A Cortactin-CD2-associated Protein (CD2AP) Complex Provides a Novel Link between Epidermal Growth Factor Receptor Endocytosis and the Actin Cytoskeleton*

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Growth factor regulation of the cortical actin cytoskeleton is fundamental to a wide variety of cellular processes. The cortical actin-associated protein, cortactin, regulates the formation of dynamic actin networks via the actin-related protein (Arp)2/3 complex and hence is a key mediator of such responses. In order to reveal novel roles for this versatile protein, we used a proteomics-based approach to isolate cortactin-interacting proteins. This identified several proteins, including CD2-associated protein (CD2AP), as targets for the cortactin Src homology 3 domain. Co-immunoprecipitation of CD2AP with cortactin occurred at endogenous expression levels, was transiently induced by epidermal growth factor (EGF) treatment, and required the cortactin Src homology 3 domain. The CD2AP-binding site for cortactin mapped to the second of three proline-rich regions. Because CD2AP is closely related to Crbl-interacting protein of 85 kDa (CIN85), which regulates growth factor receptor down-regulation via complex formation with Crbl and endophilin, we investigated whether the CD2AP-cortactin complex performs a similar function. EGF treatment of cells led to transient association of Crbl and the epidermal growth factor receptor (EGFR) with a constitutive CD2AP-endophilin complex. Cortactin was recruited into this complex with slightly delayed kinetics compared with Crbl and the EGFR. Immunofluorescence analysis revealed that the EGFR, CD2AP, and cortactin co-localized in regions of EGF-induced membrane ruffles. Therefore, by binding both CD2AP and the Arp2/3 complex, cortactin links receptor endocytosis to actin polymerization, which may facilitate the trafficking of internalized growth factor receptors.

Subcellular compartmentalization and trafficking of signal transduction complexes and a variety of dynamic cellular responses to extracellular stimuli require regulated interactions between specific components of signaling pathways and the cytoskeleton. These interactions may be direct or mediated by particular adaptor or scaffolding proteins. Among these, cortactin was identified as a v-Src substrate associated with the cortical actin cytoskeleton approximately a decade ago, although insights into its cellular function and the underlying mechanisms have only been obtained recently (1).

In line with an adaptor role, cortactin is a multidomain protein, with the individual modules capable of mediating specific protein-protein interactions (1). The N-terminal region mediates binding to the Arp2/3 complex, a highly conserved regulator of the assembly and structure of actin networks (2), and contains a DDW motif characteristic of Arp2/3-interacting proteins such as WASP, Myo5p, and AetA (2, 3). This is followed by six and a half copies of a 37-amino acid repeat, with the fourth repeat necessary for binding to F-actin in vitro (2). Downstream of the repeats is a predicted helical domain and a region rich in serine, threonine, and proline residues. The latter is a target for both tyrosine and serine/threonine phosphorylation (4–6). The C terminus of cortactin is characterized by an SH3 domain. Several binding partners for this module have been identified, including CortBP1/Shank 2 (1, 7), ZO-1 (8), and dynamin 2 (9).

Cortactin localizes with the Arp2/3 complex at sites of dynamic cortical actin assembly such as lamellipodia (2). Cortactin binding leads to a relatively weak stimulation of the actin nucleation activity of the Arp2/3 complex (10), but it can enhance actin polymerization induced by co-activators of Arp2/3 such as WASP family proteins (3, 10, 11). Furthermore, cortactin promotes the formation of branched actin networks and their stabilization (10). One function of cortactin is to regulate the organization and subcellular localization of transmembrane complexes. The cortactin SH3 domain target CortBP1/Shank 2 performs a scaffolding function in the organization of receptor complexes at post-synaptic sites of excitatory syn-

