Identification and Characterization of an Actin-binding Site of CapZ

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Abstract. A mAb (IE5) that binds the COOH-terminal region of the β subunit of chicken CapZ inhibits the ability of CapZ to bind to the barbed ends of actin filaments and nucleate actin polymerization. CapZ prepared as fusion proteins in bacteria or nonfusion proteins by in vitro translation has activity similar to that of CapZ purified from muscle. Deletion of the COOH-terminus of the β subunit of CapZ leads to a loss of CapZ’s ability to bind to the barbed ends of actin filaments. A peptide corresponding to the COOH-terminal region of CapZ β, expressed as a fusion protein, binds actin monomers. The mAb IE5 also inhibits the binding of this peptide to actin.

These results suggest that the COOH-terminal region of the β subunit of CapZ is an actin-binding site. The primary structure of this region is not similar to that of potential actin-binding sites identified in other proteins. In addition, the primary structure of this region is not conserved across species.

Chicken CapZ is an actin-binding protein that binds the barbed ends of actin filaments in vitro (9) and nucleates actin polymerization (5). CapZ is a member of the “capping protein” family, which are heterodimers with subunits of 31–36 kD (α) and 28–32 kD (β) (19). Members of this family have been cloned from chicken (6, 10, 12), Dicyostelium (15), Xenopus (2), and S. cerevisiae (1), and protein has been purified from a wide variety of eukaryotes (19). Capping protein purified from chicken muscle (18) has been called CapZ because it localizes to the Z-line (9), suggesting that it binds the barbed ends of the actin filaments in the sarcomere. In chicken, the genes encoding muscle CapZ are also expressed in nonmuscle tissues (6, 12), and capping protein has been localized to structures that contain actin filaments in epithelial cells (Schaefer, D. A., M. B. Heintzelman, M. S. Moosiker, and J. A. Cooper; manuscript submitted for publication). Deletion of the gene for the β subunit of capping protein in yeast leads to an alteration in the actin cytoskeleton (1).

To analyze the relationship between the structure of CapZ and its function, specifically its interaction with actin, we prepared mAbs to CapZ and cloned full-length cDNAs for CapZ α and β into expression vectors. We report here that a mAb against the COOH-terminal region of CapZ β inhibits the ability of CapZ β to bind actin, that deletion of this region leads to loss of activity in expressed CapZ, and that this region on its own binds actin. The results indicate that this region is an actin-binding site of CapZ.

Materials and Methods

Unless otherwise stated, chemicals were from Sigma Chemical Co. (St. Louis, MO), solvents were from Fisher Scientific (St. Louis, MO), and DNA restriction and modifying enzymes were from Promega Biotec (Madison, WI). Protein concentration was determined by the method of Bradford with BSA as the standard (4). Recombinant DNA manipulations were performed as described (21).

Bacterial Fusion Protein Constructs

A cDNA for the complete β subunit of chicken skeletal muscle CapZ (277 amino acids) was obtained as described (6) and in this study is called pBS-β(1–277). This cDNA is cloned into the EcoRI site of the pBlueScript SK(−) (pBS, Stratagene, La Jolla, CA) cloning vector, in frame with β-galactosidase. pBS-β(1–265) was constructed by digesting pBS-β(1–277) with HindIII (this restriction enzyme recognition site is at nucleotide 794 of the β subunit cDNA (6)) to excise the 3’ region of the coding sequence, the 3’ untranslated sequence, and a portion of the polylinker. The large fragment was isolated and religated. An unintentional loss of a base introduced a stop codon after V365. pBS-β(1–267)β-gal was constructed identically without loss of a base, resulting in a 3’ fusion protein with vector sequence. This construction restored the normal residues L366, T367, and R369 but changed Q354 to S and added additional residues from the vector as shown in Fig. 1.

