Alternative Splicing of the Actin Binding Domain of Human Cortactin Affects Cell Migration*

Agnes G. S. H. van Rossum‡§, Jos H. de Graaf‡, Ellen Schuuring-Scholtes*, Philip M. Kluin‡¶, Ying-xin Fan¶, Xi Zhan¶, Wouter H. Moolenaar‡, and Ed Schuurung§**

From the §Department of Pathology, Leiden University Medical Center, 2300 RC, Leiden, The Netherlands, ¶Division of Cellular Biochemistry, The Netherlands Cancer Institute and Centre for Biomedical Genetics, 1066 CX, Amsterdam, The Netherlands, ¶Department of Pathology, University Medical Center Groningen, 9700 RB, Groningen, The Netherlands, and |Department of Experimental Pathology, Holland Laboratory, American Red Cross, Rockville, Maryland 20855

Cortactin is a filamentous actin (F-actin)-binding protein that regulates cytoskeletal dynamics by activating the Arp2/3 complex; it binds to F-actin by means of six N-terminal “cortactin repeats”. Gene amplification of 11q13 and consequent overexpression of cortactin in several human cancers is associated with lymph node metastasis. Overexpression as well as tyrosine phosphorylation of cortactin has been reported to enhance cell migration, invasion, and metastasis. Here we report the identification of two alternative splice variants (SV1 and SV2) that affect the cortactin repeats: SV1-cortactin lacks the 6th repeat (exon 11), whereas SV2-cortactin lacks the 5th and 6th repeats (exons 10 and 11). SV-1 cortactin is found co-expressed with wild type (wt)-cortactin in all tissues and cell lines examined, whereas the SV2 isoform is much less abundant. SV1-cortactin binds F-actin and promotes Arp2/3-mediated actin polymerization equally well as wt-cortactin, whereas SV2-cortactin shows reduced F-actin binding and polymerization. Alternative splicing of cortactin does not affect its subcellular localization or growth factor-induced tyrosine phosphorylation. However, cells that overexpress SV1 or SV2-cortactin show significantly reduced cell migration when compared with wt-cortactin-overexpressing cells. Thus, in addition to overexpression and tyrosine phosphorylation, alternative splicing of the F-actin binding domain of cortactin is a new mechanism by which cortactin influences cell migration.

Cell migration is fundamental to many normal and pathological processes and plays a central role not only in embryonic development and wound healing but also in tumor cell invasion and metastasis. Cells migrate and change their shape by continuously assembling and disassembling their filamentous actin (F-actin) cytoskeletal network (1). Many actin-binding proteins regulate cytoskeletal dynamics, in particular proteins that cross-link F-actin (2). Cortactin is one of the major F-actin-binding and cross-linking proteins that regulates cytoskeletal dynamics by activating the Arp2/3 complex (3, 4).

Cortactin contains six (and one-half) 37-amino acid repeat domains that mediate F-actin binding; this is mainly mediated by the fourth repeat (5). Splice variants affecting the F-actin binding domain have been identified in rat (6), but little is known about their occurrence and function. Cortactin contains a DDW-Arp2/3 binding site in its N-terminal region that, together with the actin binding domain (ABD), is required for Arp2/3-mediated actin polymerization (3–5). Although cortactin promotes Arp2/3-mediated actin polymerization, it acts differently from other Arp2/3 activators such as members of the Wiskott-Aldrich syndrome protein, which bind monomeric G-actin as opposed to F-actin (7). As a consequence, cortactin inhibits the disassembly of cortical actin by stabilizing Arp2/3-induced F-actin branches (3). Both the ABD and the Arp2/3 binding site are necessary for the translocation of cortactin to sites of actin polymerization (5), which is regulated by the small GTPase Rac1 (8) and the serine/threonine kinase PAK1 (9). Cortactin also contains a proline-rich region with three c-Src tyrosine phosphorylation sites (10) and an SH3 Src homology domain at the COOH terminus that mediates the interaction with diverse proteins, including dynamin2 (11), ZO-1 (12) and neural Wiskott-Aldrich syndrome protein (13). Thus, cortactin functions as a scaffold protein that recruits other proteins to sites of actin polymerization.

