Mutational Analysis of Capping Protein Function in *Saccharomyces cerevisiae*

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To investigate physiologic functions and structural correlates for actin capping protein (CP), we analyzed site-directed mutations in *CAP1* and *CAP2*, which encode the α and β subunits of CP in *Saccharomyces cerevisiae*. Mutations in four different regions caused a loss of CP function in vivo despite the presence of mutant protein in the cells. Mutations in three regions caused a complete loss of all aspects of function, including the actin distribution, viability with *sac6*, and localization of CP to actin cortical patches. Mutation of the fourth region led to partial loss of only one function—formation of actin cables. Some mutations retained function and exhibited the complete wild-type phenotype, and some mutations led to a complete loss of protein and therefore loss of function. The simplest hypothesis that can explain these results is that a single biochemical property is necessary for all in vivo functions. This biochemical property is most likely binding to actin filaments, because the nonfunctional mutant CPs no longer co-localize with actin filaments in vivo and because direct binding of CP to actin filaments has been well established by studies with purified proteins in vitro. More complex hypotheses, involving the existence of additional biochemical properties important for function, cannot be excluded by this analysis.

INTRODUCTION

Capping protein (CP) binds the barbed end of actin filaments and nucleates actin polymerization in vitro. Found in all eukaryotic organisms examined, CP is a heterodimer, with α and β subunits, each about 30–35 kDa (Cooper *et al.*, 1993). In striated muscle, CP, also known as CapZ, co-localizes with barbed ends of actin filaments at Z lines. During myofibrillogenesis, the interaction of CP with actin is essential for the proper assembly of actin filaments (Schafer *et al.*, 1994). In *Dictyostelium*, the binding of CP to barbed ends influences actin assembly and cell motility (Hug *et al.*, 1995).

In addition to actin-binding, CP may possess other activities in vivo. Phosphoinositides inhibit the actin-binding activity of CP in vitro (Heiss and Cooper, 1991; Amatruda and Cooper, 1992), which is a poten-

tial mechanism for coupling cell surface signals to changes in actin assembly and cell motility. CP forms a complex with hsc70 in *Dictyostelium*, which may affect its actin-binding activity (Eddy *et al.*, 1993; Haus *et al.*, 1993). Also, CP localizes to the Z line independent of its ability to bind actin, indicating interactions with other proteins (Schafer *et al.*, 1994). In epithelial cells and yeast, CP localizes to sites of membrane-actin contact, suggesting that CP may mediate membrane attachment of actin (Amatruda and Cooper, 1992; Schafer *et al.*, 1992). Other actin-binding proteins are also present at these attachment sites and are also candidates to mediate the actin-membrane interaction (reviewed in Welch *et al.*, 1994).

To assess the function of CP both in vivo and in vitro, we chose the budding yeast *Saccharomyces cerevisiae* as a system for a structure-function analysis of CP. The α and β subunits of yeast CP, M_r 32 and 34 kDa, are encoded by the single genes *CAP1* and *CAP2*, respectively (Amatruda *et al.*, 1990, 1992). During the

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cell cycle, CP co-localizes with actin in cortical patches, which are clustered at the site of the emerging bud and remain polarized in the growing bud (Amatruda and Cooper, 1992). CP is not associated with the cables of actin, which are found predominantly in the mother. Near the end of the cell cycle, the mother loses its cables and acquires more cortical patches, which also contain CP. Just before cytokinesis, a ring of actin cortical patches appears at the mother-bud neck; this dense structure does not stain with antibodies to CP (Amatruda and Cooper, 1992), but does contain CP by fluorescence microscopy with a green fluorescent protein derivative of Cap2p (Waddle, Karpova, Waterston, and Cooper, unpublished data). In addition, shmooos contain CP in association with the actin patches clustered at the shmoo tip (Amatruda and Cooper, 1992). Therefore, CP may interact with actin or other components of the patch to effect actin function.

Null mutants of *CAP1* and/or *CAP2* grow a bit slowly and have cell size heterogeneity, a severe deficit or complete absence of actin cables, and depolarization of the cortical actin patches (Amatruda *et al.*, 1992). Loss of one CP subunit leads to loss of protein of the other subunit; therefore, single protein subunits are probably unstable and degraded in the absence of their heterodimer partners. While null mutants of *CAP1* and *CAP2* are viable, the additional disruption of *SAC6*, a nonessential gene encoding the actin-binding protein fimbrin, is lethal (Adams *et al.*, 1993). We used these traits to assess CP function in vivo.

To investigate physiologic functions and their structural correlates for CP, we constructed a set of site-directed mutations in *CAP1* and *CAP2*, which produce nested truncations and single and double amino acid changes in conserved hydrophilic residues of Cap1p and Cap2p. One aim of this analysis was to identify regions essential for function in vivo and to determine whether substantial portions of the genes are not essential. In chicken CP, the C-terminal regions of the β subunit (Hug *et al.*, 1992) and the α subunit (Casella and Torres, 1994) are necessary for actin binding in vitro. Therefore, we wished to test whether similar results would occur with yeast CP and what the in vivo consequences would be. In addition, certain evolutionarily conserved sequences were also targeted because they have a high likelihood for functional importance.

MATERIALS AND METHODS

Reagents and Supplies

Restriction enzymes, ligase, and all other DNA-modifying enzymes were obtained from Boehringer-Mannheim (Indianapolis, IN). These were used with their accompanying commercial reaction buffers. Radioisotopes were obtained from Amersham (Arlington Heights, IL). Yeast culture media was obtained from Bio101 (La

Jolla, CA). Otherwise, chemicals, solvents, and supplies were obtained from Sigma (St. Louis, MO) or Fisher (St. Louis, MO).

Molecular Biology and DNA Sequencing

Standard molecular biology techniques were employed (Sambrook *et al.*, 1989). Bacterial transformations were done according to the Hanahan standard competent cell method with XL1 blue *Escherichia coli* (Stratagene, La Jolla, CA), and all plasmids are resident in this strain. DNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977), using modified T7 DNA polymerase (Sequenase, United States Biochemical, Cleveland, OH). Colony lifts and dot blots were done according to the method of Sambrook *et al.* (1989) without the optional SDS treatment. Zetaprobe (Bio-Rad, Richmond, CA) was used as the lifting/dotting membrane, with the recommended hybridization solution. All probes were oligonucleotides labeled with [γ - 32 P]ATP and polynucleotide kinase.

Site-directed Mutagenesis

Plasmids with wild-type genes, used as parents for the mutagenesis, are listed in Table 1. The nature of the site-directed mutations and the methods used are described below. For point mutants, the codon was chosen based on yeast codon bias for maximal expression (Wada *et al.*, 1992). The identity of the products of the mutagenesis reactions were confirmed by sequencing with primers near the site of mutation. For oligonucleotide-mediated mutagenesis, in the initial phase of the work to test for in vivo function, several individual plasmids from each mutagenesis reaction were recovered and tested, to minimize the possibility that unintended mutations altered the phenotype. Later, if protein purification was performed, one plasmid was sequenced throughout its coding region to ensure that no unintended mutations had occurred.

Mutations of *CAP1*

For *CAP1*, nucleotide base numbers refer to GenBank/EMBL entry SCCAP1G, and amino acid residue numbers refer to SwissProt entry CAPA_YEAST.