For expression, the CapZ β cDNAs were cloned into the pGex vector in which CapZ β is preceded by glutathione-S-transferase (GST) (23). pGex-β(1–277) was constructed using the polymerase chain reaction (PCR), the forward primer 1, the reverse primer 2, and 10 ng of pBS-β(1–277) template DNA. The sequence of oligonucleotide primers is given in Table I. The PCR product was digested with BamHI and EcoRI, gel purified, and ligated into similarly digested pGex-β(1–267). The BamHI–EcoRI insert of pGex-β(1–277) was also cloned into similarly digested pBS vector and designated pBS[pGex-β(1–277)]. pGex-β(1–265) was constructed by digesting pBS-β(1–265) with KpnI (nucleotide 552 of the β subunit cDNA). The 3’ fragment corresponding to the truncated coding sequence of pBS-β(1–265) was purified and ligated into KpnI-digested pBS[pGex-β(1–277)], which had been gel purified to remove the 3’ fragment of the complete coding region, to produce pBS[pGex-β(1–265)]. pBS[pGex-β(1–265)] was digested with XhoI, filled with T4 DNA polymerase, digested with BamHI, gel purified, and ligated into BamHI and Smal digested pGex-β(1–265) to produce pGex-β(1–265). pGex-β(1–265) was con-

1. Abbreviations used in this paper: GST, glutathione-S-transferase; PCR, polymerase chain reaction.
The amino acid sequence of the COOH-termini of several constructs is shown. CapZ β sequence is shown in uppercase; vector sequence is shown in lowercase. mAB IE5 reactivity results are taken from Fig. 4. For clarity, pGex-β(213-277) is not included here; it was recognized by mAB IE5. Termination codon is denoted by an asterisk.

![Figure 1. COOH-terminal truncations and peptides of the β subunit of CapZ.](image)

- Constructed by digesting pGex-β(1-277) with BamHI and HincII and ligating the released insert into pGex-1 digested with BamHI and Smal. This construct includes five vector-derived codons after L266 of the β subunit, followed by a stop codon. pGex-β(213-277) was constructed by digesting pBS-β(1-277) with Spel (nucleotide 636 of the β subunit cDNA) and EcoRI, and purifying and ligating this fragment, which encodes the COOH-terminal 65 amino acids of the β subunit, into pGex-1 digested with BamHI and Smal.
- pGex-β(1-265) was constructed by digesting pGex-β(1-277) with BamHI and Spel, and ligating the fragment encoding the NH2-terminal 212 amino acids into BamHI- and Smal-digested pGex-1. This construct includes four vector-derived codons following L132 of the β subunit, followed by a stop codon. pGex-β(253-277) was constructed using the PCR with the forward primer 4, the reverse primer 5, and the released insert into pGex-1 digested with BamHI and Smal. This construct is shown.

**Table 1. Sequences of Oligonucleotides, Listed 5' to 3'**

| 1. CGG CGG ATC CGA TCG AAG AGT GTC TGA TGA GTC ACC AGC AGC TG |
| 2. AAT AAG ACT CAC TAT AG |
| 3. CGG CGG ATC CTA TCG AAG GTC GTT GTC AAA AGT ATA AGC AGT TG |
| 4. AAG AAG ACT TGG CAA GGT TGG ACC CAG |
| 5. GAT CCC ATC GAA GGT CG |
| 6. CAT GCC ACC TTC GAT GG |

![Figure 2. mAbs IE5 and IB1 recognize the β and α subunit of purified CapZ on immunoblot (lanes 1 and 2), respectively. Both subunits are recognized by an affinity-purified polyclonal antibody (lane 3). Preimmune IgGs from the same animal (lane 4) do not bind CapZ. Each lane contains 20 ng of purified chicken CapZ. Molecular mass standards are indicated in kilodaltons. DF, dye front.](image)
Antibodies. 12 µl of 600 nM CapZ in buffer B with 0.1 mg/ml BSA was added to 108 µl mAb at various concentrations in PBS with 0.1 mg/ml BSA in silanized microfuge tubes and incubated for 1 h. 100 µl of this CapZ/antibody solution was added to 2,350 µl Ca2+-G-buffer in a fluorometer cuvette. 60 µl of 50 µM, 70% pyrene-labeled actin filaments was vortexed for 10 s, and 50 µl was added to the cuvette. The cuvette was stirred for 15 s. The capping activity of each sample was calculated by dividing the fluorescence at 150 s by the initial fluorescence, and subtracting the fluorescence at 15 s due to actin and buffer alone. A standard curve for CapZ alone was constructed by varying the concentration of CapZ in the incubation between 0 and 100 nM.