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1 The abbreviations used are: Arp, actin-related protein; ASAP, ARF GTPase-activating protein containing Src homology 3, ankyrin repeat, and pleckstrin homology domains; CD2AP, CD2-associated protein; CIN85, Crbl-interacting protein of 85 kDa; CMS, Cas ligand with multiple Src homology 3 domains; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; FCS, fetal calf serum; FISH, five Src homology 3 domains; GFP, green fluorescent protein; GST, glutathione S-transferase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; μLC-MS/MS, microcapillary liquid chromatography tandem mass spectrometry; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay; SH3, Src homology 3; TBS, Tris-buff- ered saline; WASP, Wiskott-Aldrich syndrome protein.
aposes, whereas a further SH3 binder, ZO-1, interacts with the transmembrane proteins claudin and occludin at epithelial tight junctions (1). A second functional role for cortactin is regulation of receptor-mediated endocytosis, via its interaction with dynamin 2, a mechaenochemical GTPase that participates in the scission of endocytic vesicles at the plasma membrane (9, 12). In addition, cortactin localizes with the Arp2/3 complex at cytoplasmic spots associated with endosomal vesicles, suggesting a role in driving vesicle trafficking (13). Therefore, cortactin is representative of a growing list of proteins that physically link the endocytic machinery with components or regulators of the actin cytoskeleton. These include mAbp1, which binds F-actin and dynamin, and yeast Pnm1p, which links clathrin-binding proteins to the Arp2/3 complex (14). However, the cortactin-dynamin 2 complex is also implicated in actin reorganization, including the growth factor-induced formation of wave-like structures on the dorsal cell surface that precede lamellipodial protrusion (9, 15, 16). In addition to its role in normal cells, cortactin is also implicated in the progression of certain human cancers. The gene encoding cortactin, EMS1, localizes to chromosomal locus 11q13, a region commonly amplified in breast cancers and squamous cell carcinomas of the head and neck (17, 18). The functional properties of cortactin suggest a link with tumor invasion and/or metastasis, and this is supported by the observation that overexpression of cortactin increases cell motility and invasion in vitro (5, 19) and enhances bone metastasis of MDA-MB-231 breast cancer cells in a nude mouse model (20). Because a variety of cellular processes are regulated by cortactin, it is unlikely that all of its binding partners have been identified. Furthermore, the two published studies aimed at identifying cortactin SH3 targets focused on those expressed in brain (7, 21), and proteins expressed more highly in other tissues would not have been isolated. Because cortactin plays a key role in breast cancer progression, we have utilized a proteomics-based approach to identify binding partners for the cortactin SH3 domain from the breast cancer cell line MDA-MB-231. This has identified the scaffolding protein CMS/CD2AP (22, 23) as a novel target for the cortactin SH3 region. Importantly, a close relative of CD2AP, CIN85, has been shown recently (24, 25) to bind both Chl and the endophilins and thereby regulate growth factor receptor endocytosis. Recruitment of cortactin into a similar complex formed around CD2AP suggests related roles for CD2AP and CIN85 and reveals a new link between the actin cytoskeleton and the endocytic apparatus.

EXPERIMENTAL PROCEDURES

Tissue Culture—MDA-MB-231 human breast cancer cells and HEK 293 cells were maintained as described previously (26, 27). Prior to treatment with EGF (100 ng/ml, R & D Systems, Minneapolis, MN) the cells were starved for 16–20 h in serum-free medium.

Metabolic Labeling of Cells—MDA-MB-231 cells grown to 70% confluency were metabolically labeled for 4 h in methionine- and cysteine-free RPMI 1640 medium (ICN, Costa Mesa, CA) containing 2 mM glutamine, 10% dialyzed FCS, and 50 μCi/ml Trans35S-Sulphate (ICN) prior to cell lysis.

Transfection Transfections—These were performed using FuGENE 6 transfection reagent (Roche Applied Science) as described previously (27).

