The heterodimeric actin-capping protein (CP) regulates actin assembly and cell motility by binding tightly to the barbed end of the actin filament. Here we demonstrate that myotrophin/V-1 binds directly to CP in a 1:1 molar ratio with a $K_d$ of 10–50 nM. V-1 binding inhibited the ability of CP to cap the barbed ends of actin filaments. The actin-binding COOH-terminal region, the “tentacle,” of the CP $\beta$ subunit was important for binding V-1, with lesser contributions from the $\alpha$ subunit COOH-terminal region and the body of the protein. V-1 appears to be unable to bind to CP that is on the barbed end, based on the observations that V-1 had no activity in an uncapping assay and that the V-1 CP complex had no capping activity. Two loops of V-1, which extend out from the $\alpha$-helical backbone of this ankyrin repeat protein, were necessary for V-1 to bind CP. Parallel computational studies determined a bound conformation of the $\beta$ tentacle with V-1 that is consistent with these findings, and they offered insight into experimentally observed differences between the $\alpha$1 and $\alpha$2 isoforms as well as the mutant lacking the $\alpha$ tentacle. These results support and extend our “wobble” model for CP binding to the actin filament, in which the two COOH-terminal regions of CP bind independently to the actin filament, and bound CP is able to wobble when attached only via its mobile $\beta$-subunit tentacle. This model is also supported by molecular dynamics simulations of CP reported here. The existence of the wobble state may be important for actin dynamics in cells.

Myotrophin is a 12-kDa protein identified in hypertrophied rat hearts (1) and dilated cardiomyopathic human hearts (2). Myotrophin was named for its ability to stimulate hypertrophy in cardiac myocytes when added to cells in culture (1). Transgenic mice overexpressing myotrophin in cardiac myocytes develop cardiac hypertrophy and heart failure (3). Myotrophin was found to be identical to the protein V-1 (4), which had been identified based on increased expression in granule cell neurons during development of the rat cerebellum (5). V-1 expression correlates with morphogenetic changes of cerebellar differentiation. V-1 is expressed in many vertebrate cells and tissues (6).

V-1 homologues are present and highly conserved across vertebrates, and similar protein sequences are predicted by the genomes of other animal species, but not by those of fungi or plants. V-1 is important for expression of catecholamine-synthesizing enzymes (7), differentiation and regeneration of skeletal muscle (8), folliculogenesis and corpus luteum formation in the ovary (9), and regulation of insulin secretion (10). We will refer to the myotrophin/V-1 protein as V-1 for simplicity.

Structurally, V-1 is a small ankyrin repeat protein. A solution structure of V-1 shows two full ankyrin repeat motifs in tandem, with additional incomplete repeats at the amino and carboxyl termini (11, 12). An ankyrin repeat consists of a $\beta$ hairpin loop followed by a pair of anti-parallel $\alpha$ helices connected by a short turn sequence (13). Tandem repeats stack on each other, based on hydrophobic interactions between the helices. The loops protrude from this structural backbone, and their more variable sequences impart specificity to interactions with other proteins.

V-1 was found to bind and inhibit actin-capping protein, known as CapZ (14). Capping protein binds to the barbed ends of actin filaments with high affinity ($K_d < 1 \text{ nm}$), which prevents the addition and loss of actin monomers at the end (15). In the cell, CP can cap the barbed ends of filaments created by nucleation. CP is a key component of the dendritic nucleation model (16), and CP is necessary for reconstitution of actin-based motility from pure proteins in vitro (17). In yeast, Drosophila, Dictyostelium, and mouse cells, loss of CP results in abnormalities of actin assembly, morphogenesis, and motility (15).

CP is an $\alpha/\beta$ heterodimer (molecular mass of $\alpha \sim 36 \text{ kDa}$, $\beta \sim 32 \text{ kDa}$). Vertebrates have two major isoforms of each subunit. In striated muscle cells, the $\beta_1$ isoform is found at the Z-line, the $\beta_2$ isoform is found elsewhere, and the two $\beta$ isoforms cannot functionally substitute for one another (18). Nonmuscle cells generally express only the $\beta_2$ isoform (19). The COOH-terminal regions of the $\alpha$ and $\beta$ subunits are on the surface of the protein (20), and both contribute to high affinity capping of the barbed end of actin (21). In previous work, CP was pulled down by TAP-tagged V-1 expressed in cultured 293T cells (14). The $\alpha$ subunit was the $\alpha_1$ isoform, and the $\beta$ isoform was not determined. In those studies, purified V-1 was found to bind and inhibit recombinant CP $\alpha_1/\beta_1$ (14).