To determine the effect of mAb IE5 on CapZ bound to actin filaments, 60 µl of 5.0 µM 70% pyrene-labeled actin filaments was added to 60 µl of 120 nM CapZ in a silanized microfuge tube for 5 min. A sixfold molar excess of mAb IE5 to CapZ (48 µl of 900 nM in PBS) was added to 2.4 µl Ca2+-G-buffer in a fluorometer cuvette. 100 µl of the CapZ/actin filament solution was added to the cuvette, which was stirred for 15 s. In a control experiment, CapZ and mAb IE5 were added to Ca2+-G-buffer in a fluorometer cuvette and preincubated for 5 min. 50 µl of 5.0 µM pyrene actin filaments was added at t = 0.

Recombinant Proteins. To determine the capping activity of CapZ mutants expressed as fusion proteins in bacteria, depolymerization assays were performed with the following changes. 60 µl of test solution in buffer B was added to 60 µl of 5.0 µM actin filaments and incubated for 2 min. 100 µl was added to 2,240 µl Ca2+-G-buffer in a fluorometer cuvette. The activity of each sample was calculated as above and divided by the value obtained for 130 nM CapZ (defined as 100% capping activity). The fractional capping activity was plotted versus the final concentration of added protein. The concentration of recombinant protein required for 50% maximal capping activity was determined from this plot.

Nucleation Assay

The effect of antibodies on the nucleation activity of CapZ was assayed by incubating 70 µl of 1.8 µM CapZ in buffer B with 0.1 mg/ml BSA plus 961 µl of antibody in PBS with 0.1 mg/ml BSA in silanized microfuge tubes for 1 h. 1,207 µl of 10 µM CapZ monomers (5% pyrene labeled) in Mg2+-G-buffer was primed for 5 min in a fluorometer cuvette by adding 12 µl of 2 M MgCl2. At t = 0 the CapZ/antibody solution and 250 µl 10X MKEI buffer were added. The slope of the tangent at the inflection point of the plot of fluorescence versus time was calculated. To prepare a CapZ standard curve, this slope minus the slope for actin alone was plotted against the final concentration of CapZ. This standard curve was used to determine the fractional CapZ activity in samples containing varying ratios of mAb to CapZ.

Low-shear Viscosity Assay

Low-shear viscosity assays were performed with a falling-ball apparatus (18). Actin was polymerized at high concentration, diluted to 12 µM, and added to an equal volume of test solution immediately before entry into the capillary tube. Apparent viscosities were plotted versus concentration of added test protein. The relative activity of each CapZ mutant was determined from these plots by dividing the concentration of added protein that gave a 50% maximal reduction in viscosity by the concentration of CapZ required for an equivalent reduction in viscosity.

Critical Concentration Assay

To determine the activity of COOH-terminal peptides on the critical concentration of actin, 0–30 µM actin filaments (5 or 50% pyrene labeled), were incubated with 0–1.5 µM purified fusion protein in Ca2+-G-buffer with 18 µM MgCl2, 2 mM MgCl2, 1 mM EGTA, 20 mM imidazole/HCl, pH 7.0. The fluorescence was measured at 24 and 48 h. In a second experiment, 2.0 µM actin monomers, 5% pyrene labeled, were incubated with 0–14.0 µM purified fusion protein in Ca2+-G-buffer with 100 mM KCl, 2 mM MgCl2, 1 mM EGTA, 20 mM imidazole/HCl, pH 7.0.

