Association of cortactin with dynamic actin in lamellipodia and on endosomal vesicles

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SUMMARY

We have used fluorescent protein tagging to study the localization and dynamics of the actin-binding protein cortactin in living NIH 3T3 fibroblast cells. Cortactin was localized to active lamellipodia and to small cytoplasmic spots. Time-lapse imaging revealed that these cortactin labeled structures were very dynamic. In the lamellipodia, cortactin labeled structures formed at the leading edge and then moved toward the cell center. Experiments with green fluorescent protein (GFP)-tagged actin showed that cortactin movement was coincident with the actin retrograde flow in the lamellipodia. Cytoplasmic cortactin spots also contained F-actin and were propelled by actin polymerization. Arp3, a component of the arp2/3 complex which is a key regulator of actin polymerization, co-localized with cortactin. Cytoplasmic cortactin-labeled spots were found to be associated with endosomal vesicles. Association was asymmetric and approximately half of the endosomes were associated with cortactin spots. Time-lapse imaging suggested that these cortactin and F-actin-containing spots propelled endosomes. Actin polymerization based propulsion may be a common mechanism for endomembrane trafficking in the same manner as used in the plasma membrane protrusions. As cortactin is known to interact with membrane-associated signaling proteins it could have a role in linking signaling complexes with dynamic actin on endosomes and in lamellipodia.

INTRODUCTION

The formation of cell protrusions such as lamellipodia and filopodia in motile cells is dependent on actin polymerization. The actin assembly takes place at the leading edge of the protrusions and is thought to provide the force for forward movement of the plasma membrane (for review see Mitchison and Cramer, 1996). In several cell types the actin meshwork moves in a retrograde manner from the leading edge toward the cell body where actin is then depolymerized (Fisher et al., 1988; Theriot and Mitchison, 1992; Wang, 1985). This movement is known to be myosin dependent (Lin et al., 1996). It has been suggested that attachment of the actin cytoskeleton in lamellipodia to the substrate via transmembrane adhesion receptors stops the retrograde movement and leads to forward movement of the cell body (Lin and Forscher, 1995; Suter et al., 1998). This is supported by the finding that in some very rapidly migrating cell types the actin meshwork in the lamellipodia stays immobile in relation to the substrate (Theriot and Mitchison, 1991).

Actin cytoskeleton has also been shown to be crucial for transport of endocytosed molecules. The initial internalization step requires actin cytoskeleton (Lamaze et al., 1997) and the later trafficking of endocytosed molecules is affected by disruption of the actin cytoskeleton (Durrbach et al., 1996). The mechanism by which actin is involved in endosomal trafficking is not well understood. There is also evidence to suggest that endomembrane trafficking and cell motility are somehow connected. Endocytosed molecules are preferentially exocytosed at sites of plasma membrane protrusions (Bretscher and Aguado-Velasco, 1998). Endosomes could transport molecules needed for cell motility to sites of active actin polymerization at the leading edge.

Cortactin is a widely expressed actin-binding protein that was originally identified as a substrate for src kinase (Wu et al., 1991). In addition to the actin-binding domain it contains an SH3-domain, several tyrosine phosphorylation sites and a proline-rich region (Wu et al., 1991). It interacts via its SH3-domain with several PDZ-family proteins (Du et al., 1998; Katsube et al., 1998; Naisbitt et al., 1999). Cortactin has been implicated in cell motility and in linking transmembrane signaling to the cytoskeleton (Huang et al., 1998; Kinnunen et
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RESULTS

Localization of cortactin fusion proteins

To study the localization and dynamics of cortactin in living cells we produced expression vectors of cortactin fused to GFP or RFP. We made vectors wherein the fluorescent protein was fused to either the C- or N-terminus of cortactin (pCortactin-GFP, pCortactin-RFP and pGFP-Cortactin respectively). These constructs were then expressed in NIH 3T3 fibroblasts, either in transiently transfected cells or in a stably expressing cell line. The localization of all three fusion proteins was identical to that of the endogenous cortactin as revealed by immunostaining (Fig. 1). Therefore, the fluorescent protein tag did not interfere with the normal localization of cortactin. Cortactin was localized, as shown in previous studies (Wu et al., 1991), to the lamellipodia in spreading cells. It was also concentrated into small spots that were found throughout the cytoplasm. The localization of cortactin fusion proteins was clearly different from GFP alone, which was diffusely distributed (Fig. 1e,f). Co-expression of cortactin-RFP and soluble GFP in the same cells showed that the lamellipodial cortactin signal was not due to increased thickness at the leading edge but manifested true concentration of the cortactin fusion protein. Expression of the fusion proteins did not have any apparent effects on cell morphology.