Human cortactin is encoded by the EMS1 gene on chromosome 11q13 (14), a region frequently amplified in human carcinomas of the breast and head/neck region (14, 15). Gene amplification correlates with an increase in cortactin protein levels (16, 17) and with the presence of lymph node metastases and increased mortality (14, 18, 19). Cells overexpressing cortactin show enhanced migration in Boyden chamber (20) and wound healing assays (21). Overexpression of mouse wt-cortactin in MDA-MB-231 breast cancer cells leads to increased formation of metastases in bone (22). In addition, cortactin-containing complexes in invadopodial structures correlate with enhanced invasiveness of MDA-MB-231 cells (23). The available evidence, therefore, suggests that increased expression of cortactin, because of 11q13 amplification, promotes tumor cell invasion and metastasis.

Cortactin is a major substrate for the Src tyrosine kinase (24) and is tyrosine-phosphorylated in response to various stimuli, including growth factors, integrin cross-linking, bacterial invasion, and cell shrinkage (reviewed in Ref. 25). In human carcinomas of the breast and head/neck region (14, 15). Gene amplification correlates with an increase in cortactin protein levels (16, 17) and with the presence of lymph node metastases and increased mortality (14, 18, 19). Cells overexpressing cortactin show enhanced migration in Boyden chamber (20) and wound healing assays (21). Overexpression of mouse wt-cortactin in MDA-MB-231 breast cancer cells leads to increased formation of metastases in bone (22). In addition, cortactin-containing complexes in invadopodial structures correlate with enhanced invasiveness of MDA-MB-231 cells (23). The available evidence, therefore, suggests that increased expression of cortactin, because of 11q13 amplification, promotes tumor cell invasion and metastasis.
nomal cells, epidermal growth factor (EGF) induces translocation of cortactin from the cytosol to the cortical cytoskeleton, with concomitant tyrosine and serine/threonine phosphorylation of cortactin (26); translocation of cortactin is required for its tyrosine phosphorylation (27). Tyrosine phosphorylation by Src inhibits the ability of cortactin to cross-link F-actin (10), and mutation of the tyrosine phosphorylation sites in cortactin inhibits migration (21) and metastasis (22). Thus, Src-mediated tyrosine phosphorylation is a second mechanism by which cortactin may regulate cell motility.

In this paper, we report the identification of two alternative splice variants of human cortactin, termed SV1- and SV2-cortactin, which lack the 6th or 5th and 6th repeats in the F-actin binding domain, and promote Arp2/3-mediated actin polymerization in vitro. We propose that alternative splicing of cortactin represents a new mechanism to modulate actin dynamics and cell migration.

EXPERIMENTAL PROCEDURES

Antibodies—The following primary antibodies were used: monoclonal antibody against human cortactin (4F11) obtained from Dr. J. T. Parsons (University of Virginia, Charlottesville, VA); polyclonal antibody against cortactin (C001) (28); polyclonal antibody against green fluorescent protein (GFP)-epitope (29); monoclonal hybridoma supernatant antibody against myc (clone 9E10, 26C4, Santa Cruz Biotechnology, Santa Cruz, CA); monoclonal antibody against tyrosine phosphorylated proteins, PY-99, (Santa Cruz Biotechnology), anti-p116Rip antibodies (30) and anti-actin mouse monoclonal antibody (Mab1501R, Chemicon, Temecula, CA).

Cell Culture and Transfection—The mouse NIH3T3 fibroblast cell line and its derivatives were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with penicillin/streptomycin, 10% newborn bovine serum (NCS) at 37 °C, and 5% CO2. All other cell lines were grown in DMEM containing 8% fetal calf serum and antibiotics. COS7 cells were transfected using the DEAE-dextran method (31), N1E-115 cells using calcium phosphate precipitates (32), and NIH3T3 cells with LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s instructions. Medium for cell lines containing the neomycin resistance gene was supplemented with 1.25 mg/ml G418 (Invitrogen). COST, NIH3T3, and N1E-115 cells were purchased from American Type Culture Collection. UMSCC and VU squamous cell carcinoma cell lines were kindly provided by Dr. T. Carey and Dr. M. Hermens, respectively, and cultured as described previously (33, 34).

