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Cortactin Binding to F-actin Revealed by Electron Microscopy and 3D Reconstruction

Kiran Pant[1]†, David Chereau[2]†, Victoria Hatch[1], Roberto Dominguez[2] and William Lehman[1]*

[1]*Department of Physiology & Biophysics, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118 USA*

[2]*Boston Biomedical Research Institute, Watertown, MA 02472, USA*

**Corresponding author*

Cortactin and WASP activate Arp2/3-mediated actin filament nucleation and branching. However, different mechanisms underlie activation by the two proteins, which rely on distinct actin-binding modules and modes of binding to actin filaments. It is generally thought that cortactin binds to "mother" actin filaments, while WASP donates actin monomers to Arp2/3-generated "daughter" filament branches. Interestingly, cortactin also binds WASP in addition to F-actin and the Arp2/3 complex. However, the structural basis for the role of cortactin in filament branching remains unknown, making interpretation difficult. Here, electron microscopy and 3D reconstruction were carried out on F-actin decorated with the actin-binding repeating domain of cortactin, revealing conspicuous density on F-actin attributable to cortactin that is located on a consensus-binding site on subdomain-1 of actin subunits. Strikingly, the binding of cortactin widens the gap between the two long-pitch filament strands. Although other proteins have been found to alter the structure of the filament, the cortactin-induced conformational change appears unique. The results are consistent with a mechanism whereby alterations of the F-actin structure may facilitate recruitment of the Arp2/3 complex to the "mother" filament in the cortex of cells. In addition, cortactin may act as a structural adapter protein, stabilizing nascent filament branches while mediating the simultaneous recruitment of Arp2/3 and WASP.

Keywords: actin; Arp2/3; cortactin; WASP; electron microscopy

Introduction

The cortical actin cytoskeleton is a dynamic structure that is essential for vital cellular functions as diverse as cell motility, adhesion, endocytosis and cytokinesis.[1] Different types of cytoskeletal assemblies, including lamellipodia, filopodia, stress fibers and focal adhesions, form by recruiting actin filaments in response to various receptor-mediated signaling cascades, typically involving Rho family GTPases and receptor tyrosine-kinases. These pathways modulate the activities of numerous actin-binding proteins, which control filament assembly and inter-filament architecture. Thus, actin-binding proteins connect extra- and intracellular stimuli to cytoskeleton modeling and reorganization. A key participant in this scheme is the Arp2/3 complex, which mediates the nucleation and branching of actin filaments at the leading edge of the cell. The activity of the Arp2/3 complex is stimulated by filament nucleation promoting factors (NPFs), including members of the extensively investigated Wiskott–Aldrich syndrome protein (WASP) family.[1–3] These proteins share a C-terminal WASP-homology 2 (WH2) domain adjacent to a central-acidic region (C-A); WH2-C-A represents the minimal sequence needed for activation of the Arp2/3 complex by WASP.[4] Cortactin, an additional activator of the Arp2/3 complex, also has attracted considerable attention.[5,6] Cortactin is an F-actin-binding protein that appears to bind to the Arp2/3 complex and specifically activate Arp2/3 filamentogenesis at the cell cortex, but by a mechanism distinct from that of WH2-C-A-containing NPFs.

Like most cytoskeletal proteins, cortactin is built from several distinct domains.[7–10] Its N-terminal

† K.P. & D.C. contributed equally to the work.

Abbreviations used: EM, electron microscopy; WASP, Wiskott–Aldrich syndrome protein; IHRSR, iterative helical real space reconstruction.

E-mail address of the corresponding author: wlehman@bu.edu

acidic (NTA) domain binds to the Arp2/3 complex and contributes to its activation.[5,6] The ^{19}DDWE^{23} sequence, within this region, resembles ^{498}DEWD^{501} in the acidic region of WASP and mutations in the sequence[5,11,12] perturb the activation of the Arp2/3 complex. However, further analogy between cortactin and WASP is limited, since cortactin lacks a WH2, G-actin-binding domain. Instead, a series of 6.5 tandem repeats (37 amino acid residues per full repeat) located C-terminal to the NTA region, form an F-actin binding module. Because the protein sequence is unique to cortactin, the entire modular ensemble is referred to as the "cortactin-repeating" domain.[13] However, it appears that only the fourth in the series of six repeats and its associated flanking sequences are needed for binding to F-actin *in vitro*.[14] Nevertheless, the NTA region, as well as the cortactin-repeating domain, is required for cortactin-activation of the Arp2/3 complex[5], and cortactin variants that lack either of these distinct domains fail to localize at the cell periphery.[14] The cortactin molecule also contains three additional domains, which may be needed to bind to other protein partners or respond to tyrosine phosphorylation. These consist of an α-helical spacer, followed by a proline-rich motif and a Src homology (SH3) domain at the protein's C-terminus. Pro-rich sequences, which are abundant among cytoskeletal proteins, often mediate the binding of proteins with SH3 or WW signaling adapter domains and profilin; Ser and Tyr phosphorylation sites within this region may regulate the activity of cortactin. In fact, cortactin was initially characterized as a substrate of Src kinase, possibly connecting tyrosine kinase signaling and cytoskeleton organization.[7–9] Interestingly, the C-terminal SH3 domain of cortactin binds to several targets,[7] including N-WASP,[15,16] hence linking cortactin and WASP function.[12]

