Cortactin Associates with the Cell-Cell Junction Protein ZO-1 in both Drosophila and Mouse*

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Takanori Katsube‡, Manabu Takahisa, Ryu Ueda, Naoko Hashimoto, Mieko Kobayashi, and Shin Togashi§

From the Neurogenetics Research Project, Mitsubishi Kasei Institute of Life Sciences, Minamiooya 11, Machida-shi, Tokyo 194-8511, Japan

Cortactin is an actin filament-binding protein localized at cortical regions of cells and a prominent substrate for Src family protein-tyrosine kinases in response to multiple extracellular stimuli. Human cortactin has been identified as a protein product of a putative oncogene, EMS1. In this report, we describe the identification of a Drosophila homolog of cortactin as a molecule that interacts with Drosophila ZO-1 using yeast two-hybrid screening. Drosophila cortactin is a 559-amino acid protein highly expressed in embryos, larvae, and pupae but relatively underexpressed in adult flies. Deletion and substitution mutant analyses revealed that the SH3 domain of Drosophila cortactin binds to a PXPF motif in the proline-rich domain of Drosophila ZO-1. Colocalization of these proteins at cell-cell junction sites was evident under a confocal laser-scanning microscope. In vivo association was confirmed by coimmunoprecipitation of cortactin and ZO-1 from Drosophila embryo lysates. We also demonstrate an association for each of the murine homologs by immunoprecipitation analyses of mouse tissue lysates. Our previous work has demonstrated the involvement of ZO-1 in a signaling pathway that regulates expression of the emc gene in Drosophila. The potential roles of the cortactin-ZO-1 complex in cell adhesion and cell signaling are discussed.

Cell-cell adhesions are essential for the development of the multicellular organisms. Among the proteins composing the cell-cell adhesion complexes, members of the membrane-associated guanylate kinase homologs (MAGUKs) are widely found in Hydra, Caenorhabditis elegans, Drosophila, and mammals (1–3). MAGUKs have distinctive domains including one or three copies of the PDZ domain, an SH3 domain, and a homologous to guanylate kinase (GUK) and implicated in both the SH3 domain of cortactin and a domain containing the SH3 domain of ZO-1, cortactin may play important roles in the association between cortactin and ZO-1 in mouse tissues. In association with ZO-1, cortactin may play important roles in the formation and/or regulation of cell-cell adhesion and communication during growth, differentiation, and tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**pBD-DZO885–1367 was constructed as follows. The XhoI end of the 1.5-kb NcoI-XhoI fragment of the DZO-1 cDNA (12) was blunt-end ligated to pAS2–1 (CLONTECH) digested with NcoI and Smal. Deletion variants were constructed by double digesting pBD-DZO885–1367 with some pairs of restriction enzymes, one cut it within the DZO-1 coding region and another within the multi-cloning site, followed by blunt-ending before self-ligating. The Smal, PstI, HindIII, and AspI sites within the DZO-1 coding region and the BamHI, NcoI, and NdeI sites within the multi-cloning site were used for these construction. Point mutations were introduced into pBD-DZO1115–1253 by polymerase chain reaction-mediated site-directed mutagenesis (20). The synthetic oligonucleotide primers used for mutagenesis were as follows: 5’-gggttgctgcaacgctggggtt-3’ and 5’-tcaggggtgcaacgctgggt-3’.

1 The abbreviations used are: MAGUKs, membrane-associated guanylate kinase homologs; GUK, guanylate kinase; DZO-1, Drosophila ZO-1; emc, extramacrocraeaeata (emc) mutation. The emc gene encodes a helix-loop-helix type transcriptional regulator and negatively regulates specification of sensory organ precursor cells (13–16). We have previously shown that DZO-1 locates at cell-cell junctions and is involved in the signaling pathway, which activates the transcription of emc (12). Toward the elucidation of the DZO-1 functions in the signaling pathway, we performed a yeast two-hybrid screen to identify the Drosophila proteins that interact with DZO-1. One of the obtained cDNA clones is encoding a protein highly homologous to vertebrate cortactin.

Cortactin is an F-actin binding protein initially discovered as a prominent substrate for Src protein-tyrosine kinase (17, 18). A human homolog was identified as a protein product of a putative oncogene, EMS1, and has been implicated in both cell adhesion and cell signaling (19). We now report the primary structure of Drosophila cortactin (DCortactin) and show multiple lines of evidence suggesting that DCortactin interacts with DZO-1. We also present results demonstrating the association between cortactin and ZO-1 in mouse tissues. In association with ZO-1, cortactin may play important roles in the formation and/or regulation of cell-cell adhesion and communication during growth, differentiation, and tumorigenesis.