Cell Lysis, Immunoprecipitation, and Immunoblotting—Preparation of cell lysates and immunoprecipitations utilized RIPA buffer as described previously (27). CD2AP (H-290 or N-20), ASAP1 (N-18), FISH (M-20), Endophilin II/HSH3GL3 (C-18), Chl (C-15), and Dynamin 2 (C-18) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The cortactin monoclonal antibody 4F11 was purchased from Upstate Biologies Inc. (Lake Placid, NY), and horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20 was from BD Biosciences. The anti-N-WASP polyclonal antibody was the kind gift of Dr. T. Takenawa (28). Denaturing geometry was performed using IF Lab Gel software (Signal Analytics Corp., Vienna, VA).

Plasmids—Plasmids encoding GST fusion proteins corresponding to the cortactin helical and proline-rich regions (amino acids 352–490) and the SH3 domain (amino acids 485–550) were as described previously (6). Mammalian expression vectors for human cortactin, the 3SH3 mutant, and GFP-EGRF were also as described previously (6, 29). The construct encoding a GST fusion protein of the endophilin A1 SH3 domain was obtained from Dr. Ivan Dikic (University of Frankfurt Medical School). An expression vector encoding Myc-tagged mouse CD2AP was provided by Dr. Andrey Shay (Washington University School of Medicine).

Complementary oligonucleotides encoding the three proline-rich regions of CD2AP (23), designated P1 (amino acids 336–352), P2 (amino acids 410–422), and P3 (amino acids 490–500), were used with the primers 5′-ATGATGATCTTGTAGATGCTTCGCTTCG-3′ and 5′-TCCTCTTTTGATTTTGATTTTTGATTAGTAATG-3′ to amplify genomic DNA fragments from human CD2AP cDNA cloned into pcDNA3.1(+). (22). Three PXXP motifs (P1, P2, and P2B, Fig. 8) were individually mutated to AXXA using the QuikChange® multi-site-directed mutagenesis kit (Stratagene, La Jolla, CA). The double mutant P1/P2B was also generated. One mutagenic oligonucleotide was utilized per PXXP site. Details can be provided upon request. Mutated plasmids were sequenced in both directions to confirm that the required mutations had been introduced.

Expression and Purification of GST fusion Proteins—Expression constructs were used to transform Escherichia coli DH5α to ampicillin resistance. Fusion proteins were purified from isopropyl-β-D-thiogalactopyranoside-induced bacterial cultures by affinity chromatography on glutathione-Sepharose beads (Sigma) (32). Affinity Chromatography—GST and GST fusion proteins were cross-linked to glutathione-agarose beads using dimethylimidelimate (Sigma) (33). Prior to incubation of radioabeled lysate with fusion protein, the beads (0.5 mg of total protein) were pre-cleared by exposure to 15 μg of immobilized GST for 1 h at 4 °C. Immobilized fusion proteins were also blocked by incubation with 5% skimmed milk in TBS/Tween (10 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h at 4 °C and then washed three times in lysis buffer containing 1% Triton X-100 (34). The blocked affinity matrix (5 μg of fusion protein) was then incubated with the cleared lysate for 2 h at 4 °C and then washed five times in RIPA buffer. Samples were separated by 4–12% gradient SDS-PAGE, transferred to a PVDF membrane, and subjected to autoradiography.

The protocol was scaled up prior to analysis of bands by mass spectrometry by incubating 10 mg of unlabeled, precleared lysate protein with 30 μg of blocked, immobilized fusion protein. Following incubation, the bands were washed 10 times in RIPA buffer prior to analysis by SDS-PAGE (10% gel).

For experiments in which bound proteins were detected by Western blotting, 0.5–1 mg of unlabeled lysate was incubated for 2 h at 4 °C with 5 μg of fusion protein. The bands were then washed four times in RIPA buffer prior to SDS-PAGE.

