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The Actin-binding Domain of Cortactin is Dynamic and Unstructured and Affects Lateral and Longitudinal Contacts in F-actin

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Abstract

Cortactin is an F-actin- and Arp2/3 complex-binding protein, implicated in the regulation of cytoskeleton dynamics and cortical actin-assembly. The actin-binding domain of cortactin consists of a 6.5 tandem repeat of a 37-amino acid sequence known as the cortactin repeat (residues 80-325). Using a combination of structure prediction, circular dichroism and cysteine crosslinking, we tested a recently published three-dimensional model of the cortactin molecule in which the cortactin repeat is folded as a globular helical domain (Zhang et al., 2007). We show that the cortactin repeat is unstructured in solution. Thus, wild type and mutant constructs of the cortactin repeat, containing pairs of cysteines at positions 112 and 246, 83 and 112, 83 and 246, and 83 and 306, could be readily crosslinked with reagents of varying lengths (0–9.6 Å). Using yeast actin cysteine mutants, we also show that cortactin inhibits disulfide and dibromobimane crosslinking across the lateral and longitudinal interfaces of actin subunits in the filament, suggesting a weakening of inter-subunits contacts. Our results are in disagreement with the proposed model of the cortactin molecule and have important implications for our understanding of cortactin regulation of cytoskeleton dynamics.

Keywords

cortactin structure; circular dichroism; crosslinking; actin dynamics

INTRODUCTION

Cortactin is a cytoskeletal protein that interacts with F-actin (Wu et al. 1991), the Arp2/3 complex [Urano et al. 2001], deacetylase HDAC6 [Zhang et al., 2007] and with a growing list of cytoskeletal proteins [Selbach, Backert 2005; Cosen-Binker, and Kapus 2006; Huang et al. 2006; Boguslavsky et al. 2007; Le Clainche et al. 2007]. Cortactin is composed of three major domains: an N-terminal acidic (NTA) region implicated in the binding and weak activation of Arp2/3 complex, a central 6.5 tandem repeat of a 37-amino acid sequence involved in F-actin binding, and a C-terminal Src-homology 3 (SH3) domain that binds

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various cytoskeletal proteins, including N-WASP, WIP and MIM. Inserted between the cortactin repeat and SH3 domains, cortactin contains a segment predicted to be α -helical (although this has not been demonstrated) and a proline, serine, threonine (PST)-rich segment [Cosen-Binker and Kapus 2006]. Recently, it was reported that the cortactin repeat is acetylated *in vivo* and is a substrate for histone deacetylase 6 (HDAC6) [Zhang et al., 2007]. Acetylation of nine lysine residues within this region abolishes binding to F-actin. This effect was found to be additive, because reduced binding to F-actin occurred only when at least four of the acetylated lysine residues were mutated simultaneously to glutamine. To rationalize these findings, the authors generated a three-dimensional model of the cortactin molecule (PDB Code: 2F9X) in which the cortactin repeat is folded as a globular helical domain, with the acetylated lysine residues grouped into two patches on opposite sides of this domain (1A-B). Here, we tested this model and the effect of cortactin binding on the structure of the actin filament using a combination of structure prediction, circular dichroism (CD), and crosslinking of pairs of cysteine residues in the cortactin repeat. Our structure prediction analysis reveals a number of inconsistencies within the proposed model, while the CD spectrum of the cortactin repeat disagrees with the suggested helical fold of this domain.

Actin binding domain of cortactin appears to be dynamic and can form intramolecular crosslinkings (Shvetsov et al 2006). We found that two WT cortactin cysteines separated by 133 amino acids (C112-C246) could form a copper-catalyzed disulfide bond, suggesting close proximity of the cysteines and/or dynamic nature of the actin-binding repeats of cortactin. Recent study [Cowieson et al. 2008] reported five intramolecular cross-links between lysine residues (including cross-links within ABD) of full domain cortactin splice isoform 1 with 5.5 actin-binding repeats that are separated by more than 20 amino acids - also indicating dynamic nature of the cortactin repeats. To examine a putative dynamic nature of the cortactin ABD repeats and their interaction with actin, we created four new cysteine mutants within the actin binding domain of the cortactin construct 83-306 (consisting of the six complete repeats of mouse cortactin). We used increasing length cross-linkings such as disulfide (zero span), DBB (4.4 Å) and MTS-6 (9.6 Å) as molecular rulers for assessing the range of the proposed dynamic motions of cortactin repeats.