In Vitro Translation

Vectors for in vitro translation were constructed by splicing the coding region of the α or β subunit of CapZ into the NcoI site of a plasmid containing the 475/Jun-D hybrid (kindly provided by Dr. Daniel Nathans, Johns Hopkins School of Medicine) from which the Jun-D coding sequence had been removed (Casella, J. F., and M. A. Torres, 1991, manuscript in preparation). These constructs result in the substitution of the 5' untranslated sequence of plasmid 475/In-D for the 5' untranslated sequences of...
RNA was produced from these constructs using the SP6 promoter and a transcription kit (pGEMExpress Core; Promega Biotec). 5 F+g of template DNA was used per 50-pl reaction. RNA was capped during transcription with 0.3 mM cap analogue 5'7Me GpppG 5' (Stratagene). Translation was carried out in nuclease-treated reticulocyte lysate (Promega Biotec). A typical 50-wl reaction contained 1/10 of the RNA from a single transcription reaction for each subunit (combined before translation), 11A1 of amino acid mixture lacking methionine (final concentration 20 pM), 35 U of RNasin, 35 A1 reticulocyte lysate, and 50 A10 of 3'S-methionine. The translation mixtures were stored at 4°C and used without further purification.

**Results**

**Identification of mAb 1E5 Epitope**

COOH-terminal deletions of the β subunit of chicken CapZ were constructed using a full-length cDNA clone and two different expression vectors, pBS and pGex (Fig. 1). Immunoblots were performed on total bacterial lysates containing the recombinant fusion proteins with mAb 1E5 (Fig. 4). An affinity-purified polyclonal antibody to the β subunit and a monoclonal antibody to the α subunit of CapZ were used as positive and negative controls, respectively. A deletion that removed the last twelve amino acids and introduced a stop codon, pBS-β(1-265), abolished recognition by mAb 1115; the same results were obtained with pGex-β(1-265) and a deletion of the COOH-terminal 65 amino acids (data not shown). Removal of ten amino acids with continuation of the polypeptide into vector sequence (pBS-β(1-267)/β-gal) allowed binding of mAb 1115, as did removal of the COOH-terminal 11 residues with addition of 5 vector-derived amino acids (pGex-β(1-266); data not shown). The COOH-terminal 165 amino acids (pGex-β(1-277)) and the COOH--terminal 25 amino acids (pGex-β(253-277)) were recognized by mAb 1E5, but the COOH-terminal 14 amino acids (pGex-β(264-277)) were not. All constructs were recognized by the polyclonal antibody.

**Cosedimentation of Radiolabeled CapZ Translation Products with Actin**

Approximately 5 µl of a translation reaction containing 54,000 cpmp of precipitable 35S-methionine-labeled α2-β(1-277), α2-β(1-266), or β(1-265) in pGEMZ vectors (Promega Biotec). RNA was produced from these constructs using the SP6 promoter and a transcription kit (pGEM Express Core; Promega Biotec). 5 µg of template DNA was used per 50-µl reaction. RNA was capped during transcription with 0.3 mM cap analogue 5'Me GpppG 5' (Stratagene). Translation was carried out in nuclease-treated reticulocyte lysate (Promega Biotec). A typical 50-µl reaction contained 1/4 of the RNA from a single transcription reaction for each subunit (combined before translation), 1 µl of amino acid mixture lacking methionine (final concentration 20 µM), 48 U of RNasin, 35 µl reticulocyte lysate, and 50 µCi of 35S-methionine. The translation mixtures were stored at 4°C and used without further purification.

**Figure 4.** The epitope of mAb 1E5 is contained within the COOH-terminal 25 amino acids of the β subunit of CapZ. Bacterial expression constructs (rows) were probed with primary antibodies (columns) by immunoblot. Only the portion of the blot corresponding to the immunoreactive peptide of the appropriate size is shown. Additional bands presumably due to degradation or incomplete synthesis are seen for several constructs. Negative controls, including mAb IB11 and pre-immune polyclonal IgGs, were performed for each construct and showed no reactivity (data not shown).

**Figure 5.** Inhibition of CapZ's nucleation activity by mAb 1E5. (A) CapZ was incubated with varying concentrations of mAb 1E5 or IB11. The fluorescence of pyrene-labeled actin is plotted versus time. The final CapZ concentration was 50 nM and the final actin concentration those in A were converted to CapZ activity using a standard curve for CapZ activity (data not shown). (Open circle) mAb 1E5; (closed circle) mAb IB11.
Effect of mAb IE5 on Actin Nucleating Activity of CapZ

Capping protein increases the rate of polymerization of monomeric actin into filaments in a manner consistent with an increase in the rate of nucleation (5, 11). Preincubation of CapZ with mAb IE5 inhibited the nucleating activity of CapZ (Fig. 5 A). Preincubation of CapZ with mAb IB11, which recognizes the α subunit of CapZ, had no effect on the nucleating activity.

