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Identification of *Xenopus* Cortactin: Two Isoforms of the Transcript and Multiple Forms of the Protein

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ABSTRACT—Cortactin was initially identified as a substrate for Src tyrosine kinase. It interacts with the filamentous actin in the cell cortex through the tandem repeats of 37-amino acid. In this report, we describe the identification of a *Xenopus* homolog of cortactin. The deduced amino acid sequence shares over 70% identity with human, mouse, and chicken cortactin. Northern and Western blot analyses revealed that *Xenopus* cortactin is widely expressed in *Xenopus* tissues. Analysis of the transcripts using polymerase chain reaction revealed two isoforms being different in the number of the tandem repeats. The major isoform has 6.5 tandem repeats but the minor one has 5.5 tandem repeats. As the sixth repeat, which is missed in the minor isoform, is encoded by a single exon flanked by introns on both sides, these two isoforms are likely to be generated by alternative splicing. We propose that cortactin regulates the construction of actin cytoskeleton by altering the number of its tandem repeats.

INTRODUCTION

The cortical cytoskeleton is a dense network of actin filaments (F-actin) and associating actin-binding proteins. Cells undergo rearrangement of the cortical cytoskeleton during cell division, cell migration, oncogenic transformation, and in response to various extracellular stimuli (Schmidt and Hall, 1998). Src protein tyrosine kinase plays a pivotal role in some of these cellular processes (Thomas and Brugge, 1997). Its constitutive activation results in transformation of cells and in a gross alteration of cell morphology. Cortactin was originally identified as a highly phosphorylated protein in v-Src-transformed chicken embryo fibroblast cells (Wu *et al*., 1991). Stimulation of its phosphorylation is also observed in response to various extracellular stimuli, which often induce rearrangement of the cortical cytoskeleton (Bhattacharya *et al*., 1995; Dehio *et al*., 1995; Huang *et al*., 1997; Liu *et al*., 1996; Maa *et al*., 1992; Zhan *et al*., 1993). Cortactin has an F-actin binding activity and primarily localizes within cortical structures, such as membrane ruffles, lamellipodia, and podosomes, which are enriched for cytoskeletal proteins (Wu and Parsons, 1993). Cortactin also has an F-actin cross-linking activity, which is down-regulated by Src mediated tyrosine phosphorylation (Huang *et al*., 1997). Thus, cortactin is thought to be involved in the Src signaling pathway regulating cytoskeleton reorganization. However, its precise function in the process is obscure.

Xenopus laevis is an attractive model animal because we can access the embryos easily to analyze gene function in development utilizing microinjection of cloned DNA, synthetic mRNA, or antibodies into the oocytes and embryos (Vize *et al*., 1991). Previously, injection of a synthetic mRNA encoding a constitutive active form of Src into *Xenopus* oocyte was revealed to induce a gross alteration in the cortex (Unger and Steele, 1992). Several observations suggest the involvement of *Xenopus* cortactin (*X*cortactin) in this process (Thorn *et al*., 1999; Unger and Steele, 1992). Here, we demonstrate the molecular cloning and characterisation of *X*cortactin. This study enables us to investigate the function of cortactin in developing organisms, which will provide further information on the function of cortactin on Src signaling pathways regulating cytoskeleton organization.

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MATERIALS AND METHODS

Isolation of *Xenopus* cortactin cDNA

A 1.4-kb mouse cortactin cDNA fragment was amplified by reverse transcription and polymerase chain reaction (RT-PCR) using a set of degenerated primers as follows: 5'-TT(C/T)GT(T/C/A/G)AA(C/T)GA(C/T)GT(T/C/A/G)AG(C/T)GA-3' and 5'-TC(A/G)AA(T/C/A/G)GA(T/A/G)AT(C/T)TC(A/G)TC(A/G)TC-3'. The mouse cortactin cDNA fragment was labeled with ^{32}P and employed as a probe to

screen the *Xenopus* embryo cDNA library constructed in a lambda ZAPII vector (Stratagene, La Jolla, CA, USA). Phagemid DNA was isolated by in vivo excision from a positive cDNA clone and subjected to nucleotide sequencing on an ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, CA, USA).