Our results show that wild-type cysteine residues 112 and 246 and cysteine residues introduced at the N- and C-terminus of construct can be crosslinked with reagents of varying lengths (0–9.6 Å). These findings suggest that the cortactin repeat is unstructured and highly dynamic in solution. In addition, we find that the crosslinking of wild-type residues C112 and C246 abolishes F-actin binding, suggesting that cortactin may undergo conformational changes upon binding to F-actin. Finally, crosslinking of cysteine residues between actin subunits in the filament is inhibited by the binding of the cortactin repeat, consistent with a weakening of lateral and longitudinal inter-subunit contacts in the filament by cortactin.

MATERIALS AND METHODS

Reagents

DNase I grade D was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Affigel-10 and Bio-Rad protein assay (Bradford assay) were obtained from Bio-Rad (Hercules, CA). Sephadex G-50, N-ethylmaleimide (NEM), ATP, phalloidin, DTT and PMSF were purchased from Sigma Chemical Company (St. Louis, MO). Peptone, tryptone, and yeast extract were from Difco (Detroit, MI). Protease inhibitors were purchased from Pierce (Pierce Protein Research Products, Thermo Fisher Scientific, Rockford, IL). 1,1-Methanediyl bismethanethiosulfonate (MTS-1-MTS), and MTS-6-MTS (1,6-Hexanediyl bismethanethiosulfonate) homobifunctional sulphydryl crosslinking reagents were purchased from Toronto Research Chemicals (North York, ON, Canada).

Protein preparation

The murine cortactin fragment 83-306, comprising the six complete repeats of cortactin, was cloned between the NdeI and EcoRI sites of vector pTYB12 (New England BioLabs) and expressed in BL21(DE3) cells as described [Pant et al 2006]. This fragment contains two endogenous cysteine residues at positions 112 and 246. Three mutants of this fragment, containing pairs of cysteine residues at positions 83 and 112 (double mutant G83C and C246S), 83 and 246 (double mutant G83C and C112S) and 83 and 306 (quadruple mutant G83C, F306C, C112S and C246S), were generated using the QuickChange mutagenesis kit (Stratagene).

BL21(DE3) cells (Invitrogen) were transformed with the cortactin constructs and grown in LB medium at 37°C until the OD at 600 nm reached a value of 0.8. Expression was induced by addition of 0.5 mM isopropylthio- β -D-galactoside (IPTG) and carried out for 5 hours at 20°C. Cells were harvested by centrifugation and resuspended in chitin-affinity-column equilibration buffer (10 mM Tris-HCl pH 7.6, 300 mM KCl, 0.5 mM DTT). After purification on a chitin affinity column (New England BioLabs manual) the proteins were released by activation of intein self-cleavage with 50 mM DTT (WT construct) or by increasing pH and temperature to 8.5 and 20°C respectively (cysteine mutants). The purified proteins were dialyzed against 10 mM Tris-HCl buffer pH 7.6, 300 mM KCl, 0.5 mM DTT and further purified on a Superdex 75 sizing column (Amersham). The proteins were then concentrated using a 10 K Vivaspin centrifugal device (Sartorius). Protein concentration was determined from the OD at 280 nm, using extinction coefficients calculated from the amino acid sequences of each construct.

Saccharomyces cerevisiae Q41C and S265C actin mutants were expressed and affinity-purified on a DNase I column as previously described [Kim et al. 2000]. Rabbit skeletal muscle actin was isolated according to Spudich & Watt [Spudich and Watt 1971]. The concentration of yeast actin was measured by the Bradford protein assay using skeletal rabbit muscle actin as a standard.

Structure prediction and model validation

Secondary structure and disorder predictions and model validation were performed using the programs WHAT-CHECK (<http://swift.cmbi.ru.nl/whatif>) [Hooft et al. 1996], Phyre (<http://www.sbg.bio.ic.ac.uk/~phyre>) [Bennett-Lovsey et al. 2008], PredictProtein (<http://www.predictprotein.org/>) [Rost et al. 2004], PONDR (<http://www.pondr.com>) [Romero et al. 2001] and HCA (<http://bioserv.rpbs.jussieu.fr/RPBS>) [Callebaut et al. 1997].