The mechanism and molecular details of the interaction of V-1 with CP and its functional implications in cells are not known. In this study, we investigate the interaction of V-1 with CP, including
Interaction of Myotrophin/V-1 with CP

a structure–function analysis with mutational and computational approaches. The results have relevance to our understanding of how CP binds to the barbed end of the filament.

EXPERIMENTAL PROCEDURES

Reagents, General Procedures, Peptides, and Proteins—Restriction endonucleases and DNA-modifying enzymes were purchased from Invitrogen, Roche Applied Science, or New England Biolabs. Oligonucleotides were purchased from the Protein and Nucleic Acid Chemistry Laboratory, Washington University Medical School (St. Louis, MO). PCR was performed according to the manufacturer’s directions (PerkinElmer Life Sciences). Bacterial media preparation, restriction digests, and ligation of DNA were performed by standard protocols. Peptides corresponding to the COOH-terminal regions of the β1 and β2 isoforms of CP (21) were generated by an automated peptide synthesizer and purified by high pressure liquid chromatography (Biomolecules Midwest, St. Louis, MO). Actin, spectrin-F-actin seeds, CP, and CP mutants were purified as described (21).

To express GST-V-1 in bacteria, a 359-bp V-1 cDNA sequence was PCR-amplified with forward and reverse primers CCG GAA TTC TTA TGT GCG ACA AGG AGT TCA TG and CCC AAG CTT CTA CTA CGG GAG AAG AGC TTT GAT TG from a human embryonic cDNA library and cloned into pGEX-KG (Amersham Biosciences) to produce pBl 1526.

V-1 mutants were generated via PCR-based mutagenesis using this plasmid as the template, as described (23). The forward and reverse primers, respectively, for the mutagenesis were as follows: V-1 Δ1–20, TAT GTG GCC AAG GGA GAA GAT GTC AAC, AAG TAT ACC ACC ACC ACC ACC GGA AAC, V-1 Δ1–94, TTC TCA TCG TGA CTG ACG ATC TGC CTC GCG CTG TTC, TTC TTT CAC CTC ATC CAA GTA CAG TCC GTT TTT CAG GCC; V-1 Mut Loop1, GCA GCA ATT ACT CCT CTT CTG TCT GCT GTC TATGAG, AGC AGC TCC GTT TTT CAG GGC; V-1 Mut Loop2, TTC TCA TCG TGA CTG ACG ATC TGC. The numbers of the resulting plasmids were as follows: V-1 Δ1–20, pBlJ 1683; V-1 Δ1–94, pBlJ 1684; V-1 Mut Loop1, pBlJ 1532; V-1 Mut Loop2, pBlJ 1587. DNA sequencing and restriction digests confirmed the identity of the construct and the lack of mutations.

Expression was induced with 100 μM isopropyl-1-thio-D-galactopyranoside, and GST-V-1 was purified as described (24) with minor changes. The concentration of GST-V-1 was determined by absorbance at 280 with a calculated extinction coefficient of 50,570 M⁻¹ cm⁻¹. GST-V-1 on glutathione-agarose beads was cleaved with thrombin (Amersham Biosciences), according to the manufacturer’s instructions. The V-1 protein was tested for purity by SDS-PAGE, and the concentration was calculated by absorbance at 280 nm with a calculated extinction coefficient of 9520 M⁻¹ cm⁻¹. Purified GST-V-1 was thrombin-cleaved V-1 inhibited CP in a similar manner in functional assays. Cleaved V-1 was used in all of the functional and physical assays described here, except the uncapping assays, which were done with GST-V-1 protein. V-1 mutant proteins were purified similarly, and their folding was tested by circular dichroism spectroscopy as described (25) with a JASCO J-600 spectropolarimeter.