By using a standard curve relating polymerization rate to the concentration of CapZ (data not shown), we were able to quantify the extent of inhibition of CapZ with different amounts of mAb (Fig. 5 B). 38 nM mAb IE5 led to a 50% reduction in the nucleating activity of 50 nM CapZ, indicating a $K_d$ for binding of mAb IE5 to CapZ of $\sim$13 nM.

Effect of mAb IE5 on Actin Filament Capping Activity of CapZ

CapZ binds to the barbed ends of actin filaments and prevents exchange of actin monomers at this end (5, 9). In an assay that measures the depolymerization of actin filaments after dilution, CapZ preincubated with mAb IE5 could not prevent depolymerization of actin filaments (Fig. 6 A). CapZ preincubated with mAb IB11 was still capable of preventing dilution-induced depolymerization of actin filaments (data not shown).

We also created a standard curve for CapZ activity in this assay using varied amounts of CapZ (data not shown), and related the effects of the mAbs added to the CapZ activity (Fig. 6 B). These data indicate a 50% loss of activity when 60 nM CapZ is incubated with 36 nM mAb IE5, which predicts a $K_d$ of $\sim$6 nM, which is in reasonably good agreement with the $K_d$ determined by inhibition of actin filament nucleating activity.

Figure 6. Inhibition of CapZ's capping activity by mAb IE5. (A) The capping activity of CapZ, as measured in an actin depolymerization assay, is inhibited by preincubation of CapZ with mAb IE5. 60 nM CapZ was incubated with mAb IE5 at the indicated molar ratios. Fluorescence of pyrene-labeled actin versus time is shown. In an identical assay, mAb IB11 and purified mouse IgG had no effect (data not shown). (B) Quantitative effect of varying amounts of mAb on 60 nM CapZ. Capping activity was calculated for curves like those in A and converted to CapZ concentration using a standard curve for CapZ activity (data not shown). (Open circle) mAb IE5; (closed circle) mAb IB11.

Effect of mAb IE5 on CapZ Bound to Actin Filaments

In the previous experiment, mAb IE5 was preincubated with CapZ before the actin was added. The results show that mAb IE5 can bind CapZ and prevent it from capping actin filaments. To determine if mAb IE5 could bind to and remove CapZ that was already bound to actin filaments, we added mAb IE5 to actin filaments that had CapZ bound to their barbed ends. The filaments were diluted to determine whether they were capped. As assayed in a depolymerization assay, mAb IE5 did not inhibit CapZ bound to actin fila-

Figure 7. Effect of mAb IE5 on CapZ bound to actin filaments, as assayed in a depolymerization experiment. (A) CapZ was incubated with actin filaments for 5 min before dilution into a cuvette containing mAb IE5. (B) The same amounts of CapZ and mAb IE5 as in A were incubated in the cuvette for 5 min before addition of actin filaments. (C) Actin filaments alone. B was normalized to the same initial fluorescence as A.
Table II. Activity of Fusion Proteins in Depolymerization and Low-shear Viscometry Assays

| Depolymerization assay for capping | Low-shear viscometry | mAb IE5 reactivity |
|-----------------------------------|----------------------|--------------------|
| % relative CapZ                    | % relative CapZ      |                    |
| Muscle CapZ                       | 100                  | 100                | +                  |
| α/β(1-277)                        | 52                   | 36                 | +                  |
| α/β(1-266)                        | 43                   | 23                 | +                  |
| α/β(1-265)                        | <6                   | <4                 |                 |
| α/β(1-212)                        | <6                   | <4                 |                 |
| α1                                | <3                   | <3                 |                 |
| β                                   | >0.2                 | >0.2               | +                  |
| β(264-277)                        | NA                   | <0.2               |                 |
| β(253-277)                        | <0.1                 | 46                 | +                  |

All constructs are fusion proteins with GST. N/A, not assayed. These results are derived from data such as those in Fig. 6 (depolymerization assays). The low-shear viscometry data are not shown. mAb IE5 reactivity is derived from the immunoblots shown in Fig. 4.

ments (Fig. 7 A). In a control experiment under the same conditions, mAb IE5 did inhibit CapZ if it was added before actin filaments (Fig. 7 B).