Circular dichroism

Circular dichroism (CD) spectra of the cortactin repeat were collected in the range of 200 nm to 260 nm using a Jasco J-810 spectropolarimeter and a 1mm wide cuvette. The experiments were performed at 20°C at a protein concentration of 18 μ M in 10 mM Tris-HCl buffer pH 7.6, 300 mM KCl and 0.5 mM DTT.

Crosslinking and SDS PAGE analysis of yeast actin mutants Q41C and S265C

Immediately prior to each crosslinking reaction, DTT was removed from actin on a Sephadex G-50 spin column equilibrated with 10 mM HEPES pH 7.2, 0.2 mM CaCl_2 and 0.2 mM ATP. DTT-free actin, at the concentration of 10 μ M, was polymerized for 20 min at room temperature with addition of 3.0 mM MgCl_2 . Cysteine residues 374 and 41 (Q41C mutant) and 374 and 265 (S265C mutant) were crosslinked in F-actin with dibromobimane (DBB) at room temperature and at varying time intervals (2–60 minutes), in the absence and the presence of cortactin 83-306 (used at a molar ratio of 1:1 actin:cortactin). Disulfide bond formation (on ice) was catalyzed by addition of 5 μ M CuSO_4 . Aliquots of the reactions were

taken at selected time intervals. Free cysteine residues were blocked by addition of 2.0 mM NEM. Actin samples were analyzed on 7.5–10% SDS-PAGE in the absence of mercaptoethanol. Crosslinking of cysteines residues Q41C and S265C within the actin filament resulted in the appearance of actin oligomers with lower electrophoretic mobility on SDS PAGE.

Crosslinking and SDS PAGE analysis of wild-type and mutant cortactin repeat

Freshly prepared 10–20 μ M DTT-free cortactin repeat constructs (passed through Sephadex a G-50 spin column equilibrated with 20 mM Tris-HCl or HEPES buffer pH 7.2–7.5, 200–300 mM KCl, 100 μ M PMSF) were used in the crosslinking experiments. Pairs of cysteines at positions 112 and 246, 83 and 112, 83 and 246, and 83 and 306 were crosslinked at varying time intervals with reagents of increasing span (0–9.6 Å). The reactions included direct disulfide bond formation (using 10–40 μ M CuSO_4 to catalyze air oxidation within 7–30 min on ice), 20–40 μ M DBB (for 30 min at room temperature) or 20–30 μ M MTS-6 (7–30 min on ice). Aliquots of these reactions were taken and free cysteine residues were blocked with 2.0 mM NEM. The samples were analyzed on non-reducing 12–15% SDS-PAGE. The crosslinking of cysteine residues in cortactin resulted in species with higher electrophoretic mobility.

Co-sedimentation assays of cortactin binding to F-actin

To probe whether the cortactin repeat undergoes a conformational changes upon binding to F-actin, the endogenous cysteine residues 112 and 246 were crosslinked by reversible disulfide, MTS-1 or MTS-6 (as described above) and then co-sedimented with F-actin. The disulfide and MTS crosslinked cortactin repeat were mixed with rabbit skeletal muscle F-actin at 1:1 and 1:6 molar ratios and centrifuged in a Beckman airfuge at 25 psi for 30 min at room temperature. The pellet and supernatant fractions were analyzed on 12% SDS PAGE. The uncrosslinked wild-type cortactin repeat was used as a control in co-sedimentation experiments.

Reversal of disulfide and MTS-6 crosslinking of the cortactin repeat

The disulfide bond and MTS-6 crosslinks of the cortactin repeat were reduced by incubation in 10 mM DTT, 20 mM HEPES pH 7.8, 0.2 mM ATP, 0.2 mM CaCl_2 , 300 mM KCl and 100 μ M PMSF for 30–60 min. The actin-cortactin samples were then pelleted in a Beckman airfuge at 25 psi for 30 min, at room temperature. The formation of cortactin-actin complexes was determined by SDS PAGE analysis.