While the importance of cortactin as determinant in cytoskeleton dynamics is emerging, little is known about the structural interactions between cortactin and actin filaments; thus the behavior and action of cortactin cannot be adequately described. Here, we addressed this deficiency by carrying out electron microscopy and 3D reconstruction of F-actin complexed with a construct representing the actin-binding repeating domain of cortactin. In addition to determining its binding site on F-actin, we found that the six-module cortactin-repeating domain modified the global structure of the actin filament. Such a change in structure might mark a filament as a target for Arp2/3-binding or stabilize preformed filaments.

Results and Discussion

Electron microscopy of F-actin–cortactin complexes

F-actin was complexed with a construct containing the 224 amino acid residue long repeating domain of cortactin under conditions

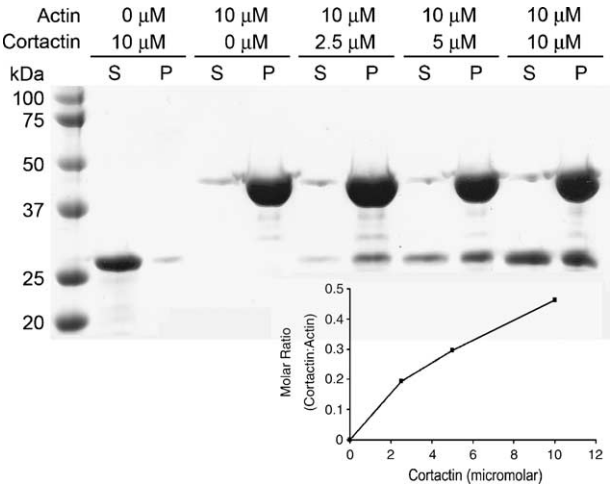

Figure 1. Assessing the purity and F-actin-binding activity of the cortactin six-repeat region. The cortactin-repeating domain runs as a single band on SDS-PAGE and co-sediments with F-actin after ultracentrifugation. As indicated, 0, 2.5, 5 or 10 μM purified cortactin-repeating domain construct (residues 83–306) incubated with 0 or 10 μM F-actin (in 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 50 mM KCl, 1 mM EGTA, 0.2 mM ATP, 0.2 mM DTT) for 30 min at room temperature was centrifuged at 400,000*g* for 30 min. (To preclude measuring non-specific binding, higher ratios of cortactin to actin were not used.) The pellet was gently washed and resuspended in a volume of fresh buffer equal to that of the supernatant. Equal aliquots of supernatant (S) and pellet (P) were analyzed by SDS-PAGE followed by Coomassie blue staining. Binding was quantified by densitometric scanning and plotted (see inset).

that should maximize binding of the construct on filaments (see Materials and Methods and Figure 1). Electron micrographs of negatively stained preparations showed that the decorated filaments were well dispersed and approximately 10% wider than undecorated control F-actin (Figure 2). When compared to control F-actin, the actin subunit structure of the decorated filaments appeared less well defined, due to the binding of additional protein, yet the filaments showed no obvious visual evidence of the shape and orientation of cortactin. To detect the binding of cortactin and its impact on F-actin structure with greater clarity, image processing and 3D reconstruction were carried out.

3D Reconstruction of F-actin–cortactin

Electron micrographs of F-actin complexed with two different preparations of the cortactin construct were analyzed independently. Since each data set yielded virtually the same results, the information was combined. Density maps of reconstituted filaments were calculated from the averages of the Fourier transform layer-line data (not shown). All the maps obtained showed typical actin subdomain structure (Figure 3(a) and (d)). When compared with maps of F-actin controls (Figure 3(b) and (e)),