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Yeast Two-Hybrid Screen and β-Galactosidase Assay—Two-hybrid screening was performed using a Matchmaker two-hybrid system (CLONTECH). Each of the bait constructs and a pcT407A. The nucleotide sequences of the plasmid clones were determined for all three reading frames downstream for the reverse transcriptase-polymerase chain reaction were 5’-cattgatctgatcagtc-3’ and 5’-ctggctgctgccagcc-3’. Yeast Two-Hybrid Screen and β-Galactosidase Assay—Two-hybrid screening was performed using a Matchmaker two-hybrid system (CLONTECH). Each of the bait constructs and a pcT407A. The nucleotide sequences of the plasmid clones were determined for all three reading frames downstream for the reverse transcriptase-polymerase chain reaction were 5’-cattgatctgatcagtc-3’ and 5’-ctggctgctgccagcc-3’. Yeast Two-Hybrid Screen and β-Galactosidase Assay—Two-hybrid screening was performed using a Matchmaker two-hybrid system (CLONTECH). Each of the bait constructs and a pcT407A. The nucleotide sequences of the plasmid clones were determined for all three reading frames downstream for the reverse transcriptase-polymerase chain reaction were 5’-cattgatctgatcagtc-3’ and 5’-ctggctgctgccagcc-3’.
mutation that substitutes two proline residues of the central PXXP within the three overlapping motifs at amino acid residues 1192–1201 (PFKAVPAPKP, DOI1115–1253M1) abolished the interaction with the D Cortactin SH3 domain, whereas mutations altering other isolated PXXP motifs did not. These results show that the D Cortactin SH3 domain binds to Fig. 3. A DCortactin cDNA and the predicted amino acid sequences. The 2413-base pair cDNA sequence includes a 173-base pair 5'-untranslated region, a 1677-base pair open reading frame, and a 548-base pair 3'-untranslated region. The nucleotide sequence of the 0.85-kb cDNA derived from the clone pcT407A matched the boxed region exactly. The deduced protein contains four direct repeats of a 37-amino acid unit (indicated by an underline), an approximately 50-amino acid region similar to the predicted alpha-helical domain of vertebrate cortactin (broken underline), a proline-rich domain (21% proline content in 167 amino acids; dotted line), and an SH3 domain (double-underline). This feature is similar to vertebrate cortactin. The consensus polyadenylation signal sequence is shown as white letters on black background.
the PXXP motif located at the center of the DZO-1 C-terminal proline-rich domain, where three PXXP motifs are clustered.

**DCortactin Colocalizes with DZO-1 at Cell-Cell Junction Sites**—Rabbit and rat antisera were raised against a GST-DCortactin fusion protein. Western blot analysis with the rabbit antiserum showed two prominent proteins with molecular masses of 105 and 110 kDa in all developmental stages (Fig. 6). The preimmune serum did not react with them.3 The expression of these proteins in adults was substantially less than those in other developmental stages. Western blotting with the rat antiserum gave almost the same profile.3 The molecular weights of these proteins determined by SDS-PAGE are significantly larger than the predicted value (61 kDa). Similar observations of two forms of protein products with anomalous electrophoretic mobility have also been reported for vertebrate cortactin and HS1. The sequence of the C-terminal region of DDCortactin (amino acid residues 498–559) was aligned with that of human cortactin (492–550) and human HS1 (428–486). Conserved amino acid residues are represented by dots.

To examine the cellular localization, DDCortactin was immunostained in epithelial cells of imaginal discs. The expression of DDCortactin in adults was substantially less than those in other developmental stages. Western blotting with the rat antiserum gave almost the same profile.3 The molecular weights of these proteins determined by SDS-PAGE are significantly larger than the predicted value (61 kDa). Similar observations of two forms of protein products with anomalous electrophoretic mobility have also been reported for vertebrate cortactin and HS1. The sequence of the C-terminal region of DDCortactin (amino acid residues 498–559) was aligned with that of human cortactin (492–550) and human HS1 (428–486). Conserved amino acid residues are represented by dots.

**Fig. 4.** DDCortactin is structurally related to vertebrate cortactin and HS1. a, schematic representation of the comparison of DDCortactin (DCort) with human cortactin (H-Cort) and human HS1 (H-HS1). Vertebrate cortactin and DDCortactin contains the 37-amino acid repeats (indicated by the shaded boxes), a predicted alpha-helical domain (dotted boxes), and a C-terminal SH3 domain (striped boxes). HS1 does not have a predicted alpha-helical domain but has a proline-glutamate repeat (closed box) just behind the proline-rich domain. This feature is not found in either vertebrate cortactin or DDCortactin. The total numbers of amino acid residues for the three proteins are shown at the right. The percentage of identities of the N-terminal region, the 37-amino acid repeated units, and the SH3 domain of DDCortactin with those of human cortactin or human HS1 are shown between the respective bars. Scores between human cortactin and human HS1 are indicated below the bar of HS1. b, the SH3 domain of the DDCortactin is more closely related to that of cortactin than to that of HS1. The sequence of the C-terminal region of DDCortactin (amino acid residues 498–559) was aligned with that of human cortactin (492–550) and human HS1 (428–486). Conserved amino acid residues are represented by dots.