RESULTS AND DISCUSSION

Analysis of three-dimensional model of cortactin reveals inconsistencies

In a recently reported three-dimensional model of the cortactin molecule [Zhang et al., 2007, PDB Code: 2F9X], the cortactin repeat is folded as a globular helical domain, with the acetylated lysine residues grouped into two patches on opposite sides of this domain (Figure 1A–B). An analysis of this model reveals a number of inconsistencies. First, the presence of two independent patches suggests the existence of two actin-binding sites on opposite sides of the cortactin repeat and a potential filament crosslinking function, which has not been shown conclusively. Second, according to the model of Zhang et al. [Zhang et al., 2007], the last 22 amino acids of each repeat form 6-turn α -helices (Figure 1A–B). However, this region of the repeats is charged and lacks hydrophobic amino acids that would form the core of a globular helical domain. Thus, analysis of the model with the program WHAT_CHECK [Hooft et al. 1996] reveals an unusual distribution of residues, with many charged amino acids buried in the core region, whereas a number of hydrophobic amino acids appear

exposed at the surface (Figure 1B). Analysis of the cortactin sequence using various structure prediction programs, including Phyre [Bennett-Lovsey et al. 2008], PredictProtein [Rost et al. 2004] and HCA(16), suggests that other than the C-terminal SH3 domain, the cortactin molecule is mostly disordered. Interestingly, the program PONDR [Romero et al. 2001], which predicts naturally disordered regions in proteins, suggests that while disordered the repeat region of cortactin could undergo a disorder-to-order transition upon binding to a partner molecule (Figure 1C). This is consistent with the presence of conserved clusters of hydrophobic amino acids within the N-terminal portion of each repeat identified with the program HCA, which display the signature pattern of α -helices [Callebaut et al. 1997]. However, the hydrophobic clusters identified by HCA are not continuous and coincide with the location of four conserved glycine residues within each repeat. This suggests that in the absence of stabilizing interactions with a partner molecule (possibly F-actin) the repeat region is disordered. Given these inconsistencies, we set out to test the model of Zhang et al. [Zhang et al 2007] experimentally. To this end, we employed CD spectroscopy, site-specific crosslinking of the cortactin repeat and co-sedimentation assays with F-actin.

The cortactin repeat appears disordered in solution but could undergo ligand-induced folding

We analyzed the secondary structure of the repeat region of mouse cortactin (residues 83–306, comprising the six complete repeats of mouse cortactin) in solution using CD. Fig. 2 shows the far-UV CD spectrum recorded at 20°C and pH 7.6. The spectrum is characterized by a minimum of ellipticity near 205 nm. This is unlike the characteristic spectra of α -helices (minima near 209 and 222nm), β -strands (minimum near 218 nm) or totally disordered structures (minimum near 196 nm). Instead, the CD spectrum of the cortactin repeat resembles that of the so-called molten globule state [Kelly and Price 1997]. The molten globule state has been identified as a protein folding intermediate, structurally and thermodynamically distinct from both the native state and the denatured state of a protein [Haynie and Freire 1993; Kuwajima 1996]. Because the CD spectrum of the cortactin repeat was collected under non-denaturing conditions, we believe that its resemblance with the spectrum of the molten globule suggests that the cortactin repeat is partially disordered. It may be speculated, as suggested above by the program PONDR, that cortactin undergoes ligand-induced folding. The total density associated with the F-actin-bound cortactin repeat in an EM reconstruction was less than expected from its molecular mass [Pant et al. 2006], suggesting that even when bound to F-actin the cortactin repeat is at least partially disordered. Although our results do not rule out the possibility that the acetylated lysine residues of the cortactin repeat are grouped in patches, another possibility would be that the cortactin ABD binds to F-actin in an extended conformation, with each lysine residue contributing additively to the binding energy.

Crosslinking of cysteines 112 and 246 in the cortactin repeat abolishes F-actin binding

A number of proteins, including spectrin [An et al. 2006], plectin [Garc 'a-Alvarez et al. 2003], and thymosin- β 4 [Domanski et al. 2004], undergo conformational changes when they bind to F-actin. To test whether this is also the case for the cortactin ABD, we crosslinked cortactin cysteine residues 112 and 246 either directly, by disulfide bond formation, or with MTS-6. The results shown in Fig. 3A demonstrate that this cysteine pair is readily crosslinked intramolecularly by direct disulfide bond formation. Similar results were obtained with bifunctional MTS reagents (data not shown). One way to rationalize these results is that the first and fifth actin binding repeats, where these two cysteine residues are located, are in close proximity to each other in the unbound cortactin repeat. Alternatively, the cortactin repeat may have a dynamic structure, making the crosslinking reaction more favorable. Unlike the unmodified cortactin repeat, the crosslinked protein fails to co-

sediment with F-actin (Fig. 3A). This loss of actin binding activity is reversible. Indeed, the binding to F-actin was fully restored upon reduction of the disulfide bond by incubation with 10mM DTT (see Materials and Methods for buffer conditions). Similar results were observed for the MTS-6 crosslinked cortactin repeat (data not shown).