Effect of Truncation of the COOH Terminus of CapZ β on Activity of Expressed Protein

Recombinant α (GST-α) and β (GST-β(1-277)) subunits were made using the pGex expression vectors. These proteins contain GST fused to the NH₂-terminus of the CapZ subunit, which permits purification by affinity chromatography. The α and β subunits bind to each other, permitting separation of the heterodimer from monomer by gel filtration chromatography. The heterodimeric protein was active in a depolymerization assay and a low-shear viscometry assay (Table II). GST protein alone was inactive in both assays (data not shown). On a molar basis the activity of the heterodimeric fusion protein in a depolymerization assay was 52 %, and in a low-shear viscometry assay 36 % of the activity of CapZ purified from chicken muscle. In a depolymerization assay, the individual α and β subunits had very little activity (Table II and Fig. 8).

Recombinant COOH-terminal truncations of the β subunit made in the pGex vector (Fig. 1) were combined with GST-α, and tested for activity using the same assays. In general, activity correlated with mAb IE5 binding (Table II). GST-α/GST-β(1-266), which lacks 11 COOH-terminal residues and has several vector-derived residues, had a small loss of activity compared to the intact protein, GST-α/GST-β(1-277), and was still recognized by mAb IE5. GST-α/GST-β(1-265), which lacks 12 COOH-terminal residues and has no vector-derived residues, had a complete loss of functional activity, as did GST-α/GST-β(1-212), which lacks the COOH-terminal 65 residues. Neither of these two constructs were recognized by mAb IE5.

Sedimentation of In Vitro Cotranslated Subunits with Actin Filaments

To confirm the above results (obtained with fusion protein preparations), we constructed α and β subunits in an expression vector lacking a fusion protein. The complete α and β subunits, and COOH-terminally truncated β subunits, were transcribed and cotranslated in vitro. Binding of heterodimeric CapZ to actin filaments was assayed in a cosedimentation assay. Constructs that maintained mAb IE5 binding (α/β(1-277) and α/β(1-266)) cosedimented with actin filaments (Fig. 9). A construct that was not recognized by mAb IE5 showed marked diminution in cosedimentation with actin filaments (α/β(1-265), Fig. 9, pellet 5). Addition of mAb IE5 or unlabeled CapZ inhibited cosedimentation (Fig. 9, "+" lanes), while addition of a mAb that recognizes vinculin had no effect (data not shown). Equal amounts of F-actin were precipitated with or without added CapZ, as determined by SDS-PAGE.

In a separate quantitative analysis, varied amounts of unlabeled muscle CapZ were added to compete for binding of the labeled translation products. Dixon plots showed that the Kₐ of α/β(1-266) relative to that of α/β(1-277) was 5.5. The binding of α/β(1-265) was not detectable, which implies a Kₐ greater than 15 times that of α/β(1-277) for these data.

Effect of the COOH-terminal 25 Amino Acids of the β Subunit on the Apparent Critical Concentration of Actin

These data indicate that the COOH-terminus of CapZ β might be an actin-binding site. To test this hypothesis, we determined whether the COOH-terminal residues of CapZ β, prepared as a fusion protein with GST, were able to bind actin. The COOH-terminal 25 amino acids, as the fusion protein GST-β(253-277), had viscosity-lowering activity in a low-shear viscometry assay. This activity was abolished by preincubation with mAb IE5 (data not shown), indicating that the structure of the peptide is similar to its structure in native CapZ. However, this fusion protein had very little activity in a depolymerization assay (Fig. 8 and Table II).