Table 1. Plasmids used as vectors and carrying wild-type copies of CP genes

pBJ#	Description
006	<i>PstI/HaeII</i> fragment of <i>CAP2</i> (Amatruda <i>et al.</i> , 1990) in the <i>EcoRV</i> site of pBS
071	<i>PstI/BamHI</i> fragment of <i>CAP2</i> (Amatruda <i>et al.</i> , 1990) in the <i>PstI/BamHI</i> sites of pBJ 080 (pRS314; Sikorski and Hieter, 1989) [<i>TRP1</i> , CEN]
107	<i>CAP1</i> (Amatruda <i>et al.</i> , 1992) in the <i>EcoRI</i> site of pBJ 080 (pRS 314; Sikorski and Hieter, 1989) [<i>TRP1</i> , CEN]
108	<i>CAP1</i> (Amatruda <i>et al.</i> , 1992) in the <i>EcoRV</i> site of pBJ 082 (pRS 316; Sikorski and Hieter, 1989) [<i>URA3</i> , CEN]
143	AAB 132, pT5V31A. ROP shuttle vector [<i>URA3</i> , <i>ADE3</i> , <i>amp^r</i> , <i>LacZ</i> , 2 μ] from A. Adams and M. Tibbetts
198	<i>XbaI/BamHI</i> fragment of <i>CAP2</i> (Amatruda <i>et al.</i> , 1990) in <i>XbaI/BamHI</i> sites of pBJ 143 [<i>URA3</i> , 2 μ]
217	<i>CAP1</i> in the <i>EcoRI</i> site of pBJ 079 (pRS 313; Sikorski and Hieter, 1989) [<i>HIS3</i> , CEN]
503	<i>XbaI/BamHI</i> fragment of <i>CAP2</i> (Amatruda <i>et al.</i> , 1990) in the <i>BamHI/XbaI</i> sites of pBJ 082 (pRS 316; Sikorski and Hieter, 1989) [<i>URA3</i> , CEN]
519	<i>PstI/BamHI</i> fragment of <i>CAP2</i> (Amatruda <i>et al.</i> , 1990) in the <i>PstI/BamHI</i> sites of pBJ 214 (pRS 424; Christianson <i>et al.</i> , 1992) [<i>TRP1</i> , 2 μ]
520, 1	<i>CAP1</i> in the <i>EcoRI</i> site of pBJ 213 (pRS 423; Christianson <i>et al.</i> , 1992) [<i>HIS3</i> , 2 μ]

cap1-1. Description: deletion of Cap1p residues R239 through A264. A threonine residue is substituted, and the sequence continues with residues 265 through 268. Method: pRS 314/CAP1 (pBJ 107) was cut with *Afl*III and *Bsm*I to remove bases 1911–1988, filled in with T4 polymerase, and religated to give the deletion (pBJ 128). The insert was transferred to pRS 313 (*HIS3* marker) to make pBJ 219. Strain YJC 324 transformed with pBJ 128 was used for protein purification. The insert of pBJ 128 was transferred to a 2 μ plasmid, pRS 213, to produce pBJ 522 and 523.

cap1-2. Description: deletion of Cap1p residues L225 through A264. A proline residue is substituted, and the sequence continues with residues 265 through 268. Method: pRS 314/CAP1 (pBJ 107) was cut with *Bgl*III and *Bsm*I to remove bases 1869–1988, filled in with T4 polymerase, and religated to give the deletion (pBJ 129). The insert was transferred to pRS 313 (*HIS3* marker) to make pBJ 220.

cap1-3. Description: deletion of Cap1p residues S2 through E21. Method: bases 1201–1260 of pRS 313/CAP1 (pBJ 217) were deleted with oligonucleotide 1076, GAGCGACAGAAAATGTTAAGG-GAAGTATAC, by the method of Deng and Nickoloff (1992) to produce pBJ 231–233.

cap1-4. Description: deletion of Cap1p residues S2 through S37. Method: bases 1201–1308 of pRS 313/CAP1 (pBJ 217) were deleted with oligonucleotide 1077, GAGCGACAGAAAATGAAAAATAC-TATTTTG, by the method of Deng and Nickoloff (1992) to produce pBJ 234–236.

cap1-5. Description: point mutations changing Cap1p residue E188 to K. Method: bases 1758–1760 of pRS 313/CAP1 (pBJ 217) were changed from GAA to AAG with oligonucleotide 1078, GTCCAC-TATTACAAGGACGGTAACGTT, by the method of Deng and Nickoloff (1992) to produce pBJ 237–240. Strain YJC 324 was transformed with these plasmids for protein purification. The insert of pBJ 237 was transferred to a 2 μ plasmid, pRS 213, to produce pBJ 524 and 525.

cap1-6. Description: point mutations changing Cap1p residues RR 239 & 240 to EE. Method: bases 1912–1916 of pRS 313/CAP1 (pBJ 217) were changed from AGAAG to GAAGA with oligonucleotide 1090, TAAGGCCTTAGAAGAAAGATTACCAG, by the method of Deng and Nickoloff (1992) to produce pBJ 263–265, 362, and 363. The coding region of pBJ 362 was sequenced, and pBJ 362 in strain YJC 324 was used for protein purification. The insert of pBJ 264 was transferred to a 2 μ plasmid, pRS 213, to produce pBJ 526 and 527.

Mutations of CAP2

For CAP2, nucleotide base numbers, minus the value 1536, refer to GenBank/EMBL entry SCCAP2, and amino acid residue numbers refer to SwissProt entry CAPB_YEAST.

cap2-1. Description: deletion of Cap2p residues S267 through L287. Method: introduction of a stop site by changing bases 2659–2661 in pBJ 071 from TCC to TAA using antisense oligonucleotide 1032, CCTCAGCAGATTAAGCAATGGC, according to the method of Kunkel *et al.* (1991) to produce pBJ 086, which was transformed into YJC 108 for protein purification.

cap2-2. Description: deletion of Cap2p residues Q277 through L287. Method: introduction of a stop site by changing bases 2689–2690 in pBJ 071 from CAG to TAG using antisense oligonucleotide 1033, CCTCTGCCTATGCGTCTTT, according to the method of Kunkel *et al.* (1991) to produce pBJ 091.

cap2-3. Description: deletion of Cap2p residues F257 through L287. Method: introduction of a stop site by changing bases 2629–2631 in pBJ 071 from TTC to TAA using antisense oligonucleotide 1031, TTTGTTTGTTGTTAGATATCTC, according to the method of Kunkel *et al.* (1991) to produce pBJ 103. The coding region of pBJ 103 was sequenced, and pBJ 103 in strain YJC 108 was used for protein purification.

cap2-4. Description: deletion of Cap2p residues S2 through T20, with preservation of M1. Method: deletion of bases 1864–1920 in pBJ 071 using antisense oligonucleotide 1029, ATTCTCCTGTAA-CATTACGGTGGT, according to the method of Kunkel *et al.* (1991) to produce pBJ 105, the coding region of which was sequenced.

cap2-5. Description: deletion of Cap2p residues S2 through S53, with preservation of M1. Method: deletion of bases 1864–2019 in pBJ 071 using antisense oligonucleotide 1030, GTTTGAATCGGC-CATTACGGTGGT, according to the method of Kunkel *et al.* (1991) to produce pBJ 140, and the method of Deng and Nickoloff (1992) to produce pBJ 269–272.

cap2-6. Description: change of Cap2p residue E119 to K. Method: change of bases 2215–2217 in pBJ 071 from GAA to AAG using antisense oligonucleotide 1079, GGAGATACCGCCCTTATAATA-GAGATC, according to the method of Kunkel *et al.* (1991) to produce pBJ 203 and 364. The coding region of pBJ 364 was completely sequenced, and pBJ 364 in YJC 108 was used for protein purification.