Preparation of nucleic acid and protein samples

Tissue samples of brain, lung, heart, liver, skeletal muscle, kidney, ovary with immature oocytes, and testis were removed from adult *X. laevis* females or males. Ovarian tissues with mature oocytes were removed from adult *X. laevis* females pretreated with gonadotropin. Genomic DNA and total RNA samples were purified from these tissue samples using a TRIZOL Reagent (GIBCO BRL, Rockville, MD, USA). Tissue samples were lysed in 2 x SDS gel loading buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 2% 2-mercaptoethanol). The protein concentrations were quantified by the Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Northern and Western blot analyses

10 μg of total RNA samples were separated on 1.2% formaldehyde-agarose gel and transferred to a Hybond-N^+ nylon membrane. Probes were labeled with digoxigenin (PCR DIG Probe Synthesis kit, Roche molecular biochemicals, Mannheim, Germany). The hybridised probes were detected using a DIG Luminescent Detection kit (Roche molecular biochemicals). Western blot analysis was done as described previously (Katsube *et al.*, 1998). Anti-chicken cortactin (p80/85) monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-*Drosophila* cortactin polyclonal antibody was described previously (Katsube *et al.*, 1998).

RT-PCR

1 μg of total RNA samples were treated with DNase I (GIBCO BRL) and reverse transcribed by SuperScript II reverse transcriptase (GIBCO BRL) using the oligonucleotide primer RT. One-twentieth part of the cDNA products was subjected to a PCR using the oligonucle-