Fig. 1. Localization of GFP or RFP tagged cortactin is similar to endogenous cortactin. (a-c) Microphotographs of live NIH 3T3 cells expressing cortactin-GFP (a), cortactin-RFP (b) or GFP-cortactin (c) fusion protein. (d) NIH 3T3 cells fixed, permeabilized and immunostained for cortactin. (e-f) A live cell co-transfected with cortactin-RFP (e) and wild-type GFP (f). Unlike GFP, which exhibited a diffuse cytoplasmic fluorescence, cortactin-RFP was localized at filamentous structures at the leading edge. Bars, 10 μm.
Dynamics of cortactin fusion proteins

Time-lapse analysis of cells expressing cortactin fusion proteins revealed that cortactin-labeled structures behaved in a strikingly dynamic manner. In the lamellipodia, cortactin was associated with structures that moved from the leading edge toward the cell center (Fig. 2, video 1). The speed of the movement, measured by tracking features brighter than the average lamellipodia staining, was 2.5 μm/minute (s.d. ±0.33, measured from 10 different cells). The directionality and speed of this movement are consistent with the reported retrograde-flow of F-actin meshwork in the lamellipodium of fibroblasts (Fisher et al., 1988; Theriot and Mitchison, 1992; Wang, 1985). Cortactin is thus likely to be associated with the F-actin meshwork in the lamellipodia. Cytoplasmic spots were also very dynamic. They, however, moved in a random manner and did not show any consistent direction. The spots were most abundant in the perinuclear area and their frequency of occurrence decreased toward the thin, lamellar parts (Figs 1, 2).

Cortactin colocalizes with dynamic actin

To further study the localization and dynamics of cortactin in comparison to actin we cotransfected cells with expression vectors encoding for cortactin-RFP and GFP-actin fusion proteins. GFP-actin distribution in the lamellipodia was less punctate than that of cortactin-RFP (Fig. 3). The retrograde movement of structures in the lamellipodia was seen with both fusion proteins. The area exhibiting retrograde flow was the same for both fusion proteins and so was the speed of the movement (about 3 μm/minute).

Cytoplasmic spots stained by cortactin-RFP were also weakly stained by GFP-actin. This was most clear in thin areas of the cells, while in the thicker parts, possible localization of GFP-actin to the cytoplasmic spots was masked by high concentration of the soluble form of GFP-actin and by brightly labeled F-actin bundles. Actin structures such as stress fibers that remained stable during time-lapse recording (4 minutes in duration) were not stained by cortactin-RFP (Fig. 3, video 2). Cortactin is thus associated only with a sub-set of F-actin that is very dynamic. Colocalization of cortactin and F-actin was confirmed by phalloidin staining of fixed cortactin-GFP expressing cells (Fig. 4a-c). As phalloidin binds only to filamentous actin the staining shows that cortactin spots contain F-actin.

Cortactin colocalizes with arp2/3 complex

Arp2/3 complex has a crucial role in the regulation of actin polymerization and has been shown to localize to sites of dynamic actin (Machesky and Gould, 1999; Schafer et al., 1998). We therefore studied whether cortactin co-localized with the arp2/3 complex. Cells were co-transfected with vectors encoding GFP-arp3 and cortactin-RFP fusion proteins. Imaging of these cells revealed that the two proteins were co-localized at the lamellipodia and at the cytoplasmic spots (Fig. 4d-f). Cortactin-RFP fusion protein, however, gave a much stronger signal than GFP-arp3. This may be due to a higher number of binding sites for cortactin or because the GFP-arp3 fusion protein is not efficiently incorporated into the arp2/3 complex. The movement of cortactin-GFP labeled structures stopped when actin polymerization was inhibited by cytochalasin D (data not shown) as was also shown for GFP-arp3 labeled structures (Schafer et al., 1998). Thus, the cortactin fusion protein can be used as a marker for dynamic F-actin.