RNA Extraction, RT-PCR, and GeneScan Analysis on ABI377—Total RNA was isolated from cell lines or human tissues (obtained from patients with no evidence of malignancies) using TRIzol reagent (Invitrogen) or by the urea/lithium chloride method described previously (16). For cDNA synthesis, 2 µg of total RNA was incubated in buffer G (50 mM Tris-HCl, pH 8.3, 8 mM MgCl2, 30 mM KCl, 10 mM dithiothreitol), 2 mM deoxynucleoside triphosphate, 7 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), 10 units of human placental RNAse inhibitor (Amersham Pharmacia Biotech, Uppsala, Sweden), and 2 µM oligo dt18 primers (Sigma) at 37 °C for 1 h. RT-PCR was performed using cDNA in 10 µl Tris-HCl (pH 8.4), 50 mM KCl, 0.06% bovine serum albumin, 10 mM dithiothreitol, 0.2 mM deoxynucleoside triphosphate, 2 mM MgCl2, 1.5 units of TaqDNA polymerase (Invitrogen), and 6 pmol each of primers p149 FAM (5′-GTGTTGGAGACAGACAGAC-3′) and p315 FAM (5′-GACAGGAGGAGGAGGAGGAG-3′). PCR was carried out for 33 cycles at 94 °C for 1 min, 56 °C for 1.30 min, and 72 °C for 2.30 min. Because cortactin-cross-link F-actin, and promote Arp2/3-mediated actin polymerization, they significantly in their ability to induce cell migration, bind and cross-link F-actin, and promote Arp2/3-mediated actin polymerization.

Expression Constructs—The GST-wt-cortactin construct was generated by cloning the NcoI-EcoRI C-terminal fragment of the 1.8-kb EMS1/cortactin cDNA (clone nr:4203; Ref. 33) and an in-frame 5′-PCR fragment of cortactin (BamH1 site linked to ATG-Nocl site) into the pPR270 vector (derivative of pGEX-2T). GST-tagged splice variants SV1 and SV2 were generated by replacing the Nol-HincII fragment of GST-wt-cortactin construct flanking the actin binding repeat domain with the Nol-HincII fragments of the p149/p150-PCR products representing the SV1 and SV2 transcripts. The GFP- and myc-tagged cortactin constructs were generated by PCR on the different GST constructs using primer A1 containing a KpnI site, myc-epitope, and EcoRI site (5′-GGGTTGAATGATCGAGAGAAGGCAG-3′) and with p150 FAM and p573 (5′-CCAGGAAAGGCACATGGTT-3′) (see Fig. 1A).

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min) or purified proteins (e-g, cortactin or bovine serum albumin) were incubated for 1 h at room temperature with 1.5 μl of G-actin filaments. The reaction mixtures were centrifuged at 100,000 × g (high speed) to determine the F-actin binding affinity or at 10,000 × g (slow speed) for 90 min (Beckman Airfuge) to evaluate the F-actin crosslinking activity of myc-tagged cortactin proteins. As a control for actin-independent sedimentation, various proteins were also centrifuged under conditions in which F-actin was omitted from the mixture. Sedimented proteins were resolved by SDS-PAGE and detected by either Coomassie Blue staining or by Western blot analysis using the anti-myc antibody 9E10.