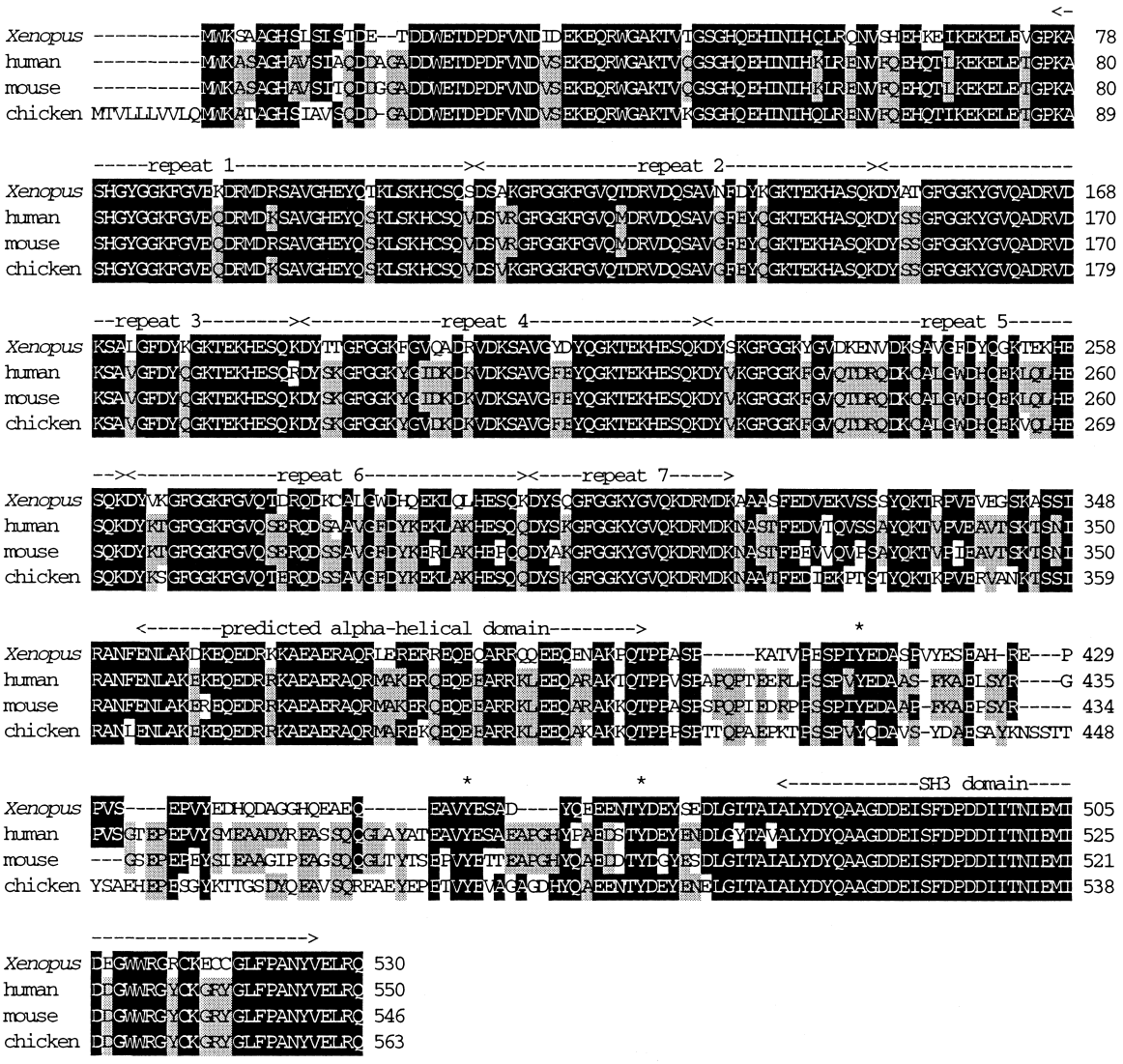

Fig. 1. Alignment of *X*cortactin with the human, mouse, and chicken homologs. Identities with *X*cortactin are shaded in black, and identities among the homologs are shaded in gray. Cortactin repeats (repeat 1-7), a predicted alpha-helical domain, and an SH3 domain are shown with broken lines above the aligned sequences. Asterisks represent conserved tyrosine residues targeted by Src tyrosine kinase (Huang *et al.*, 1998). The fourth, fifth, and sixth cortactin repeats of *X*cortactin are aligned with the repeats at the corresponding positions of the homologs, but are most related to the third, fourth, and fifth repeats of them, respectively. The region between the predicted alpha-helical domain and the SH3 domain is rich in proline residues in all proteins, although the sequence homology within the region is quite limited.

otide primers Xcort-F and Xcort-R. The reaction was processed for 30 cycles at 95°C for 30 s, 67°C for 30 s, and 72°C for 2 min. The sequences of the oligonucleotide primers were as follows: RT, 5'-CGCTTGTTAGCCAAAGCAGCCAAA-3'; Xcort-F, 5'-GGGTCTG-GACATCAAGAACACATC-3'; Xcort-R, 5'-CTGGTAGGACGAAG-AGACTTTCTC-3'.

Analysis of exon-intron structure of the *Xcortactin* gene

Two sets of oligonucleotide primers (G1, G2) and (G3, G4) were designed to amplify putative intron sequences. Each set of the primers and 0.2 μg of the genomic DNA was subjected to a 30 cycles of PCR at 95°C for 30 s and 68°C for 5 min. Nucleotide sequences of the oligonucleotide primers were as follows: G1, 5'-AGCGCTG-TTGGGTTTGACTACCAA-3'; G2, 5'-ACTCGTGCAGCTGAAGCTT-TTCCT-3'; G3, 5'-GTTTGGTGTGCAGACAGACAGACA-3'; G4, 5'-TC-TTTCTGCACACCGTACTTTCCG-3'.