cap2-7. Description: deletion of Cap2p residues L245 through L287. Method: introduction of a stop site by changing bases 2593–2595 in pBJ 071 from TTG to TAG using antisense oligonucleotide 1085, ATAAACAGTTTCCTATAGATTTCTCAT, according to the method of Kunkel *et al.* (1991) to produce pBJ 210–212. The coding region of pBJ 210 was sequenced. pBJ 210 in both strain YJC 108 and a proteinase-deficient strain YJC 1040 was used for protein purification. The insert of pBJ 210 was transferred to a 2 μ plasmid, pRS 214, to produce pBJ 517.

cap2-8. Description: change of Cap2p residues R14 to E and R15 to E. Method: change of bases 1900–1905 in pBJ 071 from AGGAGG to GAAGAA using antisense oligonucleotide 1116, GGTAGATT-TAGTTCTTCAAGAAGATCTAA, according to the method of Kunkel *et al.* (1991) to produce pBJ 289–292. The coding region of pBJ 289 was sequenced, and pBJ 289 in YJC 108 was used for protein purification. The insert of pBJ 289 was transferred to a 2 μ plasmid to produce pBJ 518.

cap2-9. Description: change of Cap2p residue L16 to I. Method: change of bases 1902–1906 in pBJ 071 from GAGGC to AAGAA using antisense oligonucleotide 1092, GTAGATTATTCTTCTAA-GAAGAAC, according to the method of Kunkel *et al.* (1991) to produce pBJ 266–268. This mutation was unintentional, derived from a mistake in synthesis of the oligonucleotide for *cap2-8*. We analyzed it as an additional control.

cap2-10. Description: change of Cap2p residue Q5 to K. Method: change of bases 1873–1875 in pBJ 071 from CAA to AAG using antisense oligonucleotide 1122, ATGTCTGATGCTAAGTTTCGAT-GCTGCT, according to the method of Deng and Nickoloff (1992) to produce pBJ 349–353.

cap2-11. Description: change of Cap2p residue K252 to E. Method: change of bases 2614–2616 in pBJ 071 from AAG to GAA using antisense oligonucleotide 1123, GTTATTTCGAAGAAACAAGA-GATATC, according to the method of Deng and Nickoloff (1992) to produce pBJ 354–358.

Yeast Strains and Techniques

Yeast strains are listed in Table 2. Transformations were done by the lithium acetate method, and yeast were cultured by standard methods (Kaiser *et al.*, 1994).

Growth Rates

Growth rates in shaking liquid culture were measured for each strain at 30° and 37°, in rich medium (YPD), and in synthetic medium (SD). Absorbance was determined with a spectrophotometer. Doubling time was calculated from the slope of log absorbance versus time. In rich medium, the difference between null and wild-type strains was greater at higher temperatures, as observed previously (Amatruda *et al.*, 1990). No difference in growth rate between null and wild-type was observed in synthetic medium.

Data are reported for rich medium at 37°, where the greatest difference was observed. For these measurements, error was assessed in two ways. First, the doubling time for the wild-type strain was measured in five independent experiments. The SD was 6% of the value of the mean, which was 3.3 h. Second, the uncertainty in the doubling time for each strain was obtained in the linear regres-

Table 2. Yeast strains used in this study

YJC #	Relevant genotype
Derivatives of W303 (R. Rothstein, Columbia University)	
108 ^a	MATa <i>CAP1 cap2-Δ1::HIS3 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>
324 ^a	MATα <i>cap1-Δ1::URA3 CAP2 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>
456 ^b	MATα <i>CAP1 cap2-Δ1::HIS3 cas6::LEU2 his3-11,15 leu2-3,112 trp1-1 ura3-1</i> [pBJ 198-CAP2, URA3, 2μ]
596 ^b	MATα <i>cap1-Δ1::TRP1 CAP2 sac6::LEU2 his3-11,15 leu2-3,112 trp1-1 ura3-1</i> [pBJ 108-CAP1, URA3, CEN]
1070 ^b	MATα <i>CAP1 cap2::HIS3 sac6::LEU2 his3-11,15 leu2-3, 112 trp1-1 ura3-1</i> [pBJ 503-CAP2, URA3, CEN]
Derivative of BJ 5457 (E. Jones, Carnegie-Mellon University)	
1040 ^c	MATα <i>cap2-Δ1::URA3 ura3-52 trp1 lys2-801 leu2-Δ1 his3-Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL</i>

^a Used for tests of actin cables, CP immunofluorescence, and CP purification.

^b Used in plasmid shuffle assay for *sac6* viability.

^c Used for CP purification.

sion analysis. These errors ranged from 5 to 15% of the value. Therefore, we consider a value of 10% for the error to be conservative.

Test for Synthetic Lethality with *sac6*

Mutated CP genes on plasmids were shuffled in exchange for plasmid-borne wild-type ones in strains carrying chromosomal null mutations of *sac6* and either *cap1* or *cap2*. These strains require a functional *CAP1* or *CAP2* gene for viability. Strain YJC 596 was transformed with each *cap1* mutant plasmid. Strain YJC 456 was transformed with each *cap2* mutant plasmid. 40–200 transformants for each plasmid were transferred to His[−] (*cap1* mutants) or Trp[−] (*cap2* mutants) media, conditions that are not selective for the *URA3 CAP1* or *URA3 CAP2* plasmids resident in the strains. The transformants were then replica plated onto medium containing 5-fluoroorotic acid (5-FOA; PCR, Gainesville, FL) and scored for growth to determine whether the resident plasmid, with the wild-type gene, could be lost.

In previous work, this assay gave a range of results, including no, partial, and complete rescue, as manifested by the percentage of colonies that were FOA⁺. In these experiments, the results with every *CAP* allele were either near 0% or 100% FOA⁺. To analyze the mutations that rescued more carefully and to look for partial loss of function, we recovered seven independent FOA⁺ colonies and tested them for growth at 15°, 25°, 30°, 37°, and 39° on YPD, and on 450 mM NaCl in YPD (OSM) at 30°. *cap2* alleles -1, -2, -3, -6, -8, -9, and -11 were tested. For each allele, under every condition, there was no difference between the mutant and wild-type allele. Thus there was no partial loss of function by this test.

Mutations that gave a loss of function in vivo, with protein present by immunoblot of whole-cell extracts, were also tested on a multicopy 2μ plasmid. The rationale was that because many of these mutations resulted in CP that was unstable during purification, we were concerned that the loss of function was due to a reduction in the cytoplasmic concentration of heterodimer. Therefore, we over-expressed the mutant protein to maximize the concentration of heterodimer. In these experiments, strains YJC 596 and 1070, which carry *CAP1* and *CAP2* on *URA3* CEN plasmids, were used to test *cap1* and *cap2* mutations, respectively. CEN plasmids were used to avoid possible recombination with the 2μ plasmids being tested.

Fluorescence Microscopy

Actin was localized using rhodamine-phalloidin (Amatruda and Cooper, 1992) or using affinity-purified goat anti-yeast actin antibodies (Karpova *et al.*, 1993). Cells with cables were counted with a Zeiss Axiovert fluorescence microscope (Thornwood, NY) with a 100× objective. CP was localized with affinity-purified rabbit anti-CP antibodies as described (Amatruda and Cooper, 1992). For anti-CP immunofluorescence, images were collected on Kodak Tmax ASA 400 film and scanned onto Kodak PhotoCDs (Rochester, NY). For rhodamine phalloidin staining, images were collected with an Optronics 470-DEI-T video CCD camera (Optronics, Goleta, CA) on an Olympus Bmax-60F microscope equipped with a 1.35 NA 100× UPlanApo objective (Olympus, Lake Success, NY). Custom macros and NIH Image 1.58 (written by Wayne Rasband at the National Institutes of Health and available by anonymous ftp at zippy.nimh.nih.gov) coordinated a stage and shutter controller (MAC2000, Ludl Electronic Products, Hawthorne, NY), a frame-grabber (AG-5, Scion Corporation, Frederick, MD), and a Power Macintosh 8100/80.