**Fig. 5.** The C-terminal SH3 domain of DDCortactin interacts with a PXXP motif in the proline-rich domain of DZO-1. Schematic representation of the DZO-1 C-terminal region variants used for the two-hybrid assay to identify the domain necessary for the interaction with the DDCortactin C-terminal region. Each plasmid expressing one of these variants fused to the GAL4 DNA binding domain was co-introduced into yeast strain Y187 with pCT407A, and β-galactosidase reporter gene induction was measured. Numbers refer to the amino acid residues that define the boundaries of each construct. DZO885–1367 corresponds to the original construct used for the yeast two-hybrid screen as a bait. PXXP motifs are represented by P or *P. Three overlapping PXXP motifs are clustered (PPKPVPPKKPK) at the site represented by P*. In DZO1115–1253M1, PFKPVPPPKPK motif starting at amino acid residue 1192 was substituted to PFKAKVPAPPKK. In DZO1115–1253M2–5, PXXP motifs starting at amino acid residues 1230, 1239, 1118, and 1130, respectively, were substituted to AXA. The sites of modified PXXP motifs are represented by A or A*. β-Galactosidase activities relative to the activity for original DZO885–1367 construct are shown by the symbols ++ (>50%), + (10–50%), and − (<10%).

**Fig. 6.** Western blot analysis of Canton-S wild type. Canton-S wild-type whole body samples were lysed in 20 volumes of SDS sample buffer. The protein concentrations of the lysates were quantitated by the Bradford protein assay (Bio-Rad). 40 μg of total protein samples from 0–16-h embryos (lane 1), third-instar larvae (lane 2), pupae (lane 3), and adult flies (lane 4) were resolved by SDS-PAGE and analyzed by Western blot with rabbit anti-DDCortactin antiserum. Positions of molecular weight markers are shown on the left in kDa.

3 T. Katsube, M. Takahisa, R. Ueda, N. Hashimoto, M. Kobayashi, and S. Togashi, unpublished results.
FIG. 7. Localization of DCortactin, DZO-1, F-actin, and DEcadherin in wing imaginal disc. Wing imaginal discs were stained with anti-DCortactin antiserum (green, a, d, and g). The same discs were also stained with anti-DZO-1 antiserum (red, b), phalloidin (red, e), or anti-DE-cadherin antibody (red, h). Merged images are shown in c (a + b), f (d + e), and i (g + h). Yellow staining indicates colocalization of DCortactin and DZO-1, F-actin, or DE-cadherin. DE-cadherin (red)-DCortactin (green) merged images corresponding to apical (j) and basal (k) regions of the adherens junction and more baso-lateral region (l) of the same area are also shown. Bar, 20 μm.

FIG. 8. DCortactin associates with DZO-1 in Drosophila embryo. Lysate from Canton-S wild-type 0-16 h embryos was divided and immunoprecipitated with rabbit anti-DCortactin antiserum (lane 3), rabbit anti-DZO-1 antiserum (lane 6), or their respective control preimmune sera (lanes 2 and 5). The precipitates were analyzed by Western blot with affinity-purified rabbit anti-DZO-1 antibody (lanes 1-3) or rat anti-DCortactin antiserum (lanes 4-6). To localize DZO-1 or DCortactin, a DZO-1-enriched fraction (by immunoprecipitation) of embryo lysate (lane 1) and a diluted embryo lysate (lane 4) were also loaded. Positions of molecular weight markers are shown on the left in kDa.

serum (lane 3) but not by the corresponding preimmune serum (lane 2). An extra band of about 100 kDa, detected in lanes 2 and 3, was not precipitated by the anti-DZO-1 antiserum and is thought to be not specific for the anti-DZO-1 antibody. Western blotting with rat anti-DCortactin antiserum revealed that the DCortactin 105-kDa form was specifically coprecipitated by the anti-DZO-1 antiserum (compare lanes 5 and 6). These results clearly proved that DCortactin associates with DZO-1 in Dro-
osophila embryo cells. The absence of the DCortactin 110-kDa form in the precipitate of the anti-DZO-1 antiserum is thought to be due to the instability of that form in lysate because it also disappeared in the diluted lysate (lane 4). However, we cannot rule out the possibility that the 110-kDa form cannot associate with DZO-1.