RESULTS

Isolation of a *Xenopus* cortactin cDNA

To isolate a *Xenopus* cDNA encoding a cortactin homolog, a *Xenopus* embryo cDNA library was screened using a 1.4-kilobase (kb) mouse cortactin cDNA fragment as a probe. Several positive clones were isolated and the clone with the longest insert was sequenced. The clone showed significant homology to the previously reported cortactin cDNAs. It contained a 3578-basepair (bp) cDNA sequence including a 39-bp 5'-noncoding region, a 1593-bp open reading frame (ORF), and a 1946-bp 3'-noncoding region. Translation of the ORF revealed that the cDNA encodes a 530-amino acid protein with a calculated molecular mass of 59.8 kilodalton (kDa). This predicted *Xenopus* protein shares over 70% identity with the previously reported vertebrate cortactin and represents unique structures characteristic for cortactin, which include 6.5 tandem repeats of 37 amino acid (we will refer to these as cortactin repeats in this manuscript) at the N-terminal region, a predicted alpha-helical domain, a proline-rich domain, and a C-terminal src homology 3 (SH3) domain (Fig. 1). Thus, we concluded that the cDNA clone encodes a *X*cortactin. It should be noted that careful examination of each repeating unit revealed the unique organization of the cortactin repeats of *X*cortactin. Among the human, mouse, and chicken homologs, each repeating unit of a homolog represents highest homology to the one at the corresponding position of the others. The first, second, and third cortactin repeats of *X*cortactin also represent highest homology to the ones at the corresponding position of the other vertebrate homologs. However, the fourth, fifth, and sixth cortactin repeats of *X*cortactin represent highest homology to the third, fourth, and fifth ones, respectively, of the other vertebrate homologs. The nucleotide sequence of the *X*cortactin cDNA has been submitted to the DDBJ/EMBL/GenBank with accession number AB027611.

Two isoforms of the Xcortactin transcript.

To examine the expression of the *X*cortactin transcript in *Xenopus* tissues, Northern blot analysis was conducted. A transcript of about 3 kb was observed in the brain, lung, heart, liver, skeletal muscle, kidney, and testis (Fig. 2). This is consistent with the result of the mouse cortactin transcript, which is expressed in most tissues other than B lymphocytes or plasma cells (Miglarese *et al.*, 1994). We further analyzed the *X*cortactin transcript by RT-PCR. A set of primers designed to amplify the region encoding the cortactin repeats domain amplified two PCR products (Fig. 3). The larger product was dominantly amplified from all tissues examined. A moderate amount of the smaller product was amplified from lung, heart, and skeletal muscle, but in brain, liver, and ovary, the smaller product was hardly or not detected. Another set of primers, which also cover the cortactin repeats coding region, gave a consistent result (data not shown). Molecular cloning and nucleotide sequencing of the products revealed that the larger product (870-bp) matched the above-described *X*cortactin cDNA. On the other hand, the smaller product missed the 111-nucleotide stretch corresponding to the sixth cortactin repeat, while the other portion matched the cDNA. The smaller product demonstrated an isoform of *X*cortactin with 5.5 cortactin repeats.

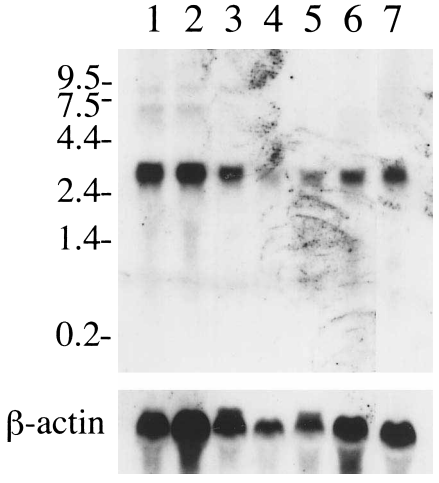

Fig. 2. The *X*cortactin transcript is widely expressed in *Xenopus* tissues. 10 μg total RNA samples from brain (lane 1), lung (lane 2), heart (lane 3), liver (lane 4), skeletal muscle (lane 5), kidney (lane 6), and testis (lane 7) of *Xenopus laevis* were loaded. The blots were hybridised with *X*cotactin (upper panel) or β-actin (lower panel) cDNA probes. Positions of RNA marker are indicated on the left in kb.