Exposure times were selected to be sufficient to generate a broad range of values for intensity in the digital image files. Adobe Photoshop was used to assemble the image files into collage figures with labels. Photoshop processing included minimal changes in grey scale intensity values. Exposure times and intensity value processing were identical for all samples within one experiment.

Proteins

SDS-PAGE, immunoblots, and preparation of anti-yeast CP antibodies were as described (Amatruda and Cooper, 1992). Acrylamide gels (10% or 12.5%) were used for SDS-PAGE. Gel-filtered actin from chicken pectoralis muscle was prepared and stored as described (Caldwell *et al.*, 1989). Actin buffer G was 2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl₂, 0.1 mM dithiothreitol, 1 mM Na₂S₂O₃.

Partial Purification of CP

We felt that a partial purification was sufficient for these studies for several reasons. First, a systematic search of a yeast whole-cell extract for activities that decrease the low-shear viscosity of actin filaments has identified only two—cofilin (Moon *et al.*, 1993) and CP (Amatruda and Cooper, 1992). The initial anion exchange column in the purification procedure separates cofilin and CP well (Amatruda and Cooper, 1992; Moon *et al.*, 1993). Therefore, none of the observed activity should be due to contaminating cofilin. Second, for several mutants the purification procedure yielded either extensively proteolyzed or no CP, as detected by immunoblot. In these cases, no actin-binding activity was detected around the expected elution position of CP. Third, the specific activity of partially purified wild-type CP was identical to that of wild-type CP purified to homogeneity. Therefore, partially purified CP was not contaminated with other actin-binding activities.

Strains with plasmids carrying mutated forms of CP were grown to an OD₆₀₀ of 6–8 in 2 liters of 2× SD medium with appropriate nutritional selection. Cells were harvested (typically 20 g wet weight), washed once in homogenization buffer (10 mM Tris/HCl, pH 8.0, 10 mM EDTA, pH 8.0, 125 mM NaCl, 0.5 mM dithiothreitol, 340 mM sucrose), and suspended in an equal volume of homogenization buffer plus protease inhibitors (0.1 mM benzamide, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were disrupted using 0.5-mm glass beads in a bead-beater (Bio-Spec, Bartlesville, OK) with eight 1-min cycles separated by 1-min cooling periods. The bead-beater was jacketed in ice water, and all manipulations were done at 4°C. Cell lysis was monitored by phase microscopy and was >90% complete. After disruption, the beads were washed with an equal volume of homogenization buffer, and the wash was combined with the lysate.

The cell lysate was spun at $12,000 \times g$ for 10 min to remove glass beads and undisrupted cells. The supernatant (~ 2 g total protein) was spun at $100,000 \times g$ for 50 min. CP partitions to this supernatant under these conditions (Amatruda and Cooper, 1992).

CP was then partially purified using two of the four column chromatography steps that were used previously (Amatruda and Cooper, 1992) to purify CP to homogeneity. The supernatant was removed, $0.2 \mu\text{m}$ filtered, and applied to a MonoQ HR16/10 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mM dithiothreitol, 1.5 mM NaN_3 . The column was eluted with a 300-ml linear gradient from 150 mM to 400 mM NaCl, and 5-ml fractions were collected. The fractions were rapidly analyzed by dot blots with rabbit anti-CP antibodies (Amatruda and Cooper, 1992) to identify fractions (typically 250–280 mM NaCl) containing CP. These fractions were pooled and applied to a 1.5×7 cm hydroxylapatite column (Bio-Gel HT, Bio-Rad, Hercules, CA) equilibrated in 10 mM KPO_4 , pH 7.0, 100 mM NaCl, 0.5 mM dithiothreitol, 1.5 mM NaN_3 . Protein was eluted with a 120-ml linear gradient to 250 mM KPO_4 , and 2-ml fractions were collected. Fractions containing CP (typically 50–60 mM KPO_4) were identified with a dot blot as above, pooled, and concentrated 10-fold with a centricon-30 microconcentrator (Amicon, Beverly, MA). The OD_{280} of the final product was typically 1.3–1.5.

SDS-PAGE analysis of the pooled CP at various stages during the purification is shown in Figure 1. The pool from the hydroxylapatite column (HAT pool) was used for activity assays. Both the MonoQ and hydroxylapatite columns provide appreciable degrees of purification. However, note that none of the Coomassie-stained bands in the region of CP are in fact CP, which was confirmed by high-resolution side-by-side immunoblots with anti-CP and protein stains of the HAT pool and purified CP.

To calculate specific activity, the amount of CP in each sample was determined by a quantitative immunoblot of an SDS gel. The sample and a purified CP standard were serially diluted and ana-

lyzed on the same gel and blot. For mutations in CAP2, the first antibody was anti-Cap1p, and vice-versa, to ensure that the antibody recognized mutant and wild-type protein equally well. The blot was probed with ^{125}I -labeled second antibody, and imaged with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Counts were obtained for each band, and counts for the standards and samples were plotted. The linear region of the graph, which generally included the entire data set, was used for calculation of the slope by linear regression. The slopes were used to calculate the concentration of CP in the sample.

Actin-binding and PIP_2 Inhibition Assay

To determine the specific activity of the partially purified protein, we quantified both the actin-binding activity and the amount of CP in the partially purified material. CP purified to homogeneity (Amatruda *et al.*, 1992) was used as a standard in both determinations. Actin-binding activity was measured by inhibition of low-shear viscometry of actin filaments (Pollard and Cooper, 1982), and the amount of CP was measured by quantitative immunoblots.

In the low-shear (falling ball) viscometry assay, 50 μl of 0.25 mg/ml chicken skeletal muscle G-actin in buffer G was incubated with 125 μl sample in MKEI (2 mM MgCl_2 , 100 mM KCl, 1 mM EGTA, 20 mM imidazole, pH 7.0) for 20 min before apparent viscosity was determined as the time for the ball to roll 10 cm. Each sample of CP was serially diluted over the concentration region that gave a 50% decrease in apparent viscosity. Wild-type CP purified to homogeneity from an overexpression strain (Amatruda *et al.*, 1992) as described (Amatruda and Cooper, 1992) was used as the standard, and serial dilutions were assayed under conditions identical to those for the mutant CP.

Inhibition of CP activity by PIP_2 was also measured by low-shear viscometry, as described (Heiss and Cooper, 1991). CP was dialyzed into buffer B (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM 2-mercaptoethanol, 1 mM NaN_3 , 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride). The reaction buffer was MKEI without MgCl_2 and EGTA.

Calorimetry

Differential scanning calorimetry was performed with a MicroCal MC-2 calorimeter (MicroCal, Northampton, MA) in the Department of Biochemistry and Molecular Biophysics at Washington University. Wild-type CP, purified from the overexpression strain (Amatruda and Cooper, 1992; Amatruda *et al.*, 1992), was present at 1–2 mg/ml in buffer B. Samples were heated at a rate of $60^\circ/\text{h}$ to a preset temperature, cooled, and heated again.

Sequence Analysis

The program MACAW, v.2.03 for Mac68K, was used to search for blocks of sequence similarity (Schuler *et al.*, 1991). The segment pair overlap method was used with BLOSUM 62 and PAM120. The sequences were the protein sequences of the α and β subunits of CP from chicken, nematode, *Dictyostelium*, and yeast (Waddle *et al.*, 1993).