The inability of the crosslinked cortactin repeat to bind F-actin could be due to either, blockage of a conformational change required for binding, or residues C112 and C246 being part of the F-actin-binding interface. To distinguish between these two possibilities we examined the binding of NEM-labeled cortactin repeat to F-actin. The cortactin repeat was labeled with 3-fold molar excess of NEM in the absence of DTT. Co-sedimentation of the NEM-labeled cortactin repeat with F-actin at a 1:6 cortactin:actin molar ratio, followed by SDS PAGE analysis of pellet and supernatant fractions revealed that NEM labeling of cysteine residues 112 and 246 does not interfere with the ability of cortactin to bind F-actin (Fig. 3B). This finding and the observation that the loss of actin binding resulting from crosslinking is reversible are consistent with a conformational change taking place in the cortactin repeat upon binding to F-actin that is inhibited by the crosslink.

Crosslinking of the cortactin repeat suggests a dynamic structure

To assess the conformation of the cortactin repeat in the unbound and F-actin-bound states, we generated a series of constructs containing pairs of cysteine residues at positions 83 and 112, 83 and 246, and 83 and 306 (Materials and Methods). In the model of Zhang et al. [Zhang et al. 2007] the C α atoms of these pairs of residues are 37.36 Å (G83-C112), 19.53 Å (G83-C246), 22.64 Å (C83-F306) and 37.45 Å (C112-C246) apart. To probe the actual distances between cysteine pairs and the dynamic motions within the domain, we used crosslinks of varying lengths, including zero length (disulfide bond), 4.4 Å (DBB) and 9.6 Å (MTS-6). Fig. 3C shows that all the pairs of cysteine residues, as well as the endogenous cysteine pair C112-C246, were effectively intramolecularly crosslinked by disulfide bonds, DBB and MTS-6. The intramolecularly crosslinked species are characterized by faster electrophoretic mobility on non-reducing SDS PAGE. The results suggested that either all the cysteine pairs are within the 0 to 10 Å distance span defined by the bifunctional crosslinking reagents or that the conformation of the cortactin repeat in solution is extremely dynamic. The latter appears more likely, in particular since all the crosslinked species fail to bind F-actin (data not shown), i.e. all the crosslinks interfere with structural changes necessary for binding to F-actin. This is inconsistent with the proposed model of the cortactin repeat [Zhang et al. 2007], and suggests that regulation of the cortactin functions by acetylation may not depend on the formation of two localized patches on the opposite sides of the cortactin repeat.

Whether the conformation of the cortactin repeat is compact or extended in the F-actin bound state is unclear. Pant et al. [Pant et al. 2006] could not account for the total mass of the cortactin repeat in their electron microscopy (EM) reconstruction with F-actin, suggesting that at least part of the repeat region is disordered in the F-actin-bound state. We note also that vertebrate cortactin, with molecular weight ~61 kDa, migrates as multiple bands with apparent molecular weights between 80 and 85 kDa SDS PAGE [Wu et al 1991; Schuuring et al. 1993]. This observation was interpreted as evidence for multiple conformational states, because cortactin migrates as a single band in 5M urea [Huang et al. 1997]. Cortactin also migrates as a single band upon deletion of the predicted α -helical and PST segments (but not the SH3 domain), indicating that these two segments are at least in part responsible for the polymorphism of cortactin [Campbell et al. 1999].