Contact associates with endosomes

The cytoplasmic cortactin spots were seen in some cells to be associated with vesicular structures that were visible as dark spheres against the diffuse cytoplasmic fluorescence due to the soluble pool of the fusion protein (Fig. 5a-i). The vesicles were most clearly seen in cells that expressed high levels of cortactin fusion protein and therefore had an intense cytoplasmic

Fig. 2. Dynamic behavior of cortactin labeled structures. (a) a general view of a cortactin-RFP expressing cell. Cell center is on the left and a cortactin-RFP labeled lamellipodium is on the right. (b) Time-lapse series reveals the retrograde movement of cortactin in the lamellipodium (boxed area on the right in a). Arrowhead points to a bright spot that moves from the leading edge toward the cell center. (c) Time-lapse series showing the random movements of the cytoplasmic spots (boxed area on the left in a). Images were taken at intervals of 6 seconds. Bar, 5 μm.
staining. The vesicles were not completely covered by cortactin-GFP but were asymmetrically associated with one or a few cortactin-GFP spots. When these vesicles moved, they were associated with short comet tails of cortactin-GFP at the trailing edge of these vesicles. Fig. 5a-i (see also video 3) shows a representative time-lapse series. The upper vesicle (arrowhead in 5a) moved initially downward while its cortactin-GFP tail pointed upward. Then the vesicle turned 180 degrees and the cortactin spot now pointed downward (5d). This was followed by an upward movement of the vesicle (5d-i). Two lower vesicles (arrows in 5a) moved slowly toward each other until they fused into one. Fusion was accompanied by an increase in the vesicular diameter. The two cortactin spots associated with the fused vesicle coalesced shortly after the fusion event (5e). The cortactin spot then turned downward and the vesicle started moving upward (5e-i). Cortactin spots were, however, not seen to be associated with sites of vesicle fusion.

To characterize the identity of the vesicles associated with cortactin, we loaded the cells with markers of endocytosis. Cortactin-GFP-expressing cells were incubated with fluorescently labeled transferrin. Transferrin binds to cell surface transferrin receptors and is endocytosed through receptor-mediated endocytosis via coated pits. It is then recycled back to the plasma membrane via recycling endosomes (Robinson et al., 1996). Imaging transferrin and cortactin-GFP together clearly revealed that endosomal vesicles were associated with cortactin-GFP spots (Fig. 5i). Over half of transferrin-labelled vesicles were associated with cortactin-GFP tails (59.0%, s.e.m. ±4.3%, calculated from randomly chosen microscopic fields from 10 different cells). The majority of vesicles associated with cortactin-GFP had only one asymmetric cortactin-GFP tail (74.3%, s.e.m. ±1.9%) and fewer had two or more tails (25.7±1.9%). No vesicles were found to be totally covered by cortactin-GFP. Similar results were obtained when the cells were loaded with fluorescent BSA, which functions as a marker for fluid-phase endosytosis (Fig. 5k).

F-actin tails were also seen when non-transfected cells were loaded with fluorescent transferrin, fixed and stained with fluorescent phalloidin (Fig. 5l). This excludes the possibility that the tails were artifacts caused by cortactin-GFP expression. Imaging of endosomal F-actin tails in live cells using GFP-actin expression was hampered by the high cytoplasmic...
endocytic vesicles in a manner similar to its involvement in derived from actin polymerization is used for trafficking dynamic actin patches suggests that the propulsive force propelling plasma membrane protrusions and intracellular pathogens. Asymmetric association of F-actin patches around the vesicles, which is obviously necessary for movement, may be a spontaneous property of dynamic actin network as suggested by recent experiments using cell extracts and polystyrene beads coated with bacterial protein that induces actin polymerization (van Oudenaarden and Theriot, 1999). F-actin is required for the initial formation of endocytic vesicles at the plasma membrane (Lamaze et al., 1997; Durrbach et al., 1996). Recently it has been shown, using GFP-actin expression, that macropinosomes generated at plasma membrane ruffles of cultured mast cells, are transiently associated with actin tails when they move from the plasma membrane towards the cytoplasm (Merrifield et al., 1999). Actin cytoskeleton is, however, also required for later cytoplasmic trafficking of endosomes (Durrbach et al., 1996). Actin rocketing of endosomes was also very recently shown in *Xenopus* oocytes (Taunton et al., 2000) and in cultured mammalian cells activated by overexpression of phosphatidylinositol phosphate 5-kinase or by pervanadate application (Rozelle et al., 2000). Actin comet tails described in these studies were more robust but less frequently observed than those shown here. Our results suggest that actin rocketing of endosomes is, in fact, a common mechanism for endosome trafficking.