RESULTS

Strategies for Choosing Site-directed Mutations

The first strategy was to truncate each protein from each end to attempt to identify portions of the protein not necessary for function in vivo. The second was to make charge changes in evolutionarily conserved hydrophilic residues, especially clusters of basic residues, which are important for binding actin in other proteins (Friederich *et al.*, 1992). This strategy was

Figure 1. SDS-PAGE with Coomassie blue staining of protein pools through the partial purification of CP. The lane labeled "CP" contains a sample purified to homogeneity from a different preparation. Molecular weight markers are at left. DF, dye front. The amount of protein loaded in each lane was adjusted to demonstrate the profile of proteins in each sample. As explained in MATERIALS AND METHODS, the CP bands are not seen in the HAT pool.

successful in site-direction mutagenesis of actin (Wertman *et al.*, 1992). The third was to make charge changes in regions conserved in both α and β subunits, to test the hypothesis that each subunit binds in a similar fashion to each protofilament of actin.

Scheme for Evaluation of Site-directed Mutations

The scheme was to introduce a site-directed mutation in one of the genes for CP, *CAP1* or *CAP2*, and then test the ability of the mutant gene to function in vivo, i.e., to rescue traits or features of the null mutant phenotype. These traits included decreased growth rate, the absence of actin cables, depolarization of actin cortical patches, loss of localization of CP to actin cortical patches (Amatruda *et al.*, 1990, 1992), and inviability in a *sac6* background (Adams *et al.*, 1993; Karpova *et al.*, 1993). Depolarization of actin cortical patches always correlated with loss of actin cables. Depolarization was difficult to define and therefore score and quantitate reproducibly; thus, we only quantitated the presence of cables and noted patch depolarization qualitatively.

We identified and excluded from extensive additional analysis mutations that led to the absence of CP by determining the level of CP in a whole-cell extract by immunoblot. Because each subunit needs the other for stability, this determination also excluded mutations that altered protein folding. As expected, in all cases in which the level of CP was severely decreased, the mutation did not rescue any aspect of the null phenotype.

CP from mutants that displayed significant cellular levels of CP was tested for actin-binding and inhibition by PIP₂. Mutant CP was partially purified from strains that carried the mutated gene on a low-copy number plasmid and whose chromosomal copy of that gene was deleted. The protein is only stable as a heterodimer, so the mutant subunit co-purified with its wild-type counterpart.

Presentation of Data

The immunoblot analysis to detect CP for all the mutations is shown in Figure 2. Representative immuno-

fluorescence images of CP distribution are shown in Figure 3 and rhodamine-phalloidin fluorescence images of actin distribution are shown in Figure 4. The data for growth rate, formation of actin cables, and *sac6* viability are presented in Table 3, which also tabulates all the immunoblot and immunofluorescence results. Note that actin cable formation was quantitated by looking through the microscope and counting many cells. The images in Figure 4 include just a few selected cells, and long exposures were chosen to demonstrate fine detail. These results together with additional data are discussed in terms of different sections of the protein targeted for mutagenesis, as follows. Figure 5 is a diagram illustrating the locations of the mutations.

The N-Terminus of the α Subunit (*Cap1p*)

Two N-terminal deletion mutations of *Cap1p*, $\Delta 2-21$ and $\Delta 2-37$ (Figure 5A), led to severe and complete absence, respectively, of cellular CP by immunoblot of whole cell extracts (Figure 2) and by immunofluorescence (Table 3). Both mutations led to absence of function in vivo by tests for actin cables, *sac6* viability, and growth rate (Table 3). *Cap1p* has a total of 268 amino acid residues. Therefore, in this region, a very small truncation (20/268 residues, 7%) led to a severe loss of protein in vivo.

The C-Terminus of the α Subunit (*Cap1p*)

Two deletion mutations in a region of conserved sequence near the C-terminus of *Cap1p* were analyzed (Figure 5B). First, residues 239–264 were deleted and replaced with Thr. Second, residues 225–264 were deleted and replaced with Pro. Each mutant protein still includes residues 265–268, so these are not truncations but rather deletions very near the C-terminus. The smaller deletion, $\Delta 239-264$, gave a moderately reduced level of CP in whole cell extracts (Figure 2). In vivo function, tested by growth rate, actin cables, and *sac6* viability, was lost completely, and the CP immunofluorescence distribution was diffuse and of low intensity (Table 3). The larger deletion, $\Delta 225-264$, gave

Figure 2. Assay for presence of CP in whole-cell extracts of yeast strains carrying various mutations. Only the relevant regions of immunoblots, probed with antibodies specific for *Cap1p* or *Cap2p*, are shown. Approximately equal amounts of protein (45 μ g, range 39–51 μ g, for the *CAP2* mutations, and 42 μ g, range 35–49 μ g, for the *CAP1* mutations, determined by Bradford assay of the cell lysate) were loaded in each lane.

Figure 3. Immunofluorescence microscopy of CP in strains as labeled on the panels. The top two panels show a wild-type distribution of CP in cortical actin patches. The lower three panels show a diffuse distribution of staining. The bottom panel shows a

no protein in vivo (Figure 2) and no in vivo function by any test (Table 3).

The region defined by the smaller deletion included two conserved Arg residues, and clusters of basic residues have been implicated in actin-binding sites (Friederich *et al.*, 1992). Therefore, we mutated the two conserved Arg residues, 239 and 240, to Glu (Figure 5B). This mutant protein was stable by immunoblot, with loss of function in vivo by all three tests (Figure 4 and Table 3). The CP immunofluorescence was diffuse and intense (Figure 3). Partial protein purification of both mutants, $\Delta 239-264$ and RR239,240EE, was attempted, but no CP was recovered, based on immunoblots. Parallel purification of wild-type and other mutant CPs was successful, as discussed below; therefore, these two mutant CPs may be unstable or highly susceptible to protease.

To test the hypothesis that decreased protein stability led to insufficient levels of heterodimer for the $\Delta 239-264$ and RR239,240EE mutants, we determined whether overexpression of the mutant proteins would rescue the phenotype. We reasoned that increasing the expression of the mutant subunit would increase the concentration of heterodimer and might thereby produce function. On the other hand, if the mutation truly leads to a heterodimer that itself is nonfunctional, then increasing expression of the mutant subunit should have no effect. The mutated genes were placed on high-copy number plasmids, which did produce increased levels of proteins, as documented by immunoblot (Figure 6). Compare the lanes labeled "H" in Figure 6 with lanes labeled "L" for the wild-type. In each case, the levels of the mutant proteins are at or above the wild-type level. For the $\Delta 239-264$ and RR239,240EE mutants, overexpression still showed loss-of-function in vivo (Table 4). Therefore, we conclude that this region near the C-terminus of Cap1p, including the Arg residues of 239 and 240, is essential for function, and note that all assayed features—actin cables, *sac6* viability, and localization of capping protein to actin cortical patches—were lost concomitantly.

The N-Terminus of the β Subunit (Cap2p)

Two N-terminal deletion mutations of Cap2p, $\Delta 2-20$ and $\Delta 2-53$ (Figure 5C), led to the absence of protein and function in vivo (Figures 2–4 and Table 3). Therefore, in this region, as at the N-terminus of the α subunit, a very small truncation (19/287 residues, 7%) led to loss of protein.

This region also contains a cluster of evolutionarily conserved hydrophilic residues, which we hypothe-

(Figure 3 cont.) distribution and intensity similar to that of a null mutant, as expected from the immunoblot. Each panel is a collage of images taken from a few different fields in the slide. Bar, 10 μ m.