Protein Identification by Mass Spectrometry—Protein bands were excised from the Coomassie-stained gel, de-stained, vacuum-dried, and then subjected to tryptic digestion. Peptides eluted from the bands were concentrated and de-salted using a C18 ZipTip™ (Millipore, Bedford, MA). MALDI-TOF MS peptide mass mapping was performed using a PerSeptive Biosystems Voyager DE-STR (Framingham, MA) equipped with a 30 nm nitrogen laser. Lists of monoisotopic masses were compared to the masses of generated tryptic peptides for each protein band were used to search the SwissProt and TrEMBL databases via the program PeptIdent. Protein identification was also performed by LC-MS/MS (35). Following μLC-MS/MS was conducted on a Finnigan MAT TSQ equipped with an in-house built microspray device for peptide ionization. MS/MS spectra were searched with data base entries using SEQUEST.

Far Western Analysis of SH3 Domain Binding—Euluted GST or GST-SH3 fusion proteins were biotinylated, following dialysis against 0.1 M sodium borate, pH 8.8, by incubation with sulfo-NHS-biotin ester (Pierce) for 4 h at room temperature. The reaction was quenched by the addition of 1 M NH4CL, samples were then dialyzed against PBS for 48 h. GST fusion proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and blocked for 1 h in 5% skimmed milk in TBS/Tween. Membranes were incubated with the biotinylated proteins (1 μg/ml) for 30 min and extensively washed in TBS containing 1% Tween
RESULTS

Identification of Proteins Binding to the SH3 Domain of Cortactin—Amplification of EMS1 is associated with poor patient prognosis in ER-negative, but not ER-positive, breast cancers (17). In order to identify proteins interacting with the cortactin SH3 region in ER-negative breast cancer cells, we performed affinity chromatography on 35S-labeled lysates from MDA-MB-231 cells. This cell line was chosen because it lacks ERs and exhibits a motile, invasive phenotype (36). Affinity purification was performed using a matrix consisting of a GST-SH3 fusion protein (Fig. 1A) coupled to glutathione-Sepharose. We also investigated the protein-binding capability of the cortactin HP region (Fig. 1A) by the same strategy, and GST was included as a negative control. Following washing, bound proteins were separated by SDS-PAGE and detected by autoradiography. Thirty proteins were specifically bound by the SH3 domain and not by the GST control (Fig. 1B). One protein of ∼30 kDa bound the HP region.

The procedure was then scaled up and bound proteins detected by Coomassie Blue staining. Following gel excision and tryptic digestion, SH3-interacting proteins were identified by either MALDI-TOF MS or LC-MS/MS. Among the proteins identified were CMS/CD2AP, subsequently referred to as CD2AP (22, 23), FISH (37), ASAP1 (38), and N-WASP (39). The MALDI mass fingerprint obtained for CD2AP is shown in Fig. 2. The specific binding of these proteins to the cortactin SH3 domain was confirmed by Western blotting of pull-downs from unlabeled cell lysates (Fig. 3).

Association of CD2AP with Cortactin in Vivo—CD2AP, FISH, ASAP1, and N-WASP have each been implicated in regulation of cytoskeletal organization, and the former three proteins are also substrates of Src family kinases (23, 37, 38, 40). These properties suggested that their isolation as cortactin SH3 targets might reflect a functional relationship with cortactin. However, we have been unable to co-immunoprecipitate either ASAP1 or FISH with cortactin at endogenous expression levels of the binding partners, although N-WASP was recently shown to associate with cortactin in vivo in an SH3 domain-dependent manner (28). In order to investigate the association of cortactin with CD2AP in vivo, co-immunoprecipitation analysis was performed from lysates of serum-starved or EGF-treated MDA-MB-231 cells. Western blotting of cortactin immunoprecipitates from MDA-MB-231 breast cancer cells with a CD2AP-specific antibody, and the reciprocal analysis, revealed EGF-inducible co-immunoprecipitation of the two proteins, revealing that they associate at physiological expression levels (Fig. 4A).