F-actin Binding Assay—Binding of the human cortactin variants to F-actin was quantified in a co-sedimentation assay, as described previously (35). GST-cortactin proteins were purified as GST-fusion proteins as described previously (3). F-actin was prepared by incubation of rabbit skeletal-muscle monomeric actin (G-actin, Cytoskeleton) in polymerization buffer containing 5 mM Tris-HCl, pH 7.5, 134 mM KCl, 2 mM MgCl₂, and 1 mM ATP for at least 4 h at room temperature. For the F-actin binding assay, GST-cortactin proteins (80 nM) were mixed with F-actin at concentrations of 0–8.0 μM in the polymerization buffer and incubated at room temperature for 30 min as described previously for mouse GST-cortactin proteins (4). The reaction mixtures were centrifuged for 200,000 × g for 30 min, and the amount of cortactin in both supernatant and precipitant were detected by immunoblotting using antibody against cortactin (C001) and quantitated by digital scanning. The amounts were normalized to percentages. To calculate the dissociation constant, Kₐ, the amounts of cortactin splicing variants bound to F-actin were fit to a single rectangular hyperbola equation: B = B₀ (KₐC + C), where B is the bound percentage of cortactin, and C is the F-actin concentration of samples to be tested.

F-actin Polymerization Assay—Actin polymerization was monitored by measuring the increase in pyrene fluorescence using an LS50B fluorometer (PerkinElmer Life Sciences) with excitation at 365 nm and emission at 407 nm, as reported previously for mouse GST-cortactin (4).

Fluorometer (PerkinElmer Life Sciences) with excitation at 365 nm and emission at 407 nm, as reported previously for mouse GST-cortactin (4). The percentage of cortactin, and protein concentration was determined by using a Bio-Rad protein assay kit. For immunoprecipitation, lysates were incubated with antibodies that were cross-linked to protein A-Sepharose beads in a rotator at 4 °C overnight. Immune complexes were washed in lysis buffer and treated as described (35). Lysates and fractions were solubilized in Laemmli sample buffer containing 0.1 mM dithiothreitol and separated by SDS-PAGE. Proteins were blotted onto nitrocellulose and stained with Ponceau S, and filters were blocked in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat milk for at least 1 h. The filters were probed with primary antibody in blocking buffer for at least 1 h, washed three times in TBST, incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) in blocking buffer for 30 min. Proteins were visualized by using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

RESULTS

Cloning and Characterization of Alternative Splice Variants of Human Cortactin—RT-PCR analysis on mRNA from human squamous cell carcinoma (SCC) cell lines using primers that flank the region encoding the entire actin binding domain (Fig. 1A) revealed at least two products (Fig. 1B): the expected 835-bp wt product and an ~100-bp smaller product. Sequencing of the PCR products revealed the wt transcript and two alternative splice variants: SV1-cortactin, lacking 111 bp, and SV2-cortactin, lacking 222 bp, which correspond to one and two actin binding repeats (37 amino acids each), respectively. To confirm that both transcripts resulted from alternative splicing, we determined the genomic structure of the human EMS1/ cortactin gene. The genomic sequence encoding the actin binding domain extends from exon 5 to exon 12 (Fig. 1A), with five exons of 111 bp (exons 6 and 8–11). Sequence analysis showed that SV1-cortactin lacks exon 11, corresponding to the 6th repeat, and that SV2-cortactin lacks exons 10 and 11, corresponding to the 5th and 6th repeats (Fig. 2). In conclusion, we identified two alternative splice variants of human cortactin: SV1-cortactin lacking the 6th repeat and SV2-cortactin lacking the 5th and 6th repeats of the actin binding domain.

Splice Variant Expression in Human Carcinoma Cell Lines and Normal Tissues—To evaluate whether prominent expression of either one of the cortactin variants correlates with 11q13 DNA amplification, we performed an RT-PCR analysis of the entire F-actin binding domain (Fig. 1A) in 23 carcinoma cell lines. Relative expression levels were determined by using semi-quantitative RT-PCR and gene-scanning analysis. Although the SV2-cortactin transcript was hardly detectable in most cell lines (Fig. 3A), wt- and SV1-cortactin were prominently expressed in all cell lines. Ratios between wt- and SV1-cortactin varied between 1:4 and 1:1 among different cell lines. There is no indication that the wt/SV1 ratio correlates with 11q13 DNA amplification. To examine whether the relative expression of wt- and SV1-cortactin is tissue-specific, we analyzed total RNA from various normal human tissues (Fig. 3B).