Figure 4. Fluorescence microscopy of rhodamine-phalloidin stained strains. Panels are labeled with *CAP* gene and allele numbers. To demonstrate cable formation in these images, long exposures were chosen; therefore, patches are somewhat overexposed. Mutants *cap1-5* E188K, *cap1-6* RR239,240EE, *cap2-5* Δ 2-53, and *cap2-7* Δ 245-287 show actin distributions like that of null mutants described previously (Amatruda *et al.*, 1992). Cable formation is severely decreased, and patches are polarized poorly. *cap2-8* RR14,15EE is the one mutant whose only defect is a partial loss of cables as detected by an observer looking through a microscope and counting cells with cables. That defect is not readily appreciated from the long-exposure images of the few cells here. *cap2-9* L161 is like wild type. Bar, 10 μ m.

sized might be important for function. Two mutations, Q5K and RR14,15EE, were tested (Figure 5C). As above, the pair of conserved Arg residues was targeted because clusters of basic residues have been implicated in actin-binding sites in other proteins (Friederich *et al.*, 1992). Both mutations had protein by immunoblot and were functional *in vivo* by tests of growth rate and *sac6* viability (Figures 2 and 3; Table 3). Q5K showed complete rescue of actin cables (81%, Table 3). However, RR14,15EE had only a partial rescue of cables (28%, Table 3). Overexpression of

RR14,15EE led to increased CP levels (L versus H in Figure 6) but did not change the level of partial rescue. In a single experiment comparing the RR14,15EE and control strains directly, the fraction of cells with cables was 29% (78/270) for the 2 μ plasmid, 28% (69/244) for the CEN plasmid, 91% (182/201) for a wild-type strain, and 1% (2/181) for a null mutant. The partial nature of defect can also be appreciated from the images in Figure 4, where the *cap2-8* RR14,15EE mutant does show cable formation and patch polarization intermediate between wild-type and null phenotypes.

Table 3. Compilation of data on phenotype rescue

Allele	Mutation	Level of capping protein ^a	Doubling time ^b	Cells with actin cables	Cells viable with <i>sac6</i>	Immunofluorescence of CP	
						Pattern ^c	Intensity ^d
<i>CAP1</i>	wild type	+++	102%	85% (118/139)	99% (97/98)	n.d.	n.d.
<i>cap1-1</i>	Δ 239-264	+	141%	9% (20/212)	0% (1/360)	diffuse	+
<i>cap1-6</i>	RR239,240EE	+++	134%	8% (38/453)	0% (1/232)	diffuse	+++
<i>cap1-2</i>	Δ 225-264	—	144%	10% (24/236)	0% (0/99)	n.d.	n.d.
<i>cap1-3</i>	Δ 2-21	—	141%	9% (39/435)	1% (5/467)	null	—
<i>cap1-4</i>	Δ 2-37	—	143%	6% (37/580)	0% (0/207)	n.d.	n.d.
<i>cap1-5</i>	E188K	++	138%	13% (124/960)	0% (0/329)	diffuse	++
<i>CAP2</i>	wild type	+++	99%	84% (122/145)	98% (367/373)	wt	+++
<i>cap2-2</i>	Δ 277-287	++	97%	60% (132/219)	93% (264/284) ^e	wt	+++
<i>cap2-1</i>	Δ 267-287	++	100%	59% (122/207)	95% (290/305) ^e	wt	+++
<i>cap2-3</i>	Δ 257-287	+	114%	76% (166/218)	99% (319/324) ^e	wt	++
<i>cap2-7</i>	Δ 245-287	+	128%	11% (21/185)	0% (0/150)	diffuse	+
<i>cap2-11</i>	K252E	+++	100%	76% (398/522)	98% (146/149) ^e	wt	+++
<i>cap2-4</i>	Δ 2-20	—	n.d.	14% (32/226)	0% (0/47)	null	—
<i>cap2-5</i>	Δ 2-53	—	149%	19% (150/801)	0% (0/322)	null	—
<i>cap2-10</i>	Q5K	++	100%	81% (731/904)	97% (146/150)	n.d.	n.d.
<i>cap2-8</i>	RR14,15EE	+	106%	28% (35/123)	100% (239/240) ^e	wt	++
<i>cap2-6</i>	E119K	++	93%	76% (146/192)	100% (30/30) ^e	wt	++
<i>cap2-9</i>	L161	++	98%	76% (184/242)	94% (412/439) ^e	wt	+++

^a The level of CP in whole-cell extracts, as detected by immunoblot, is indicated here as +++ to —, based on the data of Figure 2. If the mutation was in *CAP1*, the level of Cap2p was examined, and vice versa. A — indicates that the level was indistinguishable from that observed for control strains in which the relevant subunit was absent, that is, the level of Cap1p in a *cap2Δ* strain and vice versa.

^b Generation time normalized to the mean of five wild-type strains, two listed here as *CAP1* and three listed as *CAP2*. Error is 10%, as discussed in MATERIALS AND METHODS.

^c The pattern of immunofluorescence staining is listed as wt, diffuse, or null, corresponding to representative images in Figure 3. n.d., not determined.

^d Intensity, indicated as +++ to —, was judged from prints prepared with identical parameters for exposure and processing. A — indicates that the level was indistinguishable from that observed for null strains.

^e Seven independent isolates of YJC 456 with a mutant *cap2* plasmid substituted for wild type were tested for growth on YPD and YPD plus 450 mM NaCl at 15°, 25°, 30°, 37°, and 39°. The phenotype was indistinguishable from a control strain with a wild-type *CAP2* plasmid in each case. Thus, the rescue of *sac6 cap2* synthetic lethality by these alleles is complete.

RR14,15EE had an immunofluorescence distribution of CP similar to that of wild type (Figure 3).

Because RR 14,15 EE showed a partial rescue for cables, but a complete rescue for *sac6* viability and growth rate, we analyzed the growth of strains carrying *sac6* and this mutation more carefully, looking for defects in the level of rescue. Over a wide range of temperatures, on high osmolarity and conventional rich solid medium, the mutant grew as well as wild type (Table 3; MATERIALS AND METHODS). Thus, there was no loss of function, even in part, by this test. Therefore, this is the only mutant for which the in vivo tests of function were discordant and the only certain case of partial rescue. Partial purification of RR14,15EE mutant protein was attempted; however, no protein was recovered.

The C-Terminus of the β Subunit (Cap2p)

This region is of particular interest because our previous work using chicken muscle CP (CapZ) implicated

this area as necessary for actin-binding and perhaps the actin-binding site itself (Hug *et al.*, 1992). In that work, truncation of the C-terminus of the β 1 subunit by 12 amino acid residues led to a loss of actin-binding. The primary structure of this region of the protein is remarkable because it is the only region, throughout the sequences of both α and β subunits, that is *not* conserved across species (Waddle *et al.*, 1993). In addition, this region is the only difference between the β 1 and β 2 isoforms of vertebrates, which have different subcellular distributions (Schafer *et al.*, 1994). Therefore, we hypothesized that this region is not the actin-binding site, but rather a structural element necessary for actin-binding.

Because of the lack of sequence conservation between chicken and yeast in this region, we could not choose a mutation directly analogous to the 12-residue truncation of the chicken β 1 subunit, therefore, we made truncations of 11, 21, 31, and 43 amino acids (Figure 5D). Truncations of 11, 21, and

A Cap1p N-terminus **B Cap1p C-terminus** **C Cap2p N-terminus** **D Cap2p C-terminus**

Figure 5. Diagram to summarize the mutations near the ends of the coding regions. Each diagram shows the predicted amino acid sequence of the *CAP1* or *CAP2* gene, labeled Cap1p or Cap2p, respectively. * indicates a stop codon. Residues on the line labeled "Conserved" are found in all CP sequences, including yeast, *Dictyostelium*, nematode, and chicken (Waddle *et al.*, 1993). Gray bars indicate the deleted amino acid residues, and allele names are indicated on the right side. For point mutations, the result of the mutation is indicated in the line labeled "Point Mutants," under the main amino acid sequence, with allele names underneath.