The requirement for the cortactin SH3 domain in CD2AP association was investigated by expressing a truncation mutant of cortactin lacking the SH3 domain (ΔSH3) in HEK 293 cells. Cells were also transfected with an expression vector for full-length cortactin as a control. The expressed cortactin, but not the ΔSH3 mutant, could readily be detected in CD2AP immunoprecipitates (Fig. 4B), indicating that the cortactin SH3 domain is essential for interaction of these proteins in vivo. As with the association of the endogenous proteins, co-immunoprecipitation was increased by short term EGF treatment.

The Cortactin-CD2AP Complex Is Implicated in Regulation of Receptor Endocytosis—The same molecular architecture and significant sequence homology indicate that CD2AP and CISN5 are members of the same subfamily of adaptor proteins (41). Recent studies (24, 25) have demonstrated a role for CISN5, in a complex with endophilins and Cbl, in receptor-mediated endocytosis. In this complex, the CISN5 SH3 domains bind to receptor-associated Cbl, whereas the endophilin SH3 region binds a specific proline-rich motif in CISN5. This raised the possibility that CD2AP may also participate in growth factor receptor endocytosis.

To test this hypothesis, we investigated the different protein-protein interactions involving CD2AP following EGF stimulation and their kinetics. MDA-MB-231 cells were serum-starved and then treated with EGF for varying times. Because CD2AP association with Cbl is dependent on Cbl tyrosine phosphorylation (42), first we investigated the tyrosine phosphorylation status of proteins co-immunoprecipitated with CD2AP. Blotting with anti-phosphotyrosine antibodies revealed three protein bands in the range of 50 kDa, which were detected between 1 and 5 min of EGF stimulation (Fig. 5). A tyrosine-phosphorylated protein of ∼80 kDa was only detected at 2 and 5 min post-stimulation. Western blotting with specific antibodies identified the 180- and 120-kDa bands as the EGFR and Cbl, respectively. CD2AP association with endophilin was constitutive and unaffected by EGF stimulation. Consequently, like CISN5, CD2AP associates with both receptor-bound Cbl and endophilin.
Western blotting with a cortactin-specific antibody revealed a low basal level of association in serum-starved cells. Following EGF stimulation, there was a marked but transient increase in complex formation that peaked at 5 min and returned to the basal level following 30 min of EGF-stimulation (Fig. 5). Comparison of recruitment kinetics and gel mobility suggests that the 80-kDa tyrosine-phosphorylated protein detected in CD2AP immunoprecipitates at 2 and 5 min following EGF treatment is likely to be cortactin. These data are consistent with recruitment of cortactin to the endocytic complex, but association of cortactin with CD2AP is slightly delayed compared with that of Cbl/EGFR.

An alternative explanation for these data is that two independent CD2AP-based complexes exist, i.e. CD2AP-cortactin and CD2AP-Cbl-EGFR. To investigate this possibility, we examined whether Cbl and the EGFR could be detected in cortactin immunoprecipitates. Western blotting of cortactin immunoprecipitates with an anti-phosphotyrosine antibody revealed an EGF-inducible band of ~120 kDa, subsequently identified as Cbl (Fig. 6A). A faint band of 180 kDa, enhanced upon cortactin overexpression, was also detected and is likely to represent the EGFR. Because we have already demonstrated the presence of CD2AP in cortactin immunoprecipitates (Fig. 4), these data confirm that cortactin is indeed recruited to the CD2AP-Cbl-EGFR complex.

Finally, to confirm that the observed co-immunoprecipitation of cortactin and CD2AP was not explained by their simultaneous SH3 domain-dependent recruitment to the proline-rich region of dynamin 2, we investigated whether dynamin 2 could be detected in CD2AP-containing protein complexes. Although dynamin 2 was readily detected in MDA-MB-231 cell lysates, it was absent from cortactin-containing CD2AP immunoprecipitates (Fig. 6B). This indicates that the association between CD2AP and cortactin is likely to be direct.