Cortactin Associates with ZO-1 in Mouse—We found that rabbit anti-DCortactin antibody could also react with a bacterially expressed protein containing the mouse cortactin 37-amino acid repeat domain fused to maltose binding protein.3 Using this antibody, Western blot analysis of tissue lysates from a 4-day postnatal mouse detected 80- and 85-kDa proteins (Fig. 9a). These proteins are fairly abundant in brain and testis but not so in liver and kidney. Western blot analysis using the rat anti-DCortactin antiserum also yielded the same pattern as that using an anti-chicken p80/85 (cortactin) monoclonal antibody,3 which was reported to cross-react with mouse cortactin (17, 24). These results indicate that both the rabbit and rat anti-DCortactin antibodies can cross-react with mouse cortactin.

To examine the interaction between mouse cortactin and mouse ZO-1, we conducted immunoprecipitation analysis of brain and testis lysates with the rabbit anti-DCortactin antiserum. Western blotting of a mouse tissue lysate with anti-mouse ZO-1 monoclonal antibody showed one faint and two prominent bands with molecular masses of 210, 200, and 190 kDa, respectively (Fig. 9b, lane 1). A 200-kDa protein was detected in the immunoprecipitates of the anti-DCortactin antiserum from brain and testis lysates of 1-3-day postnatal mice (lanes 3 and 5) but not in those of the pre-immune serum (lanes 2 and 4). These results clearly show that mouse cortactin associates with mouse ZO-1 in vivo.

DISCUSSION

Conserved Interaction of Cortactin and ZO-1—We have identified DCortactin as a protein that interacts with the C-terminal proline-rich domain of DZO-1 by a yeast two-hybrid system. Interaction of these proteins was supported by colocalization at cell-cell junction sites in wing disc epithelial cells and confirmed by coimmunoprecipitation from embryo lysates (Figs. 7 and 8). We showed that mouse cortactin associates with mouse ZO-1 in brain and testis of 1-3-day postnatal mice as DCortactin does with DZO-1 in Drosophila embryo (Fig. 9b). This conservation suggests that the interaction of these proteins has a functional significance.
Recently, the SH3 domain of vertebrate cortactin was found to bind preferentially to peptides sharing the consensus motif +PPΨPPKPXWL (+ and Ψ represent basic and aliphatic residues, respectively) (29). A search of the prolactin-rich domain of DZO-1 revealed that the KPVPPPKNY sequence (amino acid residues 1194–1204) is the most likely ligand sequence for the SH3 domain of cortactin. This is consistent with our result showing that prolidine residues at amino acids 1195 and 1198 (PFKPVPPPKP) are necessary for the interaction with DCor-
tactin in yeast (Fig. 5).

Possible Function of Cortactin in the Signaling Path-
way.—We have previously shown that DZO-1 is involved in a signaling pathway that activates transcription of emc and proposed that mammalian ZO-1 is involved in a similar signaling pathway that activates transcription ofId genes (12).Id genes encode transcriptional regulators homologous to emc and are known to play an important role in the regulation of fate determination, proliferation, and transformation in several cell lineages (30–33).

Cortactin, a substrate for Src protein-tyrosine kinase, is phosphorylated in response to multiple extracellular stimuli and implicated in cell signaling (17, 18, 23, 34–38). It is known that cortactin is up-regulated during osteoclast differentiation and megakaryocyte maturation (39, 40). DCor-
tactin is not as abundant in adult flies as in embryos, larvae, and pupae, in which developmental stages cells are actively proliferating (Fig. 6). Further, the result of our preliminary experiment indicates that cortactin is abundant in highly proliferating cultured human cells but not in adult human tissues. These observations suggest that cortactin is involved in both cell differentiation and proliferation.

Human cortactin encoding gene EMS1 is amplified and overexpressed in subsets of breast carcinomas (19, 41–43). Oncogenic effects of Id-1 overexpression in mammary epithelial cells were demonstrated by the analysis using a cell line (44). We hypothesize that cortactin is included in the ZO-1 signaling pathway regulating the expression of emc/Id gene(s).

Possible Function of Cortactin in Cell-Cell Junctions—Cortactin is an F-actin binding protein primarily localizing at cortical structures and thought to be important for microfila-
ment-membrane interactions (18, 34). Cortactin also has an SH3 domain of cortactin. This is consistent with our result showing that proline residues at amino acids 1195 and 1198 (Fig. 6). Further, the result of our preliminary experiment indicates that cortactin is abundant in highly proliferating cultured human cells but not in adult human tissues. These observations suggest that cortactin is involved in both cell differentiation and proliferation.

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Perspectives.—We found that the DCor-
tactin gene is situated on the chromosome segment between 93B3 and 93B7.3 The isolation of mutant alleles of DCor-
tactin gene is currently under progress using deficiency lines encompassing this region. Molecular genetic studies of DCor-
tactin will provide further information on the conserved roles of cortactin in cell adhesion and signaling required for cell fate determination and cell proliferation.