Immunofluorescence Microscopy—NIE-115 neuroblastoma cells, transiently transfected with the GFP-cortactin variants, were grown on uncoated glass coverslips. After 24 h of serum starvation, cells were stimulated with 5 ng/ml insulin (Sigma). Cells were fixed in 3.7% formaldehyde/PBS for 20 min, permeabilized in 0.1% Triton X-100/PBS, and blocked with 1% bovine serum albumin in PBS. Cells were stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR). Images were collected with confocal microscopy (Leica). Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) was used to process the images.

Immunoblot Analysis—COST cells were serum-starved overnight and treated 10 min with 40 ng/ml EGF (BD Biosciences). Cells were washed in ice-cold PBS and lysed in radioimmune precipitation assay buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM KCl, 0.1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 1 mM Na₃VO₄, 5 mM NaF, and 1 μg/ml leupeptin) and were tumbled at 4 °C for 45 min. Cell extracts were pre-cleared by 15-min centrifugation, and protein concentration was determined by using a Bio-Rad protein assay kit. For immunoprecipitation, lysates were incubated with antibodies that were cross-linked to protein A-Sepharose beads in a rotator at 4 °C overnight. Immune complexes were washed in lysis buffer and treated as described (35). Lysates and fractions were solubilized in Laemmli sample buffer containing 0.1 mM dithiothreitol and separated by SDS-PAGE. Proteins were blotted onto nitrocellulose and stained with Ponceau S, and filters were blocked in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat milk for at least 1 h. The filters were probed with primary antibody in blocking buffer for at least 1 h, washed three times in TBST, incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) in blocking buffer for 30 min. Proteins were visualized by using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).
Again, SV2 expression was hardly detectable in most normal tissues, except in the frontal cerebrum. Splice variants lacking the critical fourth repeat, which is essential for F-actin binding (5), were not detected. These results suggest that the relative expression levels of wt- and SV1-cortactin are determined by tissue origin rather than by gene amplification.

**F-actin Binding, Cross-linking, and Activation of the Arp2/3 Complex**—To investigate how alternative splicing may influence F-actin binding and cross-linking, we used co-sedimentation assays using lysates of COS7 cells transfected with myc-tagged cortactin variants. We found that wt-cortactin and SV1-cortactin co-sedimented with F-actin (Fig. 4A, left panel). In control experiments, we found that myc-tagged delABD-cortactin, which lacks the entire F-actin binding domain, remained in the supernatant. Fig. 4 (left panel) also shows that SV2-cortactin co-sediments with F-actin for ∼50%, suggesting that SV2 has reduced affinity for F-actin. To investigate the F-actin cross-linking activity of these variants, we performed low speed co-sedimentation assays (Fig. 4A, right panel). A considerable amount of wt-cortactin was detected in the low-speed pellet, suggesting the induction of an actin network, which is consistent with findings with mouse cortactin (10). In contrast to wt-cortactin, myc-SV2 does not co-sediment to the pellet under similar conditions and, therefore, is most probably not involved in cross-linking F-actin. On the other hand, the amount of co-sedimented myc-SV1-cortactin is significantly decreased (faint band after longer exposure), despite the fact that myc-SV1 has a similar binding affinity to F-actin as does wt-cortactin (Fig. 4, A and B). These results indicate that SV1 has some cross-linking activity, whereas SV2 has not.

In co-sedimentation assays using total lysates, other proteins might affect the actin binding activities of the cortactin variants. Therefore, we performed a semi-quantitative F-actin binding assay using purified GST-cortactin variants. As shown in Fig. 4B, the $K_a$ of GST-wt-cortactin and GST-SV1-cortactin for F-actin is ∼0.4 μM. The binding affinity of GST-SV2-cortactin is ∼10-fold lower ($K_a$ of ∼4 μM) than that of GST-wt-cortactin, which is in agreement with the high speed co-sedimentation results (Fig. 4A, left panel).