Mapping of the Binding Site for the Cortactin SH3 Domain on CD2AP—CD2AP contains three proline-rich regions harboring potential SH3-binding sites (23) (Fig. 7A). The optimal binding sequence for the cortactin SH3 domain has been characterized as +PPΨPXKP, where + and Ψ indicate basic and hydrophobic amino acid residues, respectively (30). Examination of the CD2AP amino acid sequence did not reveal an exact match with this sequence. In order to delineate the binding site, each of the three proline-rich regions of CD2AP (P1–P3) (Fig. 7B) was expressed as a GST fusion protein. A fusion protein corresponding to the consensus binding sequence for the cortactin SH3 domain (Popt, Fig. 7B) was also generated as
positive control. These fusion proteins, and GST as a negative control, were subjected to a far Western assay with biotin-labeled GST-SH3 or GST alone. Strong binding of the SH3 domain to Popt was detected, but significant binding to P1 and P2 also occurred. Binding to P3 was undetectable (Fig. 7C).

These findings demonstrate that the cortactin SH3 domain can bind directly to CD2AP-derived proline-rich sequences, specifically P1 and P2.

Because both cortactin and endophilin bind CD2AP via SH3 domain-mediated interactions, it was of interest to compare the binding selectivity of the endophilin SH3 domain to P1–P3 with that of the cortactin SH3 region. The endophilin A1 SH3 domain bound strongly to P1 and relatively weakly to P2 and P3 (Fig. 7C). This suggests that in vivo, endophilin may predominantly bind P1, whereas cortactin is recruited to P2.

In order to delineate the cortactin SH3 domain binding site in more detail, proline to alanine substitutions were introduced into PXXP motifs in mouse CD2AP exhibiting similarity to the cortactin SH3 domain binding consensus (Fig. 8A). Epitope-tagged wild-type and mutant proteins were then transiently expressed in HEK 293 cells (Fig. 8B), and binding to the cortactin SH3 domain was determined by GST fusion protein pull-down analysis (Fig. 8C). Mutation of the P2A motif reduced binding to ~20% that of the wild-type protein, whereas mutation of P1 caused a 2-fold reduction. Mutation of P2B resulted in a small effect, which was also evident when binding of the P2A single mutant was compared with that of the P2AB double mutant.

To determine whether association of the full-length proteins in cells followed this binding profile, the Myc-tagged CD2AP proteins were immunoprecipitated and Western-blotted for cortactin (Fig. 8D). In agreement with the pull-downs, P2A was identified as the major interaction site, with a smaller contribution from P2B. However, unlike the pull-downs, a significant role for P1 could not be detected. It is possible that the pull-downs and immunoprecipitations differ in their ability to detect P1 binding because of the ability of the GST-SH3 fusion
protein to displace endogenous endophilin from this site on CD2AP.

Consequently, the mutagenesis results, in combination with the requirement for the cortactin SH3 domain for association with CD2AP (Fig. 4), and the ability of the cortactin SH3 domain to bind directly to P2 (Fig. 7), provide strong evidence that association of the two proteins in cells is largely mediated by direct binding of the cortactin SH3 domain to the P2 region of CD2AP. However, because the P2AB mutant still exhibits some residual binding activity in vivo, other molecular interactions must make a small contribution to complex formation.