These results indicate that GST-β(253-277) binds actin but does not cap actin filaments, suggesting that it may bind actin monomers. To test this hypothesis, we determined the
The effect of GST-ß(253-277) on the critical concentration for actin polymerization. Varying concentrations of actin filaments in Mg²⁺-F buffer incubated with GST-ß(253-277) exhibited a decrease in fluorescence, reflecting an increase in the apparent critical concentration of actin (Fig. 10). As negative controls, neither GST alone nor GST-ß(264-277) had any effect in this assay, which was predicted from the results in the previous sections. Similar results were obtained with 50% pyrene-labeled actin (data not shown). In a second experiment, 2 µM actin was incubated with increasing concentrations of GST-ß(253-277). Complete depolymerization was observed (Fig. 11), which indicates that GST-ß(253-277) can increase the apparent critical concentration to 2 µM. This value is greater than the critical concentration for the pointed end (0.6-0.7 µM [3, 5]), which indicates that GST-ß(253-277) does not cap barbed ends.

The results in both experiments (Figs. 10 and 11) can be explained by GST-ß(253-277) binding actin monomers with a Kd of 325 nM, which is diagramed in Fig. 11. Furthermore, the results of Fig. 10 cannot be explained by a model in which GST-ß(253-277) caps actin filaments with a single binding constant, and hence are not consistent with capping activity.

**Discussion**

**Identification and Characterization of the Actin-binding Site**

mAb 1E5 binds to CapZ and inhibits its ability to bind actin stoichiometrically. CapZ's ability to cap actin filaments, as measured in a depolymerization assay, and its ability to cap barbed ends...
nucleate actin polymerization from monomers are both inhibited. mAb IE5 does not bind to CapZ that is bound to an actin filament and remove it from the filament. These results indicate that the binding of mAb IE5 and actin to CapZ are mutually exclusive, perhaps because the binding sites are overlapping or identical. To test this idea, we located the binding site of mAb IE5 and then determined whether that region of CapZ was important for binding actin.

Epitope mapping shows that mAb IE5 binds near the COOH terminus of CapZ β. Loss of the terminal 12 residues leads to loss of binding. In a construct in which several vector-derived residues are substituted in place of the terminal 11 residues, binding is preserved, implying that the structure of the epitope is restored by these residues. The COOH-terminal 25 residues are sufficient for binding of mAb IE5, and the COOH-terminal 14 residues are not. Together these results indicate the COOH-terminal 25 residues are necessary and sufficient for antibody binding.

Deletion of the COOH-terminal 12 residues of the β subunit in recombinant expressed CapZ also leads to a loss of ability to cap actin filaments. In these experiments, both α and β subunits were required for full activity. When both complete subunits were expressed separately as fusion proteins and combined into heterodimers, the product was capable of capping actin filaments, although at a level quantitatively less than that of purified muscle CapZ. Truncation of the COOH terminus of CapZ β such that mAb IE5 did not bind led to a nearly complete loss of activity. In an in vitro translation system that expresses CapZ with no fusion protein, the expressed heterodimer bound actin filaments with high affinity. Truncation of the COOH-terminal region again led to a total loss of actin binding activity. Taken together, these results are consistent with the COOH-terminal region of CapZ β being an actin-binding site. An alternative interpretation is that the COOH-terminal region of CapZ β is important for the structure of an actin binding site, perhaps because in three dimensions the COOH-terminal region of CapZ β is adjacent to an actin-binding site of native CapZ.

To distinguish between these alternatives, we tested whether the COOH-terminal region could bind actin on its own. We prepared a peptide corresponding to the COOH-terminal 25 amino acids of CapZ β as a fusion protein. This protein binds actin monomers based on determinations of its effect on the apparent critical concentration of actin. Incubation of this fusion protein with mAb IE5 inhibited its activity in a low-shear viscosity assay, indicating that the actin-binding properties of the peptide are due to the same structure as in the native protein. A fusion protein that included the COOH-terminal 14 amino acids had no effect in the critical concentration assay or the low-shear viscosity assay.

