mCP following elution at pH 5.2 (Figure 4, lane 3; quantitative densitometry indicated that typically >95 % of the Coomassie blue signal in gels of GST-mCAH3 column eluates arises from the alpha and beta subunits of mCP). Subsequent chromatography on Mono Q removed some of the minor contaminants (Figure 4, lane 4) and concentrated mCP, but minor bands running below intact mCP were usually present to some extent in the final material. We note that these low molecular weight bands precisely co eluted with mCP from Superose-6 and Sephacryl S-300 gel filtration columns (data not shown). This observation suggests that these bands arise from a small fraction of CP that is proteolytically nicked but that otherwise maintains its integrity under native conditions. Consistent with this, micro sequencing of most of the low molecular weight bands present in one preparation showed that they were all derived from mCP (Urano and Zwolak, unpublished observation). We conclude, therefore, that the preparation of mCP obtained by affinity chromatography on the GST-mCAH3 matrix is homogeneous, although a small percentage of the CP molecules have internal cleavages. We note that in some preps we subjected the bacterial lysate to a 0–50% ammonium sulfate precipitation step before applying it to the GST-mCAH3 column. This step was not used routinely, however, because it did not significantly improve the purity of mCP, it reduced the overall yield, and it lengthened the purification procedure.

The yield of mCP is typically ~10 mg CP per liter of *E. coli* expression culture using 8–10 ml of GST-mCAH3 affinity matrix (which is close to the expected capacity of the matrix i.e. ~1 mg CP per ml matrix). We note that the final yield of mCP was often affected by the dialysis step that follows elution of mCP from the affinity matrix. It is not uncommon to see the formation of a precipitate during this dialysis step, although these precipitates tend to contain more of the contaminating bands than intact mCP (data not shown). Some modification of our dialysis conditions might improve the yield. Nevertheless, it is easy to scale the purification up to obtain tens of mgs of mCP. Moreover, the affinity matrix is reusable. With *E. coli* extracts as the source of mCP, we used the GST-mCAH3 resin as many as 10 times with only a small decrease in its capacity. Finally, the function of mCP purified by this method has been rigorously confirmed by its use in ongoing analyses of the anti-CP activities of mCARMIL-1 (Urano et al., unpublished observations).

Discussion

Here we report the two-step purification of *Acanthamoeba* CP from amoeba extracts and recombinant mouse CP from *E. Coli* extracts using as an affinity matrix the CP binding site from *Acanthamoeba* CARMIL and mouse CARMIL-1, respectively, followed by chromatography on Mono Q. This new method, which exploits the high affinity of CP for the CAH3 domain of CARMIL, possesses significant advantages in terms of both protein yield and procedure length over the traditional purification schemes for CP. While our method does involve two steps, essentially all of the purification is provided by the CAH3-domain affinity step (see Figures 3 and 4), making our procedure in essence a one step purification. Our method should facilitate the biochemical and functional characterization of CP, as well as the growing list of proteins and molecules that regulate the activity of CP (CARMIL, V1, twinfilin, PIP2; for review, see [2; 10]).

The original purification scheme for CP from *Acanthamoeba* described by Cooper and Pollard [18] involves the growth of large quantities of amoeba and a labor-intensive purification scheme involving four types of column chromatography (DEAE, gel filtration, hydroxylapatite, and phosphocellulose), all of which have to be assayed by time-consuming functional assays (immunoblotting and reduction in the low shear viscosity of F-actin). The standard yield from this procedure, which typically takes five to six days to complete (unpublished observations), is ~1 mg of CP per 500 g of amoeba cell pellet. By contrast, our new procedure yields *Acanthamoeba* CP that is essentially pure after affinity chromatography using GST-AP, although we do include subsequent chromatography on Mono Q to concentrate the protein and improve its purity slightly. Moreover, our method requires no activity assays, can be completed in less than two days, and yields ~7 mg of CP from 500 g of amoeba, corresponding to a seven-fold increase in yield over the traditional method. Although the initial generation of the CAH3 domain affinity matrix takes about two days, it can be reused at least four times without an apparent loss in performance, although it is advisable to regenerate the matrix quickly after use (see Methods).

Importantly, our affinity purification method also works extremely well for the purification of untagged mouse CP ($\alpha 1$, $\beta 2$) expressed in bacteria [19]. Although the method described by Palmgren et al. [19] to purify recombinant, untagged mouse CP from *E. coli* already represents significant advantages over the purification of CP from vertebrate tissues [17], it still involves ammonium sulfate fractionation followed by three chromatographic steps (hydroxylapatite, Mono Q, and Mono S), requires time-consuming immunoblotting to follow column fractions, and take four days to complete (unpublished observations). By contrast, our new method (affinity chromatography using the CAH3 domain of mCARMIL-1 followed by Mono Q) involves no activity measurements and can be completed in less than two days. Moreover, it can easily be scaled up for the purification of large quantities of mouse CP (we routinely purify 50 –100 mg at a time). While we did not attempt to use our new method to purify mouse CP from mouse tissue or isoforms other than mouse CP $\alpha 1\beta 2$, we see no reason why it should not work in such an application. If it does, it would again represent a major improvement over the established method for purifying CP from tissue [17], which involves four steps (ammonium sulfate fractionation, DEAE, gel filtration, and Mono Q) and functional assays to identify column fractions enriched for CP.