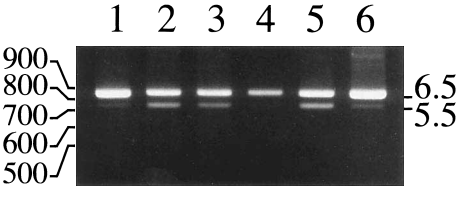

Fig. 3. Two isoforms of the *X*cortactin transcript. RT-PCR was performed to amplify the cortactin repeats coding region of the *X*cortactin transcript. The amplified products from brain (lane 1), lung (lane 2), heart (lane 3), liver (lane 4), skeletal muscle (lane 5), and ovary (lane 6) total RNA were analyzed on 2.5% agarose. Larger band corresponds to the transcript for the isoform with 6.5 cortactin repeats. Smaller band corresponds to the isoform with 5.5 cortactin repeats. No product was amplified when reverse transcriptase was excluded from the reactions (not shown). Positions of size markers in bp are shown at the left.

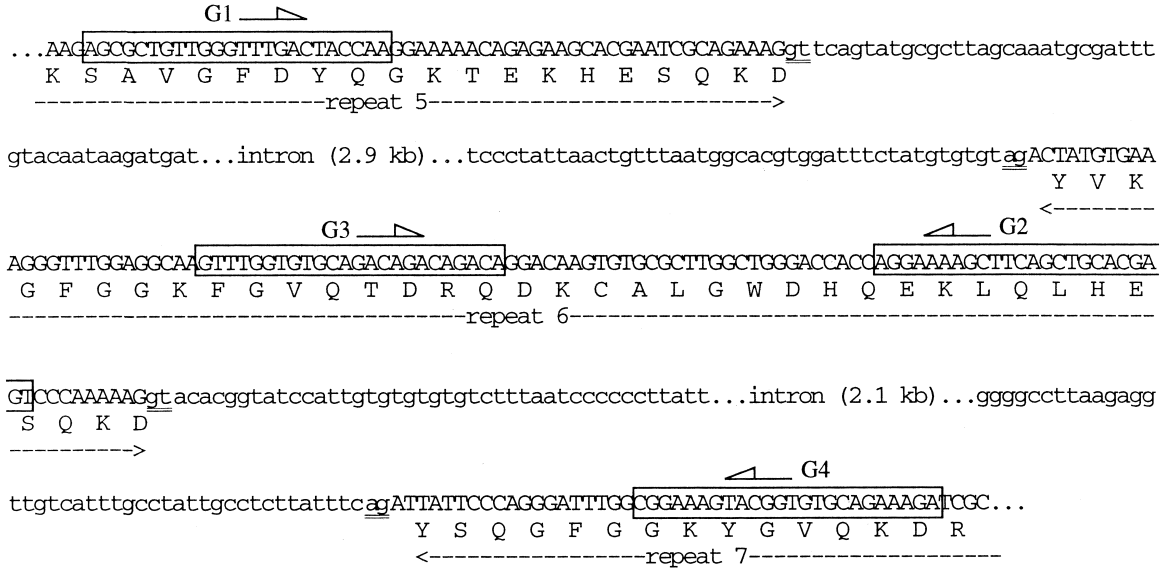

Fig. 4. The sixth cortactin repeat of *X*cortactin is encoded by a single exon. Nucleotide sequences of the sixth repeat coding exon and the 3' or 5' end of its flanking exons are shown with exon-intron junction sequences. Exons are shown by upper case letters and introns are shown by lower case letters. Positions of the oligonucleotide primers for the PCR are boxed. The GT/AG consensus sequences at exon-intron junctions are double-underlined.