The F-actin binding domain of cortactin is required for its translocation to the lamellipodial cortex (3), where it stimulates Arp2/3-mediated actin polymerization (5, 37). We monitored Arp2/3-mediated actin polymerization using pyrene-labeled monomeric G-actin (Fig. 4C). Although spontaneous actin polymerization occurs at a very slow rate, the addition of Arp2/3 alone increased the rate of actin polymerization significantly, which was further increased by adding human GST-wt-cortactin. This result is in accordance with the effect of wt mouse cortactin (4). The stimulation of Arp2/3-mediated actin polymerization by GST-SV1-cortactin is comparable with GST-wt-cortactin, whereas GST-SV2-cortactin acts significantly less efficiently. In conclusion, all three cortactin variants are able to bind F-actin and stimulate Arp2/3-mediated actin polymerization, although SV2-cortactin shows lower efficiency, most likely because of its lower binding affinity for F-actin.

**Overexpression of Cortactin Splice Variants Reduces Cell Migration**—As shown previously, overexpression of wt mouse cortactin results in increased cell migration and invasion (20, 21). To test how the cortactin splice variants may affect cell migration, we used NIH3T3 cells stably transfected with various GFP-tagged cortactin constructs in Boyden chamber migration assays. Cells overexpressing human wt-cortactin were 50% more motile than the GFP control cells (Fig. 5A). The SV1-cortactin-overexpressing cells also showed an increase in cell migration, although significantly less when compared with wt-cortactin-overexpressing cells. In contrast, in SV2-cortactin-overexpressing cells, the migration rate was not altered. Similar effects were observed in wound healing assays in vitro (data not shown). In conclusion, although overexpression of human wt-cortactin stimulates cell migration, overexpression of the splice variants leads to a reduction (SV1) or even a loss (SV2) of an induction of cell migration.

**Subcellular Localization and Tyrosine Phosphorylation of Cortactin Variants**—The stimulatory effect of cortactin on actin polymerization and cell migration depends not only on cortactin protein expression levels but also on its tyrosine phosphorylation and subcellular localization (25). We determined the translocation of cortactin from the cytosol to lamellipodia after growth factor stimulation. To this end, we used neuroblastoma N1E-115 cells because these cells show extensive lamellipodia formation in response to insulin.3 In serum-starved N1E-115 cells, endogenous cortactin is mainly present in the cytosol (not shown). Insulin treatment resulted in a redistribution of endogenous cortactin to lamellipodia, where it co-localized with F-actin (data not shown). The translocation of GFP-tagged wt-, SV1- and SV2-cortactin after insulin treatment was not significantly different from that of endogenous cortactin (Fig. 6). GFP-delABD-cortactin and GFP alone failed to translocate after the addition of insulin. Thus, because of the F-actin binding

**Fig. 2. Schematic representation of the cortactin variants and their activities.** Diagram of cortactin variants used in N-terminal myc-, GFP-, and GST-tagged expression constructs. Right, summary of assays used in this study. For F-actin binding, ++ indicates a binding affinity of 0.4 μM, and + indicates 4 μM. F-actin cross-linking was evaluated by measuring the fractions of protein in the supernatant and pellet from a Western blot. Boyden chamber migration assay was indicated as the % of the mock control. For cortical translocation, neuroblastoma N1E-115 cells were serum-starved, and translocation of cortactin into lamellipodia was induced by stimulation of insulin. The tyrosine phosphorylation was measured by immunoprecipitating cortactin after EGF stimulation of COS7 cells transfected with cortactin constructs. The epitopes of the monoclonal antibody 4F11 used to detect cortactin in experiments are represented in the figure. nd, not determined.

3 O. Kranenburg and W. H. Moolenaar, unpublished data.
domain, locating in the fourth repeat is required for translocation to the cell cortex; indeed, SV1- and SV2-cortactin behave similarly to wt-cortactin in this respect.