Chicken CP α1β1; CP truncation mutants CPα1(ΔC28)β1, CPα1β1(ΔC34), and CPα1(ΔC28)β1(ΔC34); and mouse CP α1β2 were expressed and purified as described (26) from pBlJ 994, 1533, 1534, 1536, and 1219, respectively. A mouse CP α2β2 expression plasmid, pBlJ 1603, was constructed using a similar strategy (26) in pET-24a (Novagen) by Dr. Ilgu Kang. The α2 and β2 sequences were PCR-amplified from cDNA-containing plasmids, pBlJ 601 and 622, respectively (19, 27). Primers included restriction enzyme sites for Ndel/HindIII and Ndel/PvuI. The PCR-amplified α2 sequence was inserted between the Ndel and the HindIII sites of the vector, and β2 was inserted between the Ndel and PvuI sites. Each subunit had a separate ribosomal binding site to maximize translational efficiency. PCR-induced single base errors were corrected by site-directed mutagenesis. Purified CP was stored at −20 °C in 10 mM Tris-HCl, pH 8.0, 40 mM KCl, 0.5 mM dithiothreitol, and 50% glycerol. The concentration of CP was determined by absorbance at 280 with extinction coefficients of 76,300 M⁻¹ cm⁻¹ for α1β2, 78,450 M⁻¹ cm⁻¹ for α1β1, and 79,300 M⁻¹ cm⁻¹ for α2β2, calculated with ProtParam at www.ExPASy.org, which uses values from Pace et al. (28). Actin Polymerization Assays—Actin was purified and labeled with pyrenylidooacetamide (Molecular Probes, Inc., Eugene, OR), and actin polymerization and capping assays were performed as described (29). The uncapping assay was performed as described (30), using PIP2 as a positive control.

Fitting to Determine Binding Constants—Binding constants for capping of actin filaments by CP were determined by kinetic modeling of actin polymerization assays, using Berkeley Madonna as described (21). The kinetic mechanism used is shown in Reactions 1–3, where A represents actin monomer, Nb is free barbed ends, CP is capping protein, and V is V-1. The complex generated by CP and V-1 is represented as CPV. The on- and off-rate constants were determined from least-squares fitting for the full time course of the reaction.

The initial concentration of Nb was set equal to the concentration of free barbed ends created by the spectrin-F-actin seeds alone in control polymerization reactions. The barbed end on-rate and off-rate constants for actin elongation were 11.6 μM⁻¹ s⁻¹ and 1.4 s⁻¹, respectively (31). The rate constants k⁺cap and k⁻cap in Reaction 2 were fit with results from experiments with CP added, and those values were consistent with previous studies (21). When V-1 was
added, the results were used to determine $k_{+V}$ and $k_{-V}$ in reaction 3. In this model, the CPV complex has no capping activity. CP mutants with single $\alpha$ or $\beta$ COOH-terminal truncations can cap actin, so the concentration of the CP mutant was substituted for that of WT CP in Reactions 2 and 3. The CP mutant lacking both COOH-terminal regions, referred to as CP$_{\Delta\Delta}$, does not cap actin, so we performed a competition experiment with WT CP and added Reaction 4 to the kinetic mechanism.

$$\begin{align*}
CP_{\Delta\Delta} + V & \rightleftharpoons CP_{\Delta\Delta}V \\
& \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 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For the steady-state assay, the reaction mechanisms were the same. The $K_d$ value for CP binding to the barbed end was determined first, from a CP titration experiment. Next, with a constant CP concentration, a V-1 titration was performed. Those results were used to determine the $K_d$ for V-1 binding to CP. Least-squares fitting by nonlinear regression was performed using Prism 4.0 (GraphPad Software, San Diego, CA). In this analysis, the number concentration of actin filament barbed ends is assumed to be much less than the concentration of CP, so that free [CP] is set equal to total [CP].

**Tryptophan Fluorescence of CP Binding to V-1**—Intrinsic tryptophan fluorescence emission spectra for WT and mutant CP proteins were collected as described (32). A continuous variation assay was done to determine the stoichiometry of CP $\alpha_1\beta_1/\alpha_1\beta_2$ binding to V-1. The ratios of the two proteins were varied while maintaining the total protein concentration at 10 $\mu$M, which was well above the experimentally determined $K_d$ of ~40 nm.