**CD2AP, Cortactin, and the EGF Receptor Co-localize in Membrane Ruffles Upon EGF Treatment**—Depending on cell type, CD2AP has been localized to lamellipodia and punctate cytoplasmic structures (23, 43). The inducible association between cortactin and CD2AP and the appearance of the EGFR in this complex (Figs. 5 and 6A) led us to investigate the subcellular distribution of these proteins by confocal microscopy. HeLa cells were transiently transfected with an expression construct encoding GFP-tagged EGFR, serum-starved, and then treated for either 5 or 15 min with EGF. Before stimulation, EGFRs were distributed over the plasma membrane. Following 5 min of EGF treatment, the receptors accumulated in membrane ruffles and by 15 min were concentrated into prominent vesicular structures within the cytosol, presumably early endosomes (Fig. 9). Cortactin and CD2AP were analyzed in the same cells by indirect immunofluorescence. Both CD2AP and cortactin localized to punctate cytoplasmic structures in serum-starved cells, and transiently appeared in membrane ruffles following 5 min of EGF stimulation. In resting cells CD2AP and cortactin demonstrated very little co-localization with the EGFR. However, following 5 min of EGF stimulation, all three proteins co-localized to distinct structures within the membrane ruffles, as well as a small subset of punctate cytoplasmic spots. This increase in co-localization was transient and returned to the level of resting cells following 15 min of EGF stimulation. These data are consistent with the transient nature of complex formation indicated by co-immunoprecipitation analysis (Fig. 5), and the two approaches demonstrate the formation of an EGF-induced complex containing both CD2AP and cortactin at membrane ruffles that is likely to regulate trafficking of internalized EGFRs.

**DISCUSSION**

Given the emerging role of cortactin as an adaptor linking transmembrane or intracellular protein assemblies with the Arp2/3 complex and hence sites of dynamic actin networks, we considered it likely that additional binding partners for the cortactin SH3 domain may exist. Prior to this study, immunofluorescence studies by other workers suggested that cortactin may be involved in cellular roles for CD2AP. Welsch et al. (43) detected CD2AP in cytoplasmic spots in cultured podocytes and showed that these structures also stained for F-actin, cortactin, and the Arp2/3 complex. Also, in PtK1 fibroblasts expressing...
an active mutant form of ARF6, CD2AP and cortactin localize to the same structures, including F-actin tails and foci on the ventral surface of cells (44). However, in this report we provide the first demonstration that the scaffolding protein CD2AP directly associates with the cortactin SH3 domain, and we demonstrate a functional role for this complex in EGFR endocytosis.

Binding of cortactin to CD2AP requires the cortactin SH3 domain, as evidenced by GST pull-down experiments (Figs. 1 and 3), and co-immunoprecipitation experiments using the cortactin H9004 SH3 mutant (Fig. 4). The absence of dynamin 2 in CD2AP immunoprecipitates (Fig. 6B) rules out the possibility that the observed association is due to simultaneous recruitment of CD2AP and cortactin to a dynamin 2 scaffold. The association between cortactin and CD2AP is likely to be direct, because the CD2AP P2A and P2B motifs are required for strong binding in both GST-SH3 pull-downs (Fig. 8C) and co-immunoprecipitation experiments (Fig. 8D), and the cortactin SH3 domain binds the P2 region in a far Western blot (Fig. 7). However, as observed for CIN85 (24, 25), CD2AP also associates with endophilin (Fig. 5). Upon far Western analysis, the endophilin A1 SH3 domain bound strongly to P1, which con-
tains the endophilin SH3 binding consensus sequence PKKPP-P PPP (45), and relatively weakly to P2 and P3 (Fig. 7). There- fore, it is likely that in vivo, endophilin binds to P1, whereas cortactin binds to P2A and to a lesser extent P2B.