The redistribution of cortactin is accompanied by an increased tyrosine phosphorylation of cortactin after treatment with growth factors (26, 28). Tyrosine phosphorylation of mouse wt-cortactin by Src decreases its F-actin cross-linking activity and increases cell migration (21). To investigate...
Splicing of the Actin Binding Domain of Cortactin

In this paper, we describe the identification of two alternative splice variants affecting the F-actin binding domain of human cortactin: SV1-cortactin lacking the 6th repeat (exon 11) and SV2-cortactin lacking the 5th and 6th repeats (exon 10 and 11). We show that SV1- and SV2-cortactin differ significantly in their ability to bind and cross-link F-actin, promote Arp2/3-mediated actin polymerization in vitro, and induce cell migration, when compared with wt-cortactin.

SV1-cortactin, in common with wt-cortactin, is expressed in all carcinoma cell lines and normal human tissues tested, whereas expression of SV2-cortactin is hardly detectable except in the frontal cerebrum. Alternative splicing of the F-actin binding domain is not unique to human cortactin because similar variants have been found in rat brain (6). RT-PCR experiments using other primer sets did not reveal splice variants other than SV1 and SV2 (data not shown).

We found that overexpression of human wt-cortactin in NIH3T3 cells significantly increased cell migration in both Boyden chamber and wound-healing assays, in agreement with a previous study using mouse wt-cortactin (20, 21). In contrast, cells overexpressing SV1-cortactin were significantly less mobile than wt-cortactin-overexpressing cells, and this effect was even more pronounced in SV2-cortactin-overexpressing cells. This differential effect on cell migration correlates with the F-actin binding activity (right panel) of various variants (see also Fig. 2). COST cells were transiently transfected with myc-tagged cortactin variants and subjected to in vitro co-sedimentation assays. 10 μl of total lysates were mixed with polymerization buffer and centrifuged in either the presence (+) or absence (−) of 1.5 μM pure F-actin. The myc-tagged cortactin variants were visualized by using Western blot analysis with antibody 9E10, and Coomassie Blue staining was performed to visualize non-myc-tagged proteins. At longer exposures, no signals were visible in the low speed pellet fractions of myc-SV2 and myc-delABD, whereas a weak band was visible in the myc-SV1 pellet (data not shown). α-Actinin and the recently described F-actin cross-linking protein (myc-tagged) p116Rip (30) were used as positive controls for both F-actin binding and cross-linking. Bovine serum albumin (BSA) and the myc-tagged delABD-cortactin protein, both unable to bind F-actin, were used as negative controls. B, comparison of the F-actin binding affinity of GST-cortactin variants. Each GST-cortactin splice variant (80 nM) was mixed with F-actin at increasing concentrations, and F-actin binding was determined by high speed co-sedimentation. Pellet and supernatant fractions were immuno-blotted with anti-cortactin antibody C001 and quantitated by digital scanning. The Kd value for wt- and SV1-cortactin is ~0.4 μM, whereas the Kd for SV2-cortactin is ~4 μM. The experimental data were fitted to a single rectangular hyperbola equation, B = BmaxC/(Kd + C), where B is the bound percentage of cortactin, Bmax is the maximum binding percentage at an infinitely high concentration of F-actin, C is the concentration of F-actin, and Kd is the dissociation constant. C, comparison of the activities of the cortactin splice variants in Arp2/3 complex-mediated actin polymerization. Polymerization of 2.7 μM 10% pyrene-labeled actin monomers was measured in the presence of 20 nM Arp2/3 complex and different concentrations of the GST-cortactin splice variants. The activity of cortactin was determined on the basis of the time taken to induce half-maximal activity (t1/2). Inset, representative curves in the presence of 20 nM Arp2/3 and 20 nM cortactin. Black circles, wt-cortactin; black triangle, SV1; black diamond, SV2; white triangle, Arp2/3; white circle, actin.
Fig. 5. Migration of NIH3T3 cells overexpressing cortactin variants. A, overexpression of cortactin splice variants affects cell migration. NIH3T3 cells stably transfected with GFP-tagged cortactin variants were analyzed in Boyden chamber migration assay. Cells were allowed to migrate 6 h through fibronectin-coated Transwell membranes in response to 1% NCS-containing media. To quantitate migration, stained cells were counted in four fields from each of three Transwell filters per experiment. The percentage of cell migration for each cell line was calculated relative to the number of migrated GFP-control cells (set at 100%). Data are the means ± S.D. of three independent migration experiments. GFP-wt, GFP-SV1, and GFP-delABD were significantly different from GFP-cells using analyses of variance (p < 0.05). B, relative expression levels of the cortactin variants in relation to endogenous mouse cortactin. Protein lysates of NIH3T3 cells stably transfected with GFP-tagged cortactin variants were subjected to immunoblotting using the anti-GFP-antibody (left panel) and the anticortactin antibody 4F11 (right panel). Actin staining was used as a loading control (two lower blots). Antibody 4F11 identifies endogenous cortactin (arrow), GFP-tagged wt-cortactin, and SV1-cortactin. Because the epitope of 4F11 is located in the 5th repeat of the actin binding domain, SV2- and delABD-cortactin were not detected. Therefore, the same lysates were immunoblotted with anti-GFP. GFP, mock (empty vector)-transfected cells.