Cortactin inhibits strongly lateral and longitudinal crosslinking in F-actin

One of the main goals of this study was to elucidate the effects of cortactin on interprotomer contacts in F-actin and to clarify the cortactin-induced changes in the filament structure. The EM reconstruction of the F-actin-bound cortactin repeat revealed a widening of the gap between the filament strands [Pant et al 2006]. Based on this observation, we speculated that cortactin would affect both lateral and longitudinal contacts between actin subunits in the filament. To test this possibility, we probed the longitudinal and lateral interfaces of actin subunits in the filament by crosslinking yeast actin mutants Q41C and S265C in the presence and the absence of the cortactin repeat. Indeed, the endogenous C374 of actin can be crosslinked longitudinally to C41 in the D-loop, or laterally to C265 in the hydrophobic loop of neighboring actin subunits in the filament [Kim et al. 2000]. These crosslinking reactions can occur either directly or mediated by dibromobimane (DBB) (Materials and Methods). As shown in Fig. 4, binding of the cortactin repeat to F-actin inhibits strongly the rate of copper-catalyzed disulfide crosslinking across the lateral and longitudinal interfaces of actin subunits in the filament. The inhibition of lateral (inter-strand) crosslinking was prominent (~100%) and characterized by the absence of actin dimers, or higher oligomers in the presence of the cortactin repeat, as compared to the abundance of crosslinked species in the absence of the cortactin (Fig. 4A). Similar results were obtained by crosslinking with DBB (data not shown). Strong inhibition of disulfide (and DBB) crosslinking by the cortactin repeat was also observed along the longitudinal intra-strand interface (Fig. 4B). Therefore, in agreement with the EM reconstruction [Pant et al. 2006], our results suggest that cortactin weakens lateral and longitudinal interprotomer contacts in the filaments. In particular, the inhibition of the crosslinking reactions suggests an increase in the distance and/or changes in the orientation of cysteine pairs C41-C374 and C265-C374. However, unlike cofilin, which also alters longitudinal and lateral contacts in F-actin, cortactin does not change the twist of the actin filament [Pant et al. 2006]. The cortactin-induced changes may act synergistically to affect the binding of other proteins to the actin filament. Thus, for instance, coronin, Aip1 and cofilin appear to act synergistically, such that coronin helps to create a new binding site for cofilin on F-actin [Briher et al. 2006]. Similarly, Pant et al. [Pant et al. 2006] suggested that cortactin-induced conformational changes in F-actin could help stabilize the binding of Arp2/3 complex to the side of the mother filament. Our results are consistent with this proposal, but do not provide direct evidence to support it.

CONCLUSIONS

Our results strongly suggest that the conformation of the cortactin repeat is highly dynamic in solution. However, a transition of the cortactin repeat from a disordered state in solution to a more stably folded conformation upon binding to F-actin is supported both by structure prediction with the program PONDR and by the resemblance of its CD spectrum with that of the molten globule. The binding of the cortactin repeat to F-actin strongly affects lateral and longitudinal contacts between actin subunits in the filament. Our findings are consistent with the EM reconstruction of the F-actin-bound cortactin repeat [Pant et al. 2006] and with the recent study of Cowieson et al., 2008, and are incompatible with the proposed model of the cortactin molecule [Zhang et al. 2007].

These observations and continued studies along similar lines should contribute to the understanding of the mechanism by which cortactin interacts with actin and other target proteins to affect the cytoskeleton structure and dynamics.

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Abbreviations

ABP	actin-binding protein
ABD	actin-binding domain
HDAC6	histone deacetylase 6
DBB	dibromobimane
DNase I	deoxyribonuclease I
F-actin	filamentous actin
G-actin	monomer actin
DTT	dithiothreitol
Arp2/3 complex	actin-related proteins 2 and 3 complex
MTS-1 and MTS-6	bismethanethiosulfonate, bi-functional crosslinkers

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Fig. 1. Analysis of the proposed model of the cortactin repeat

(A) Alignment of the repeats of mouse cortactin (NP_031829). Amino acids are colored according to their chemical character: green, hydrophobic; red, negatively charged; cyan, positively charged; orange, glycines. Cysteine residues 112 and 246 are highlighted in yellow. (B) Proposed model of the repeat region of cortactin [Zhang et al. 2007]. Some inconsistencies of this model include: the presence of long α -helices within each repeat that are not supported by structure prediction or the CD spectrum (see Figure 1), the presence of charged amino acids buried in the core region (atom-type color coded), and the presence of hydrophobic amino acids at the surface (green). The acetylated lysine residues are shown (purple). Although these lysine residues were described as forming two opposite patches in the model, they appear relatively scattered. (C) Most of the cortactin molecule (except the

C-terminal SH3 domain) is predicted to be disordered (blue and magenta traces) by the program PONDR [Romero et al. 2001]. However, the cortactin repeat (amino acids 80 to 325) is predicted to undergo a disorder-to-order transition upon binding to a target (red trace).