31 residues yielded mutant CP that did function in vivo by tests of growth rate, actin cables, and *sac6* viability, and retained a wild-type CP immunofluorescence distribution (Table 3). Additional tests for *sac6* viability at different temperatures and at high osmolarity also showed complete rescue. Mutant CP was partially purified for the 21-residue truncation. In contrast to the prediction from the chicken CP results (Hug *et al.*, 1992), the actin-binding activity of the mutant CP was identical to that of wild-type CP (Figure 7B). The actin-binding activity was inhibited by PIP₂, as observed for wild-type CP (Table 5). Purification of the 31-residue truncated protein yielded CP that was extensively proteolyzed by immunoblot and inactive by falling ball viscometry.

The 43-residue truncation showed loss of function in vivo, for growth rate, actin cables, and *sac6* via-

bility (Figure 4 and Table 3). The level of CP by immunoblot of a whole cell extract was moderately decreased, and the CP immunofluorescence distribution was diffuse and weak (Figures 2 and 3; Table 3). Overexpression still gave loss-of-function (Table 4) despite increased levels of protein (Table 4). No CP was recovered from attempts to purify this mutant protein. A mutation of a conserved hydrophilic residue in this region, K252E (Figure 5D), functioned in vivo and had a wild-type CP immunofluorescence distribution (Table 3).

Therefore, we conclude that the region between residues 245 and 257, but not residue 252 alone, is essential for function, and that all assayed functions—actin cables, *sac6* viability, and CP localization to actin cortical patches—are lost together.

Figure 6. Assay for protein overexpression in strains carrying high-copy number plasmids of CP genes with mutations. The relevant regions of immunoblots with antibodies specific for Cap1p and Cap2p are shown. For each mutation, a low-copy number (CEN) plasmid is labeled "L," and a high-copy number (2 μ) plasmid is labeled "H." Equal amounts of protein (48 μ g, range 44–52 μ g, determined by Bradford assay of the cell lysate) were loaded in each lane. Note that some of the charged amino acid substitutions do produce slight alterations in mobility.

Calorimetry

The observation that stability is compromised by small truncations at each end of each subunit suggests that CP is a single domain. To test this hypothesis, we determined whether CP unfolded in one or more transitions by differential scanning calorimetry. A single peak, centered at 58°, was observed (Figure 8), consistent with the single-domain hypothesis. Cooling and repeat analysis of individual samples showed that heat-induced denaturation was irreversible.

Region of α/β Similarity

One hypothesis for how CP binds to and caps the barbed end of the actin filament is that each subunit of CP, α and β , binds to the terminal protomer of one of the two protofilaments. Perhaps each CP subunit even binds to the same site on the actin molecule because each subunit contains the same actin-binding motif. However, the primary sequences of the α and β subunits of capping are not similar to each other and do not contain known actin-binding motifs found in other proteins (Am-

atruda *et al.*, 1990, 1992). MACAW, a program that detects blocks of sequence similarities (Schuler *et al.*, 1991), was challenged to align simultaneously the complete sequence of the α and β subunits from four organisms. The block shown in Figure 9 had the greatest statistical significance ($P = 2 \times 10^{-14}$). To test the importance of these regions, we reversed the charge of a conserved charged residue in each subunit.

The α subunit, E188K *cap1*-5, showed loss of function in vivo, stable protein by immunoblot, and a diffuse immunofluorescence distribution (Figures 2 and 4; Table 3). Placing the mutation on a multicopy plasmid gave increased expression (Figure 6) but still showed loss of function (Table 4). Therefore, this mutation identifies a third essential region, and all functions are lost together, as in the other two essential regions. Purification of this mutant protein was attempted but no CP was recovered.

On the other hand, a similar mutation in the β subunit, E119K *cap2*-6, showed normal function in vivo and a wild-type immunofluorescence distribution (Figure 2 and Table 3). The partially purified protein had normal actin-binding activity (Figure 7C) and was inhibited by PIP₂ (Table 5).

Therefore, the hypothesis as presented above is not confirmed, in the sense that this single region of similarity does not represent two actin-binding sites, one in α and one in β , both of which are necessary for binding. CP may still have two binding sites for actin, each interacting with one protofilament at the barbed end of the actin filament. Alternatively, CP may have one large binding site for actin, that covers the actin protomers at the barbed end of the filament.

DISCUSSION

What are the molecular interactions important for the function of CP in vivo? Binding to actin filaments is an

Table 4. Rescue of *sac6* synthetic lethal phenotype by mutated CP genes on high copy number (2 μ) plasmids^a

Allele	Description	Cells viable with <i>sac6</i>
<i>CAP1</i>	wild type	100% (30/30)
<i>cap 1-1</i>	$\Delta 239-264$	2% (1/60)
<i>cap1-6</i>	RR239,240EE	0% (0/30)
<i>cap1-5</i>	E188K	2% (1/60)
<i>CAP2</i>	wild type	100% (30/30)
<i>cap2-7</i>	$\Delta 245-287$	0% (0/30)
<i>cap2-8</i>	RR14,15EE	100% (30/30)

^a Similar to Table 3, except that high copy number plasmids, instead of low copy number, were used to express the mutated genes.

Figure 7. Assay for actin-binding activity of mutant CPs by low-shear viscometry of actin filaments. Time in seconds for the ball to roll 10 cm is plotted versus amount of CP added. Each panel represents one experiment, in which a partially purified mutant CP is compared with purified CP as a standard. The quantity of CP in the partially purified preparation is determined by quantitative immunoblot. (A) Control using wild-type CP for the partial purification. (B) Mutant *cap2-1*, $\Delta 267-287$. (C) Mutant *cap2-6*, E119K. The values for the ordinate differ among experiments, because of slight differences in the actin preparation and details of assay conditions.

obvious and very likely possibility. CP binds filamentous actin very tightly *in vitro* and co-localizes with actin in a variety of cell systems. In addition, in yeast, null mutations of CP genes show synthetic phenotypes with null mutations of *SAC6*, which encodes the actin-binding protein fimbrin (Adams *et al.*, 1993; Karpova *et al.*, 1993), and with some point mutations of *ACT1*, which encodes actin (Karpova *et al.*, 1995). Another possible molecular interaction is binding and inhibition by PIP_2 . This interaction occurs *in vitro* (Amatruda and Cooper, 1992; Heiss and Cooper, 1991)

Table 5. Effect of PIP_2 on interaction of CP with actin filaments by low-shear viscometry

Source of CP	CP:	–	–	+	+
	PIP_2 :	–	+	–	+
wild type		44	40	8	42
<i>cap2-1</i>		41	36	12	33
<i>cap2-6</i>		35	32	15	35

The value reported is time in seconds for the ball to roll 10 cm. Final concentrations in the reaction are as follows: 0.35 mg/ml actin; 25 $\mu\text{g}/\text{ml}$ PIP_2 ; approximately 100 nM CP; 25% (v/v) buffer B; 27.5% (v/v) buffer G; 100% (v/v) KI buffer.

but its physiologic significance is uncertain. A third possible interaction may be with a component of cortical patches, other than actin, which could then serve to localize actin to the patches.

To identify physiologically relevant molecular interactions, we used a combination of the technical strengths of the yeast system—molecular genetics, cell biology, and biochemistry—to identify regions of CP necessary for function *in vivo*. We then determined whether different aspects of *in vivo* function, i.e., different phenotypic traits, were lost together or singly. We made site-directed mutations, both deletions and point mutations, and assayed for function *in vivo* by several different criteria, looking for whether mutations would affect one or another trait.

As expected, some mutations led to a complete loss of protein and a complete loss of function *in vivo*.