reduced F-actin cross-linking capacity of the splice variants. As summarized in Fig. 2, the F-actin binding affinity and the ability to activate Arp2/3-mediated actin polymerization are similar for wt-cortactin and SV1-cortactin (Kd = 0.4 μM), whereas both parameters are significantly decreased for SV2-cortactin (Kd = 4 μM). Low speed co-sedimentation assays revealed that SV1-cortactin is still able to cross-link F-actin, however, at a much lower efficiency as wt-cortactin, despite their similar F-actin binding affinities. On the other hand, SV2-cortactin was not able to cross-link F-actin. Although enhanced tyrosine phosphorylation of cortactin can account for a reduction in F-actin cross-linking activity (10), we found that wt-, SV1-, or SV2-cortactin are all properly tyrosine phosphorylated, at least after EGF stimulation. This finding suggests that the reduced ability of SV1- and SV2-cortactin to cross-link F-actin cannot be ascribed to altered tyrosine phosphorylation. Finally, we found that GFP-tagged wt-, SV1-, and SV2-cortactin translocate to lamellipodia after hormone treatment in a manner similar to endogenous cortactin, suggesting that neither splice variant has a unique subcellular localization. Our study indicates that the number of cortactin repeats determines proper binding to F-actin; precisely how the cortactin variants affect actin dynamics in vivo to differentially affect cell migration remains to be elucidated. The role of SV2-cortactin might be neuron-specific, because significant mRNA levels of this particular isoform were only detected in the frontal cerebrum.

Expression of the cortactin splice variants seems to be independent of 11q13 DNA amplification. The relative expression
levels of wt- versus SV1-cortactin in 23 human carcinoma cell lines ranged between 1:4 and 4:1, with no correlation being observed between the wt/SV1 ratio and DNA amplification. Gene amplification correlates with “total” cortactin overexpression at the mRNA and protein levels (16, 17, 19, 38) and with increased invasive potential of the cells (14, 17, 18). Whether wt- and SV1-cortactin transcripts are translated or degraded with different efficiencies remains to be examined. Development of antibodies that discriminate between wt-cortactin and wt- and SV1-cortactin transcripts are translated or degraded.

In human cancer, increased cortactin levels promotes cell motility, invasion, and metastasis, but precisely how overexpression of antibodies that discriminate between wt-cortactin and wt- and SV1-cortactin transcripts are translated or degraded with different efficiencies remains to be examined. Development of antibodies that discriminate between wt-cortactin and wt- and SV1-cortactin transcripts are translated or degraded. Splicing of the Actin Binding Domain of Cortactin...
Alternative Splicing of the Actin Binding Domain of Human Cortactin Affects Cell Migration
Agnes G. S. H. van Rossum, Jos H. de Graaf, Ellen Schuuring-Scholtes, Philip M. Kluin, Ying-xin Fan, Xi Zhan, Wouter H. Moolenaar and Ed Schuuring

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