**Molecular Simulations**—The starting point for our computational work was the crystal structure of CP (Protein Data Bank code 1IZN) and the NMR structure of V-1 (Protein Data Bank code 1MYO). We first selected the COOH-terminal portion of the $\beta_1$ subunit of CP, which corresponds to the peptide used in the experiments, and simulated the molecular dynamics of the peptide structure using Gromacs 3.2 (33). Following minimization and heating to 300 K, we performed 10 ns of NVT simulations with the OPLS/AA force field, SPC waters, Particle Mesh Ewald, Berendsen temperature coupling (NVT ensemble), and 2-fs time steps. Structures were written out every 10 ps, resulting in an ensemble of 1000 structures. Each of these peptide structures was then used in docking studies using AutoDock 3.0 (34). Docking grid maps were created for 17 NMR V-1 structures in the Protein Data Bank file, and all 1000 tentacle structures were docked to each map, resulting in a total of 17,000 trajectories. The docking results were ranked by energy and clustered based on a root mean square deviation of 4 Å. The cluster analysis resulted in a single top-ranked struc-
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**RESULTS**

**Molecular Dynamics Simulation of CP**—We used molecular dynamics to investigate the range of structures that CP might occupy in solution. Beginning with the x-ray crystal structure, we performed a 20-ns molecular dynamics simulation (see “Experimental Procedures” for details). For the full-length wild type protein, the position of the COOH-terminal α-helical region of the β subunit fluctuated to the highest degree, as illustrated in Fig. 1. The COOH-terminal α-helical region of the α subunit moved much less, remaining in position on the surface of the protein, primarily through the interactions of Trp279. The loop proximal to the helix and the COOH-terminal extension distal to the helix did show some movement, but the α-helix itself moved minimally. These results are further illustrated with plots of root mean square fluctuations in Fig. 2, C and D.

We then asked how truncating the actin-binding COOH-terminal regions of the subunits affected the structure of the rest of the protein. These mutants have been used in the past and are used here, below, to analyze how CP binds to the barbed end of the actin filament. Upon removal of the α subunit COOH-terminal region, fluctuations were significantly decreased in a region of the α subunit on the other side of the protein, near the COOH-terminal region of the β subunit, whereas the β subunit showed little change apart from the helix that contacts the α subunit COOH terminus. These results are illustrated in Fig. 2. This analysis is important for several reasons. The region of the α subunit exhibiting the change in dynamics is where most of the differences between the α1 and α2 isoforms are located, and this region is part of the contact site for V-1 predicted by our docking analysis. This correspondence is in line with experimental observations, described below, that the α1 and α2 isoforms have slightly different affinities and that the truncation of the α COOH terminus has a minor effect on the affinity for V-1. The mobility of the β subunit COOH terminus in the molecular dynamics simulation strongly suggested that its movements were independent of the rest of the protein. These points are addressed further under “Discussion.”

**V-1 Inhibits the Actin-capping Activity of CP**—To investigate whether V-1 influences the capping activity of CP α1β2, which includes the major β isoform expressed in nearly all vertebrate cells and tissues, we performed seeded actin polymerization assays. CP α1β2 inhibited barbed end polymerization in a dose-dependent manner (Fig. 3A). The equilibrium dissociation constant for CP binding to barbed ends ($K_{\text{d}}$) was 0.1 nM, consistent with previous results (21). The addition of increasing concentrations of V-1 inhibited the capping activity of 2–4 nM CP, with complete inhibition at 500 nM V-1 (Fig. 3B). V-1 alone, without CP, had no effect. Kinetic modeling with a simple model of V-1 binding CP and completely inhibiting the ability of CP to cap the barbed end gave good fits (Fig. 3B, red lines), with a $K_{\text{d}}$ of 40 ± 9 nM for the V-1-CP interaction (Table 1).

The $\beta_1$ isoform of CP is specific for the Z-line of striated muscle. Assays with CP α1β1 gave similar results, with a $K_{\text{d}}$ of 45 ± 3 nM (Fig. 3, C and D, Table 1). The α1 and α2 isoforms of CP are widely expressed, in a ratio that varies among cell and tissue types (27). CP α2β2 capped actin with a $K_{\text{cap}}$ of 0.2 nM (Fig. 3E). V-1 inhibited the capping activity of