Transmembrane receptors, such as epidermal growth factor receptor (EGFR), may also be involved in endosomal trafficking. Activated EGFR can induce actin polymerization at the plasma membrane (Rijken et al., 1991). The activated receptor is endocytosed and its activity is known to persist in endosomes (Haugh et al., 1999). It is thus possible that it could also induce actin polymerization at endosomal membranes. Further, cortactin could link transmembrane signaling at endosomal membranes to F-actin in a manner proposed to occur at the plasma membrane.

As endosomes and the leading edge contain similar molecular components that are required for actin polymerization, it is conceivable that localized exocytosis of recycled endosomes may regulate direction of actin based cell motility by concentrating membrane associated molecules that induce actin polymerization. In migrating cells the leading edge is known to be the major site of exocytosis and an essential role concentration of diffuse G-actin and by other F-actin structures such as stress fibers.

**DISCUSSION**

We show in this study that cortactin localizes to two different dynamic membrane structures, lamellipodia and endosomes. Cortactin is associated in these structures with F-actin that undergoes rapid turnover. Both of these membrane domains are propelled by actin polymerization.

Myosin-driven retrograde flow of actin meshwork in the lamellipodium is a crucial mechanism for cell migration (Lin et al., 1996). Linking of the actin meshwork to the underlying extracellular matrix via transmembrane receptors is thought to result in the forward movement of the cell (Lin and Forscher, 1995; Suter et al., 1998). We have shown that cortactin is associated with the F-actin meshwork in the lamellipodium. As cortactin interacts with both F-actin and transmembrane receptors it may be involved in linking these two systems together. Cortactin binds to polyproline motifs in several different PDZ domain proteins via its SH3 domain (Du et al., 1998; Katsube et al., 1998; Naisbitt et al., 1999). PDZ proteins assemble signaling complexes at the plasma membrane, and they interact specifically with a wide variety of transmembrane proteins using their PDZ domains (Fanning and Anderson, 1999). Cortactin may function in lamellipodia as a linker of actin meshwork and transmembrane receptors via PDZ-proteins and thus connect the actin meshwork to the underlying substrate. Src kinases are known to regulate the F-actin crosslinking activity of cortactin (Huang et al., 1997) and overexpression of a cortactin mutant deficient in tyrosine phosphorylation impaired the migration of endothelial cells (Huang et al., 1998). Cortactin could thus provide a mechanism for linking src signaling with cell motility.

The actin cytoskeleton is essential at several steps in the endocytic cycle. However, the precise role of actin in endosomal traffic has been unclear. Our finding that endosomal vesicles are asymmetrically associated with and move with dynamic actin patches suggests that the propulsive force derived from actin polymerization is used for trafficking endocytic vesicles in a manner similar to its involvement in

Asymmetric association of cortactin spots.

(a-i) Movement of vesicles with cortactin-GFP tails. The total length of the time series is 4 minutes and interval between each image is 30 seconds. See text for detailed discussion.

Bar, 2.5 μm. (j) Cortactin-GFP (green) and TRITC-transferrin-labeled endosomes (red).

(k) Cortactin-GFP (green) and Texas Red-BSA-labeled endosomes (red) as visualized in a live cell. (l) Visualization of phalloidin stained F-actin (green) in a fixed and permeabilized cell preloaded with TRITC-transferrin to stain endosomes (red). Bar, 2.5 μm.
for membrane recycling in cell motility has recently been suggested (Bretscher and Aguado-Velasco, 1998).

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