Although a basal association of cortactin with CD2AP was detectable in serum-starved cells, a marked but transient inc- rease in co-immunoprecipitation of the two proteins was detected following EGF treatment (Figs. 4–6). Because SH3 do- main binding was not dependent on phosphorylation of the proline-rich binding motif (30), the most likely explanation for this effect is a phosphorylation-induced conformational change in cortactin and/or CD2AP, which enhances complex formation by increasing the accessibility of the cortactin SH3 domain and/or its target sequences on CD2AP. Although CD2AP is tyrosine-phosphorylated in cells overexpressing c-Abl, c-Fyn, or c-Src, EGF treatment of 293T cells failed to induce CD2AP tyrosine phosphorylation (23). Tyrosine phosphorylation of the related CIN85 has not been reported. The appearance of a tyrosine-phosphorylated protein of ~80 kDa in CD2AP immuno- precipitates following 2–5 min of EGF stimulation (Fig. 5) is consistent with cortactin. Moreover, EGF-induced cortactin tyro- sine phosphorylation occurs with similar kinetics to cortac- tin/CD2AP association, exhibiting a transient peak within 2 min of stimulation (46, 47). Therefore, one possibility is that cortactin tyrosine phosphorylation enhances access of its SH3 domain. A further contributory mechanism, which is not mutually exclusive, is the effect of recruitment of other proteins into a multimeric complex. In this context, it is worth noting that the association of CD2AP with Cbl is dependent on tyro- sine phosphorylation of Cbl. This is thought to be due to a conformational change in Cbl leading to increased binding of C-terminal proline-rich motifs to the second SH3 domain of CD2AP (42). Similarly, EGF-induced complex formation be- tween CIN85 and Cbl follows the rapid and transient tyrosine phosphorylation of Cbl (41). Therefore, it is possible that bind- ing of CD2AP to tyrosine-phosphorylated Cbl improves access into the P2 region of CD2AP to the cortactin SH3 domain.

To date CD2AP has been shown to play a role in the forma- tion of specialized cell or tight junctions in both the kidney, specifically in podocytes, and in T-cells. In T-cells the adhesive complex is the immunological synapse involving the transmem- brane adhesion molecule CD2 (22), whereas in the kidney the specialized tight junction is the slit diaphragm, where CD2AP assembles a complex with nephrin and podocin (48, 49). Whether cortactin is recruited to CD2AP in these contexts is not known at present, although it does associate with specific transmembrane complexes in other cell types, e.g. at the postsynaptic density, where it binds CortBP1/Shank2, and the epithelial tight junction, where it binds ZO-1 (1). Recruitment of cortactin, and hence Arp2/3, to such complexes is thought to regulate their organization and clustering. However, in view of the data presented in this paper, a further possibility is that the CD2AP-cortactin complex regulates the internalization and/or intracellular trafficking of a variety of transmembrane proteins.

In this study we identify a role for CD2AP in receptor- mediated endocytosis in a complex with Cbl and endophilin, analogous to that of CIN85 (24, 25). We also demonstrate a novel inducible link between this endocytic complex and the cytoskeleton via the interaction between CD2AP and cortactin. Therefore, we propose that Cbl-mediated ubiquitylation marks the receptor for down-regulation, and recruitment of endophilin via CD2AP leads to membrane invagination (24, 25) (Fig. 10). The subsequent binding of cortactin to this complex then provides a link to F-actin network assembly via Arp2/3. Be- cause the recruitment of cortactin to CD2AP is slightly delayed compared with that of Cbl/EGFR, it is likely that CD2AP-bound cortactin does not regulate very early endocytic events, e.g. membrane invagination, but rather vesicle movement away from the membrane. Based on the data presented in Fig. 5, CD2AP then dissociates from both cortactin and Cbl. This contrasts with the more prolonged association of CIN85 with the EGFR, which leads to degradation of both proteins in the lysosome (50). Whether these differences are cell type-specific or reflect differences between the properties of CD2AP and CIN85 is under investigation. We propose that the receptor and Cbl remain coupled and continue through the vesicle-sorting pathway (51).

In summary we have identified CD2AP as a cortactin bind- ing partner, demonstrated a functional similarity between CD2AP and CIN85, and revealed a novel link between the endocytic machinery and the actin cytoskeleton likely to play an important role in regulating the trafficking of receptor- containing vesicles.

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A Cortactin-CD2-associated Protein (CD2AP) Complex Provides a Novel Link between Epidermal Growth Factor Receptor Endocytosis and the Actin Cytoskeleton

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