**TABLE 1**

| Binding Conditions | $K_{\text{d}}$ (nM) |
|--------------------|-------------------|
| CP and V-1         |                   |
| WT CP α1β2 + V-1   | 40 ± 9            |
| WT CP α1β1 + V-1   | 45 ± 3            |
| WT CP α2β2 + V-1   | 153 ± 20          |
| CP mutants and V-1 |                   |
| CPα1(ΔC28)β1 + V-1 | 65 ± 7            |
| CPα1β1(ΔC34) + V-1 | 270 ± 6           |
| CPα1(ΔC28)β1(ΔC34) + V-1 | 262 ± 35 |
| CP β1 28-aa peptide + V-1 | 70, 100 |
| CP β1 34-aa peptide + V-1 | 64, 70 |
| CP and V-1 mutants |                   |
| WT CP + V-1 Mut Loop 1 | 4450, 4506 |
| WT CP + V-1 Mut Loop 2 | 1363, 1547 |
| WT CP + V-1 Δ1–94 | 6300, 6750        |
| WT CP + V-1 Δ1–20 | 59, 67            |

**FIGURE 4.** V-1 inhibition of CP in a steady-state assay. Pyrene-actin fluorescence, which is proportional to the concentration of filamentous actin, is plotted. A, increasing concentrations of CP α1β2, in the absence of V-1, causes the F-actin level to fall, because the critical concentration rises to that of the pointed end. B, increasing concentrations of V-1 added to 75 nM CP antagonize this inhibitory effect and promote actin polymerization.
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CP α2β2, and kinetic modeling gave a $K_d$ of 153 ± 20 nM (Fig. 3F, Table 1).

In addition to actin polymerization growth assays, we tested the binding affinity of V-1 for CP α1β2 with a steady-state polymerization assay. Increasing concentrations of CP caused the steady-state level of F-actin to decrease and plateau, characteristic of barbed end capping, which increases the critical monomer concentration to that of the pointed end (Fig. 4A). Increasing concentrations of V-1 reversed the effect of CP (Fig. 4B). The $K_d$ for V-1 binding to CP was 10–30 nM in three experiments, based on fitting to the same simple reaction mechanism used for the kinetics assays.

Some inhibitors of CP, such as CARMIL or PIP$_2$, can rapidly reverse the effect of CP on actin, implying that they can dissociate CP from the capped barbed ends in addition to preventing CP from binding to the barbed end (30, 36). We tested V-1 in an uncapping assay (Fig. 3G). The addition of GST-V-1 at concentrations up to 1 μM, which was sufficient to completely inhibit the capping activity of CP in Fig. 3B, had no effect in the uncapping assay, for two CP concentrations (Fig. 3G, curves 4 and 6). As a positive control, PIP$_2$ produced a rapid increase in the polymerization rate, approaching the maximal rate of actin polymerization in the absence of capping protein (Fig. 3G, curves 2, 3, and 5), which is consistent with complete uncapping.

Stoichiometry of CP Binding V-1—These analyses assume a 1:1 stoichiometry for CP binding to V-1, which is supported by previous results with native PAGE (14). To measure the stoichiometry, we used intrinsic tryptophan fluorescence and the continuous variation method (37). The two proteins were mixed in various ratios, with a constant total protein concentration of 10 μM, well above the $K_d$. CP has higher intrinsic fluorescence, due to its 9 tryptophan residues, than does V-1, with a single tryptophan residue. The presence of V-1 increased the intrinsic fluorescence of CP. The maximum fluorescence intensity was seen at concentrations of 5 μM for each of CP and V-1 (Fig. 5), indicating a 1:1 molar ratio for the complex.

V-1 Interaction with CP Mutants Lacking the COOH-terminal Actin-binding Regions—We asked which regions of CP bound to V-1. We tested truncation mutants of CP α1β1 lacking one or both COOH-terminal actin-binding sites with functional assays of actin capping. CPα1(ΔC28)β1, which lacks 28 amino acid residues at the COOH terminus of the α1 subunit, capped actin with a $K_{cap}$ of 640 ± 240 nM, consistent with previous studies (21). V-1 inhibited the capping activity of
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CP β COOH-terminal Peptides Bind V-1—The above results show that the CP β COOH terminus is important for CP to bind V-1. We asked whether peptides corresponding to the β subunit tentacle in CP αβ1 would be sufficient to bind V-1, using functional actin-capping assays. Two peptides, of 28 and 34 aa, were tested. Each peptide was able to cap barbed ends, with $K_{\text{cap}}$ values of 500 ± 9 nM, similar to previous findings (21). In the presence of a 5 µM concentration of the 28-aa β peptide (Fig. 7C), V-1 concentrations up to 1.5 µM were able to partially inhibit the capping activity. For the 34-aa peptide, we added 0.5 and 0.8 µM V-1 to 1.5 µM of the peptide and saw partial inhibition (Fig. 7D). Modeling gave a $K_d$ of ~65 nM for the 34-aa peptide and ~85 nM for the 28-aa peptide (Table 1), which are similar to the $K_d$ of 65 nM for V-1 binding to the CP α COOH terminus truncation mutant and greater than the $K_d$ of 40 nM for WT CP. Thus, the CP β1 COOH-terminal tentacle is sufficient as well as necessary for binding to V-1.