(D) Oblique representation of the sequences of each of the cortactin repeats, showing the distribution of clusters of hydrophobic amino acids as implemented in the program HCA [Callebaut et al. 1997]. Amino acids forming part of hydrophobic clusters are contoured and colored green. Symbols are: Gly, black diamond; Pro, red star; Thr, empty square; Ser, partially filled square. The region contoured by a black rectangle includes the conserved clusters of hydrophobic amino acids of the cortactin repeats. Although these clusters have the characteristic shape of α -helices, this region also contains four conserved glycine residues (black diamonds) and formation of α -helices will likely depend upon stabilization by interaction with a partner. Notice that the helices predicted by HCA occur at the N-terminus of each repeat, not the C-terminus as in the proposed model (part B of this figure).

Fig. 2.

Far-UV CD spectrum of the cortactin repeat. The CD spectrum was obtained at a protein concentration of 18 μ M in 10 mM Tris-HCl pH 7.6, 300 mM KCl, 0.5 mM DTT, using a Jasco J-810 spectropolarimeter. Measurements were taken at 20° C. Note that the CD spectrum of the cortactin repeat presents a minimum at 205 nm, similar to the spectrum of the molten globule state of protein folding (Kelly and Price, 1997).

Fig. 3. The cortactin repeat has a dynamic conformation in solution

(A) The endogenous cysteine residues 112 and 246 of the cortactin repeat were crosslinked and the ability of the crosslinked protein to bind F-actin was tested in a co-sedimentation assay (Materials and Methods). The pellet (P) and supernatant (S) were analyzed on 12% SDS-PAGE. Lanes 1 and 2, uncrosslinked cortactin; lanes 3 and 4, disulfide crosslinked cortactin (xl-cortactin); lanes 5 and 6, reduction of the disulfide crosslink with 10mM DTT. (B) The ability of the NEM-labeled cortactin repeat (at residues C112 and C246) to bind to F-actin was assessed using a co-sedimentation assay (Materials and Methods). The pellet and supernatant were analyzed on 12% SDS-PAGE. Lanes 1 and 2, non-labeled cortactin; lanes 3 and 4, NEM-labeled cortactin.

(C) Crosslinking of cortactin repeat constructs containing pairs of cysteine residues at positions 83 and 306 (left), 83 and 246 (middle), and 83 and 112 (right). Prior to each crosslinking reaction, DTT was removed from the samples on a Sephadex G-50 spin column equilibrated with 20 mM HEPES pH 7.0, 0.1 M KCl, and 100 μ M PMSF. The crosslinking reactions were performed on ice and catalyzed by addition of 10 μ M CuSO₄. Each cysteine pair was crosslinked either directly by disulfide bond formation (zero length crosslink), or using the crosslinking reagents DBB (4.4 Å span) or MTS-6 (9.6 Å span).

Fig. 4.

The Cortactin repeat alters inter-subunit contacts in F-actin. The cortactin repeat inhibits the formation of lateral (A) and longitudinal (B) crosslinks in the actin filament between C374 and mutant cysteine residues at position 265 and 41 of yeast actin, respectively. Note how the formation of higher molecular weight crosslinked species in the absence of cortactin is inhibited upon addition of the cortactin repeat. Conditions: DTT-free actin (10 μ M) was polymerized for 20 min at room temperature by addition of 3.0 mM MgCl₂. Cysteine residues 374 and 265 (lateral inter-subunit contact) and 374 and 41 (longitudinal inter-subunit contact) were crosslinked on ice in the presence or the absence of the cortactin repeat at a 1:1 molar ratio. These cysteine pairs were crosslinked directly, via disulfide bond formation catalyzed by addition of 5 μ M CuSO₄. Aliquots of the reactions were taken at selected time intervals and analyzed on non-reducing 7.5–10% SDS-PAGE. xl-actin, crosslinked actin.