In conclusion, the CP purification protocol described here represents a significant improvement over purification procedures currently in use and should be straightforward to establish in laboratories that are interested in biochemical analyses of CP structure, function and regulation.

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Abbreviations

GST	glutathione S-transferase
IPTG	isopropyl-1- β -galactopyranoside
TBS	Tris-buffered saline
AEBSF	4-(2-aminoethy) benzenesulfonyl fluoride
EDTA	ethylenediaminetetraacetic acid
DTT	dithiothreitol
MOPS	3-(N-morpholino)propanesulfonic acid
PBS	Phosphate-buffered saline
HRP	Horseradish peroxidase

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Figure 1. Generation of the GST-AP affinity matrix

Fractions sampled during the procedure were resolved by SDS-PAGE and stained with Coomassie Blue. Shown are lysates of *E. coli* harboring the GST-AP plasmid before (lane 1) and 3 h after induction of protein expression (lane 2), as well as the HSS (i.e. the soluble fraction) (lane 3) that was then captured on Glutathione Sepharose beads. After elution of GST-AP (lane 4) and dialysis against coupling buffer, the fusion protein was immobilized on CNBr-activated Sepharose. Coupling appeared to be complete, as the flow through from the coupling reaction (lane 5) was devoid of GST-AP. M, molecular mass standards.

Figure 2. Quantitative immunoblot analyses of the distributions of CP in ammonium sulfate fractions and small scale CP purifications using *Acanthamoeba* HSS

The HSS of lysed *Acanthamoeba* was subjected to ammonium sulfate fractionation (Panel A: 0–25% pellet, 25–55% pellet and 55% supernatant in lanes 2, 4 and 3, respectively), a single ammonium sulfate precipitation (Panel B: 0–55% supernatant and 0–55% pellet in lanes 2 and 3, respectively), or not fractionated (Panel C). The 0–55% supernatant in Panel B was then subjected to small scale affinity chromatography on GST-AP Sepharose, and the unbound and bound fractions obtained (Panel B, lanes 4 and 5, respectively). Similarly, the HSS in Panel C was subjected to small scale affinity chromatography on GST-AP Sepharose, and the unbound and bound fractions obtained (Panel C, lanes 2 and 3, respectively). All of the samples in Panels

AC were then probed by Western blotting using a polyclonal antibody raised against the α -subunit of *Acanthamoeba* CP [14]. To allow the estimation of the relative distribution of CP in various ammonium sulfate fractions and in bound versus unbound fractions, the percent of the total fraction loaded was the same for the samples in lanes 2–4 in Panel A, for the samples in lanes 2–3 in Panel B, for the samples in lanes 4 and 5 in Panel B, and for the samples in lanes 2 and 3 in Panel C.

Figure 3. Large scale purification *Acanthamoeba* CP from amoeba lysates using GST-AP as an affinity matrix

Samples taken during the purification procedure were separated by SDS-PAGE and stained with Coomassie Blue. Shown are the HSS of lysed *Acanthamoeba* (lane 1), the unbound fraction from the GST-AP Sepharose affinity step (lane 2), the low pH eluate from the GST-AP resin (lane 3), and the pooled fractions from Mono Q (lane 4). M, molecular mass standards.

Figure 4. Large scale purification of mouse CP from bacterial lysates using GST-mCAH3 as an affinity matrix

Samples taken during the purification procedure were separated by SDS-PAGE and stained with Coomassie Blue. Shown are the HSS of lysed bacteria expressing mCP $\alpha 1\beta 2$ (lane 1), the flow through from the GST-mCAH3 Sepharose affinity column (lane 2), the low pH eluate from the GST-mCAH3 resin (lane 3), and the pooled fractions from Mono Q (lane 4). M, molecular weight standards.

Table 1Quantitation of the purification of *Acanthamoeba* CP using the GST-AP affinity matrix and Mono Q.

	Amoeba HSS	MonoQ Pool	Flow Through GST-AP
Volume [ml]	192	1.5	190
Protein [mg/ml]	16.4	0.8	16.5
Total Protein [mg] ^a	3149	1.2	3135
CP [mg/ml] ^b	0.012	0.45	0.007
Total CP [mg]	2.3	0.68	1.3
Specific Protein [ug/mg]	0.73	560	0.42
Enrichment		770	
Recovery		30%	

^aTotal protein concentrations were determined using the Bradford protein assay with IgG as standard.

^bThe concentration of CP at individual steps in the purification procedure was determined by quantitative Western blotting of samples along with a CP standard of known concentration [13].