We also tested a 34-aa peptide corresponding to the COOH terminus of the β2 isof orm (21). Much higher concentrations of this peptide were needed to cap actin (Fig. 7A), presumably because the peptide folds poorly, so we were not able to analyze the data with kinetic modeling. We did observe that V-1 was able to inhibit the capping activity of the β2 peptide (Fig. 7B). V-1 binding to both the β1 and β2 peptides of CP corroborates our finding that V-1 is able to bind to both the β isof orms of CP in the context of full-length heterodimeric protein.

The Loops of V-1 Are Needed to Interact with CP—We asked which regions of V-1 are necessary for its interaction with CP. Ankyrin repeat proteins often interact with other proteins by their loop regions (13), and V-1 has two such loops, which we refer to as ANK1 and ANK2 (Fig. 8A). Loop regions from different ankyrin repeat protein families generally show little sequence similarity (13), but the sequences of the loops of V-1 are nearly identical across vertebrates (Fig. 8B).

We assayed the ability of V-1 mutants to inhibit CP in seeded actin polymerization growth assays. To investigate the importance of the ANK1 loop, we mutated four residues at the tip of the loop, Glu$^{33}$-Arg$^{36}$, to alanine. This V-1 mutant showed normal α-helical content by circular dichroism, as did all of the V-1 mutants except as noted. The mutant had very little effect on CP activity, even with a 10 µM concentration of the V-1 mutant and 4 nM CP (Fig. 8C, compare with Fig. 3A). Kinetic modeling gave fits (Fig. 8C, red lines, inset is a zoomed image of initial time) with a $K_d$ of ~4500 nM (Table 1), which is ~100-fold weaker than the $K_d$ of WT V-1 for CP. We tested the ANK2 loop in a similar way by changing the

CPα1(ΔC28)β1 (Fig. 6A, black lines), and kinetic modeling gave good fits (red lines) with a $K_d$ of 65 ± 7 nM (Table 1), slightly weaker than for full-length CP αβ1.

CPα1β1(ΔC34), lacking the COOH-terminal 34 amino acids of the β subunit, capped actin with a $K_{\text{cap}}$ value of 41.3 ± 2.2 nM, consistent with previous findings (21). V-1 also inhibited the capping activity of this mutant (Fig. 6B, black lines), and modeling gave a good fit with a $K_d$ of 270 ± 6 nM (Table 1), ~6-fold weaker than that of full-length WT CP αβ1.

A CP mutant with truncation of both COOH-terminal regions CPα1(ΔC28)β1(Δ34) had no capping activity, as in previous studies (21). To measure binding of V-1 to this CP mutant, we performed actin polymerization assays in the presence of WT CP, asking if increasing concentrations of the mutant CP would compete with WT CP for binding to V-1. The addition of the CPα1(ΔC28)β1(Δ34) mutant neutralized the inhibitory effect of V-1 and restored the activity of WT CP to a nearly normal level. We modeled the reaction assuming that V-1 can bind the mutant or WT CP. The affinity of V-1 for WT CP αβ1 was set to the value determined above. For V-1 binding to the CPα1(ΔC28)β1(Δ34) mutant, the $K_d$ value was 262 ± 35 nM (Table 1), ~6-fold higher than that of WT CP and similar to that of the CP mutant lacking only the β tentacle. Thus, the results of the actin-capping assays with the CP truncation mutants indicate that the β subunit COOH-terminal region is important for binding V-1 and that the α subunit COOH-terminal region and the body of CP make lesser but significant contributions.