Figure 8. Calorimetry of CP. Calorie/degree is plotted versus temperature. A representative experiment is shown. CP consistently gave a single peak centered at 58°. The upper line is the initial heating, the lower line is the reheating after cooling. The absence of a peak in the lower line indicates that no refolding occurs. Samples heated to above 90° showed a total loss of the peak, and even samples heated only to 65° showed a very small peak.

Alpha		<i>cap1-5</i>	188 K
	Chick	YYEDG	
	Nematode	YYEDG	
	Dictyo	YFEDG	
	Yeast	YYEDG	
Beta	Chick	YFEGG	
	Nematode	YFEGG	
	Dictyo	YFEGG	
	Yeast	YYEGG	
		<i>cap2-6</i>	K 119

Figure 9. Diagram of the single short region of similarity in the predicted protein sequences of both α and β subunits of CP. The nature of the point mutation, its residue number, and allele name are also indicated.

These mutations are not informative for this analysis, which requires that the mutant protein be present in the cell.

Number of Regions Essential for Function

Mutations that give loss of function in vivo and preserve the presence of protein identify four different regions of the primary structure necessary for CP function. The first is the C-terminal portion of the β subunit, Cap2p, identified by a small truncation. The second is the C-terminal region of the α subunit, Cap1p, identified by a double charge-change point mutation in two conserved residues and a small deletion. The third is the interior of the α subunit, defined by a charge-change point mutation in a conserved residue, Glu188Lys. The fourth is the N-terminal region of Cap2p, identified by a double charge-change point mutation in two conserved residues, Arg14Glu and Arg15Glu.

Mutations in the first three regions led to a complete loss of all cellular aspects of CP function—growth rate, formation of actin cables, polarization of cortical actin patches, *sac6* viability, and localization of CP to cortical actin patches. The mutation in the fourth region caused a deficit only in the actin distribution, including formation of cables and polarization of patches, and that deficit was partial, not complete.

Some of these mutations also led to a decreased level of CP in vivo, so we were concerned about the possibility that loss of function was secondary to a reduced level of protein instead of reflecting the function of the affected region of the protein. To address this possibility, the mutant proteins were overexpressed and achieved levels equal to or greater than those of wild-type CP. In each of the four cases, including the mutation with only a partial deficit in actin distribution, we saw no increase in the level of function in vivo upon overexpression. Therefore, we conclude that the

effects of the mutations do reflect the properties of the relevant region of the protein and not simply a destabilization of the protein overall.

Number and Nature of Molecular Interactions

The first question for interpreting these results is how many molecular interactions are revealed? If the mutations in the four different regions were each associated with a different constellation of phenotypic traits, then the interpretation would be four different molecular interactions. This was the case for a mutational analysis of calmodulin in yeast (Ohya and Botstein, 1994). However, the results here are that the mutations in three different regions show the same constellation of phenotypic traits, which are the same as and include all those seen in a null mutant. These results do not support the existence of multiple molecular interactions and can be explained most simply by a single molecular interaction. Next, the constellation of traits associated with the fourth region is a subset of the larger group of traits. There are two main possible interpretations of this result. First, there may be multiple molecular interactions and this fourth region may be important for only one interaction while the other three regions are important for all molecular interactions. Second, there may be only one molecular interaction, and mutation of this fourth region only causes a loss of a portion of that interaction, while mutations in the other three regions cause a complete loss of the interaction. An explanation for only some traits being affected by the mutation of the fourth region is that these traits are more sensitive indicators of the interaction.

Without additional evidence, we prefer the more conservative interpretation of one molecular interaction because it is the simplest model that explains all the results. Additional experimentation might help discriminate between these models. First, testing more mutants would help to choose between the two interpretations, by providing either more results like those already obtained or by providing evidence for different constellations of traits. Second, an atomic structure of capping protein would indicate whether the four regions were in the same or different places in the tertiary structure. This analysis has an important limitation, however, which has been observed in studies of actin mutants. Mutations that affect one area of the three-dimensional structure of actin can have allosteric effects on distant regions (reviewed in Egelman and Orlova, 1995). Because the calorimetry analysis here indicates that CP is a single domain, the possibility for such allosteric effects is higher.

The second question for interpreting these results, assuming the single molecular interaction model, is what is the nature of that molecular interaction? The obvious candidate is binding to actin filaments for

several reasons. CP binds actin filaments *in vitro* with high affinity, CP co-localizes with actin filaments in cells, and CP interacts genetically with actin. One aspect of the results here supports that interpretation. Mutant CP showed a diffuse localization in the cell, instead of localizing to the patches of actin filaments. The mutants still had patches of actin filaments, so the mutant CP did not bind to those actin filaments *in vivo*. The best test of this hypothesis would be whether the mutant CPs bind to actin *in vitro*, using purified proteins. Unfortunately, our attempts to purify the relevant mutant CPs were unsuccessful, despite a wide variety of technical maneuvers. The proteins were proteolyzed or unstable during the purification process. We were able to purify two mutant CPs that showed normal function *in vivo*. They bound actin normally, which does support this hypothesis.

Comparison to Results with Chicken CP

The sequence of CP is well conserved across species (Waddle *et al.*, 1993), and CP from nematode (Waddle *et al.*, 1993) and *Drosophila* (Hopmann, Cooper, and Miller, unpublished data) is able to function in yeast, rescuing the null phenotype. The results here include one interesting discrepancy in comparing how yeast and chicken CP function to bind actin *in vitro*. At the C-terminal region of the CP β subunit, a 43-residue truncation was not functional *in vivo*, but truncations of 11, 21, and 31 residues were functional. Purified CP with the 21-residue truncation bound actin normally. On the other hand, with chicken CP β 1, a 12-residue truncation caused loss of actin-binding *in vitro* (Hug *et al.*, 1992) and a loss of function, consistent with loss of actin binding, *in vivo* during myofibrillogenesis (Schaffer *et al.*, 1995). A possible explanation for the difference between yeast and chicken proteins, especially because the 43-residue yeast truncation mutant was not functional *in vivo*, is that the C-terminal region is not the actin-binding site *per se*, but rather an element adjacent to the actin-binding site and essential for its structure. The yeast and chicken proteins might then simply differ in the extent of deletion necessary to destabilize that structure. Alternatively, this actin-binding site may exist in chicken muscle CP but not yeast CP, enabling stronger binding by the chicken CP.

The second essential region is the C-terminal region of the α subunit, Cap1p, where deletion of 45 residues causes loss-of-function, with presence of protein. In addition, mutation of two conserved Arg residues to Glu in this region also leads to loss of function. With chicken CP, deletion of 55 residues at the C-terminus of the α -subunit causes a loss of actin binding *in vitro* (Casella and Torres, 1994). So in this case, loss of

function *in vivo* with yeast CP does correlate with loss of actin binding *in vitro* with chicken CP.

Instability of Mutant CPs

Complete loss of one CP subunit leads to a loss of the other subunit *in vivo* (Amatruda *et al.*, 1992). Here we found that relatively small deletions at either end of either subunit of CP lead to a loss of protein *in vivo* and that CP behaves as a single domain by calorimetry. Therefore, the two subunits depend heavily on each other for their stability, which can be compromised by relatively minor perturbations. These results *in vivo* present an interesting contrast with results *in vitro* using chicken CP prepared by *in vitro* translation. In those studies, much more extensive deletions were compatible with the subunits binding to each other tightly enough to be purified by gel-filtration chromatography at relatively low protein concentrations (Casella and Torres, 1994). This difference between *in vivo* and *in vitro* results suggests that *in vivo* short truncations are sufficient to target the protein for degradation. This degradation must be rapid and complete because proteolysis of the subunits in the immunoblots of the whole cell extracts was not observed, just the absence of protein. Further analysis of the mechanism of this degradation may provide insight into normal mechanisms of protein turnover.

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