Implications for the Mechanism of CapZ Binding to Actin

We want to emphasize that the COOH-terminal 25 amino acids, as a fusion protein, does not cap actin filaments as does CapZ, and that unlike CapZ, the fusion protein is capable of binding actin monomers. The peptide clearly does not replicate the interaction of CapZ with actin. We envision two alternative implications of this discrepancy: CapZ has one or more actin-binding sites. First, if CapZ has one actin-binding site, then the peptide may encompass part but not all of the actin-binding region. Indeed, the affinity of the peptide for actin monomers is low (Kd = 325 nM) compared to that of CapZ for barbed ends of filaments (Kd ≈ 1 nM [5]). On the other hand, if CapZ has more than one actin-binding site, the COOH terminus of the β subunit may represent one of these sites. In this case, the combination of more than one low-affinity actin monomer-binding site could yield a single high-affinity actin filament-binding site.

An argument against the multiple-site model is that the heterodimer lacking the COOH-terminal 12 amino acids of CapZ β (α/β(1–265)) does not bind actin in these experiments. No activity was detectable in the functional assay (low-shear viscometry), whereas the COOH-terminal 25 amino acids has some activity in this assay (Table II).

However, if the binding at a second site did not prevent actin polymerization, this would not have been detected in the low-shear viscometry assay. Physical binding should not depend on this function. Co-sedimentation with filaments was also undetectable, although the limits of detection of this assay were 15-fold, which is not sensitive to exclude a 300-fold difference in binding constants such as is seen between the COOH-terminal region (GST-β(253–277)) and native CapZ.

Primary Sequence of the Actin-binding Site

The primary sequence of the actin-binding site has two interesting features. First, this sequence is not conserved across species. In general, the β subunit is highly conserved, with 50% identity between yeast and other species, including chicken, Dictyostelium discoideum, and Caenorhabditis elegans (1, Waddle, J. A., J. A. Cooper, and R. H. Waterston, unpublished results, 1991). However, no similarity is found among the species in the COOH-terminal region of the β subunit. Therefore, the conventional notion that functionally important residues are conserved through evolution may not hold in this case, or, as noted above, this region may represent a portion of the actin-binding site and additional conserved residues may be involved.

Second, this site is not similar to actin-binding sites of other actin-binding proteins, implying that it represents a novel class of actin-binding sites. We noted previously that the primary sequences of capping protein alpha and beta are not similar to those of any other protein (6, 11). We have now compared the primary sequence of the COOH-terminal region of the β subunit to the database and to conserved regions of actin-binding proteins that may represent actin-binding sites and find no significant similarity. In this analysis we used the actin-binding site of coflin (27), which is similar to regions of thymosin β4 (20) and actobindin (26), and we used regions of similarity identified (24) in actin, the gelsolin family, profilin, and DNase I.

One possible feature of similarity may be positive charge. Basic residues are a prominent feature of the coflin/thymosin β4/actobindin sequences. Capping protein β does include an arginine in the COOH-terminal region in approximately the same position in all four species. In addition, truncation of CapZ β from 277 to 265 residues leads to a loss of activity, but the activity can be partially restored by a different construct (β [1–266] of Fig. 1 and Table II) in which several vector-derived residues, including an arginine and a histidine, follow β266.
We are grateful to Dr. Dorothy Schafer, Katherine Rader, and Jane Caldwell for assistance with preparation and characterization of the polyclonal and monoclonal antibodies against CapZ, to Steven Heiss for assistance with preparation of muscle CapZ, to James Amatruda for reading the manuscript, and to Rob Piper, Dr. Robert Mercer, and James Waddle for discussions.

This work was supported by grants to J. F. Casella from the National Institutes of Health (NIH) (R29 HL38855), an American Heart Association Grant in aid, and to J. A. Cooper from NIH (GM38542) and the Lucille P. Markey Charitable Trust. J. F. Casella is an Established Investigator Grant in aid, and to J. A. Cooper from NIH (GM38542) and the Lucille P. Markey Charitable Trust. J. F. Casella is an Established Investigator of the American Heart Association. J. A. Cooper is a Lucille P. Markey Biomedical Scholar.

Received for publication 21 August 1991 and in revised form 20 November 1991.

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