We also assayed the ability of V-1 mutants to inhibit CP in seeded actin polymerization growth assays. To investigate the importance of the ANK1 loop, we mutated four residues at the tip of the loop, Glu$^{33}$-Arg$^{36}$, to alanine. This V-1 mutant showed normal α-helical content by circular dichroism, as did all of the V-1 mutants except as noted. The mutant had very little effect on CP activity, even with a 10 µM concentration of the V-1 mutant and 4 nM CP (Fig. 8C, compare with Fig. 3A). Kinetic modeling gave fits (Fig. 8C, red lines, inset is a zoomed image of initial time) with a $K_d$ of ~4500 nM (Table 1), which is ~100-fold weaker than the $K_d$ of WT V-1 for CP. We tested the ANK2 loop in a similar way by changing the
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FIGURE 8. Effect of V-1 mutants on capping activity of CP. A, NMR solution structure of V-1 adapted from Yang et al. (11). The α helices are labeled as α1–α8. The loops of the complete ankyrin repeats are labeled ANK1 and ANK2. Residues EGGR (arrow; ANK1) and KHHI (arrow; ANK2) were mutated to alanine. Arrows mark the start of the truncation mutants V-1 Δ1–20 (deletion of the first 20 amino acids) and V-1 Δ1–94 (deletion of the first 94 amino acids). B, alignment of V-1 sequences from various organisms, with some features from α labeled. A period indicates a residue identical to the one in the top line, mouse V-1. The mutated loop regions, boxed, have very similar sequences among vertebrates. The alignment was performed with ClustalW. For C–F, actin polymerization is plotted versus time for a seeded growth assay. 2 μM actin, 4% pyrene-labeled, was used with indicated concentrations of V-1 mutants, lacking the first 20 residues of the NH2 terminus, V-1 Δ1–94. The mutant completely inhibited CP (Fig. 8F). Kinetic modeling gave good fits (Fig. 8F, red lines) with a $K_d$ of −60 nM (Table 1). Finally, we tested a V-1 Δ1–94 mutant, lacking the two complete ankyrin repeats. This mutant had no α-helical structure by circular dichroism, as expected. The mutant had very little effect on CP activity (Fig. 8E, Table 1), and the mutant alone had no effect on actin polymerization. Thus, the loops of the V-1 ankyrin repeats are necessary for its interaction with CP.

Molecular Docking of CP and V-1—In order to gain more structural insight into the binding of V-1 and CP, we performed molecular dynamics on the β1 tentacle and used these structures in molecular docking studies with the NMR solution structure of V-1 (see “Experimental Procedures”). Analysis of these results provided us with a clear prediction for the V-1-β1 tentacle complex. The tentacle binds between the ankyrin repeats on V-1, making significant interaction with both loop regions (Fig. 9). Therefore, this structure agrees well with the in vitro mutagenesis results. Taking this structure for the peptide-V-1 complex and fitting it back onto a full-length CP structure, generated from a molecular dynamics simulation, showed that V-1 appears to bind between the β tentacle and the main body of CP (Fig. 9).

DISCUSSION

In the present study, we characterized the binding and inhibition of the nonmuscle and sarcomeric isoforms of CP by V-1, and we investigated the regions of CP and V-1 involved in this interaction. In addition, we used computational simulation and docking approaches to construct a model for the interaction of CP with V-1. Predictions from that model agree with the results from the mutagenesis studies.

Based on physical binding assays, we found that V-1 binds to CP in a 1:1 complex. Actin polymerization assays of the capping activity of CP gave a $K_d$ value of −40 nM, using a simple model in which the CP-V-1 complex was 1:1 and had no ability to cap the barbed end. These results and conclusions are similar to those of a previous study (14).

This simple model fit all of the data well, and more complex models in which CP-V-1 complex had weak capping activity did not improve the fit. At high concentrations of V-1, the level of inhibition saturated at zero capping activity for CP. The protein CARMIL also binds and inhibits CP, but the CARMIL-CP complex has detectable, albeit weak, effect on CP activity (Fig. 8E, Table 1), and the mutant alone had no effect on actin polymerization. Thus, the loops of the V-1 ankyrin repeats are necessary for its interaction with CP.

CP is a central element of the dendritic nucleation model for
Arp2/3 complex-mediated actin assembly, which may account for much of actin-based motility in cells (16). In vitro, CP is constitutively active, binding barbed ends with high affinity. Certain results suggest that inhibitors of CP may exist within the cell. For example, the apparent affinity of CP for barbed ends in vivo is ~100-fold weaker than the affinity observed with pure proteins in vitro (40). V-1 is a candidate to play this role, along with the other protein and lipid inhibitors mentioned above. The cytoplasmic concentrations of V-1 and CP are estimated to be higher than the values, and endogenous cellular V-1 and CP can be co-immunoprecipitated (14), confirming the potential for physiological relevance.

The Role of the V-1 Loops in Binding to CP—We asked which regions of V-1 were responsible for binding CP. V-1 is an ankyrin repeat protein, with two loops protruding from the structural backbone of α-helices. Among ankyrin repeat proteins, the loops generally mediate protein interactions, and the loop sequences are not conserved (13). The sequences of the two V-1 loops are highly conserved among vertebrates, and both loops of V-1 are important for binding to CP. Mutations in either loop decreased the binding affinity by a very large amount. Truncation of 20 residues from the NH2 terminus of V-1, which should not affect the ankyrin repeats, had no effect on CP binding.

Role of the COOH-terminal Regions of CP in Binding V-1—Since V-1 inhibits actin capping by CP, and the COOH-terminal regions (“tentacles”) of the CP α and β subunits are responsible for binding actin (21), we reasoned that V-1 might bind to the COOH-terminal regions. We found that a CP mutant lacking the COOH-terminal region of CP β showed a substantial loss of binding affinity for V-1. In addition, a peptide corresponding to the CP β COOH-terminal region was able to bind V-1, indicating that this region is sufficient to bind V-1. Other regions of CP may also contribute to V-1 binding, as evidenced by the ability of CP mutants lacking the CP β COOH-terminal region to bind V-1. Truncation of the α subunit tentacle decreased the CP-V-1 affinity by a small amount, as did a change of the α subunit isoform from α1 to α2, which involves only residues on the body, not the tentacle. Based on the physical dimensions of CP, it does not seem possible that V-1 could interact with both tentacles simultaneously; however, molecular simulations were able to lend insight into these phenomena. Molecular dynamics simulations of CP with a truncated α subunit tentacle showed a distinct change in dynamics in the body of the α subunit. Interestingly, the region where this change is observed corresponds to the same vicinity of where V-1 would be predicted to interact with the α subunit, and this region is likewise an area where the α1 and α2 isoforms differ in sequence.

The β tentacle is the only region of difference between the β1 and β2 isoforms of CP, because they are produced by alternative splicing from a single gene (19). The sequences are quite different, but both are predicted to form amphipathic α-helices and appear as such in the crystal structure (20). Remarkably, we find that the β1 and β2 isoforms bind V-1 equally well, and they are known to bind the actin filament equally well (30). The sequence conservation between the β1 and β2 tentacles is greater on the hydrophobic side of the helix, so this surface may be the one that contacts both actin and V-1, which would be consistent with these results, along with the observed competitive inhibition.

Relevance to the Mechanism of Capping—These results support and refine the model for how CP binds to the barbed end of the actin filament. In the current version of the “wobble” hypothesis, the α and β COOH-terminal regions of CP bind actin independently, each with a dissociation rate constant of ~0.2/s (21). When CP is bound to the barbed end, and the α COOH-terminal region dissociates, then the CP can wobble about, because the attachment of the β COOH-terminal region to the body of the protein is mobile, like a tentacle. In this wobble state, CARMIL can bind to CP, because CARMIL does not require either COOH-terminal region (36), and thus prevent rebinding by steric inhibition. The CARMIL-CP complex should then dissociate from the barbed end with the dissociation rate constant of the β tentacle, ~0.2/s. In contrast, V-1 requires the β tentacle, at least for maximal binding, so V-1 is predicted to not bind the wobble state and thus not uncap. Indeed, we found that V-1 did not uncap and that V-1 could not bind in a ternary complex with CP and the barbed end, as predicted. Additional structural and mutational studies will be important to test this model. The
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model has potential significance for cells, where inhibition of capping and its reversal (i.e. uncapping) may be important for actin assembly and movement.

Acknowledgments—We thank Dr. Hanspeter Niederstrasser for help with V-1-CP structural analyses, Dr. Mark Crankshaw (Protein and Nucleic Acid Chemistry Laboratory, Washington University) for circular dichroism studies, Dr. Ilgu Kang for helping to construct the CP α2β2 expression plasmid, and Dr. Martin Wear for invaluable advice and assistance with experiments, data analysis, and the manuscript.

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