Alternative splicing generates Xcortactin isoforms

Isoforms of cortactin, which differ in the cortactin repeats region, were also identified in mouse and rat (Ohoka and Takai, 1998; Wu and Parsons, 1993). Alternative splicing was implicated as a mechanism generating the isoforms. To examine this possibility, we analyzed the exon-intron organization of the *Xcortactin* gene. Introns flanking the sixth cortactin repeat coding sequence were revealed by the PCR using *Xenopus* genomic DNA. A set of primers, targeted to the fifth and sixth cortactin repeat coding regions to amplify their junctional region, amplified a 3.1-kb genome DNA fragment (data not shown). Another set of primers for the junctional region of the sixth and seventh cortactin repeat coding sequences amplified a 2.2-kb genome DNA fragment (data not shown). Nucleotide sequencing of these fragments revealed that the fifth and sixth cortactin repeat coding sequences were intervened by a 2.9-kb intron, and the sixth and seventh cortactin repeat coding sequences were intervened by a 2.1-kb intron (Fig. 4). All exon-intron junctions were well matched with the GT/AG consensus sequence. This result shows that an alternative splicing of the fifth cortactin repeat coding exon to the seventh repeat coding exon generates the cortactin isoform with 5.5 cortactin repeats.

Expression of cortactin in adult *Xenopus* tissues

Western blot analyses against *Xenopus* tissue lysate samples were conducted using antibodies against chicken or *Drosophila* cortactin. The anti-chicken cortactin monoclonal antibody had been shown to cross-react with human, mouse, rat, hamster, and *Drosophila* cortactin, and the anti-*Drosophila* cortactin polyclonal antibody had been shown to cross-react with human and mouse cortactin (Katsube *et al.*, 1998; Wu *et al.*, 1991; and our unpublished data). The result of the anti-chicken cortactin antibody is shown in Fig. 5, and the anti-*Drosophila* cortactin polyclonal antibody yielded the same result (data not shown). Thus, we judged that the bands in Fig. 5 are the *X*cortactin protein. A distinct band of about 85 kDa was observed in all tissues examined. A 90-kDa band was restricted to liver and ovary with immature oocytes. A smear of bands ranging from 65 to 80 kDa was observed in most tissues. Overall, the anti-chicken cortactin antibody reacting molecules were abundant in brain, lung, liver, and testis.

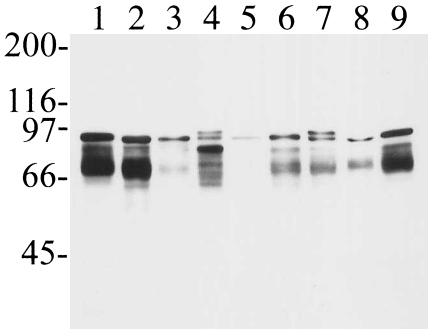

Fig. 5. Western blot analysis of *X*cortactin. 20 μg of total protein samples were resolved by SDS-PAGE as follows: lane 1, brain; lane 2, lung; lane 3, heart; lane 4, liver; lane 5, skeletal muscle; lane 6, kidney; lane 7, ovary with immature oocytes; lane 8, ovary with mature oocytes; lane 9, testis. Western blot was conducted with anti-chicken p80/85 (cortactin) monoclonal antibody. Positions of molecular weight markers are shown at the left in kDa.

DISCUSSION

We isolated a homolog of the cortactin cDNA from *Xenopus laevis*. *X*cortactin showed over 70% identity to human, mouse, and chicken cortactin, and was expressed in all the tissues examined; brain, lung, heart, liver, skeletal muscle, kidney, ovary, and testis. As previously reported for mouse and rat cortactin, *X*cortactin has isoforms which differ in the number of cortactin repeats (Ohoka and Takai, 1998; Wu and Parsons, 1993). As the sixth cortactin repeat, which presents in the isoform with 6.5 cortactin repeats but missed in the isoform with 5.5 cortactin repeats, is encoded by a single exon of 111 bp flanked by 2.9- and 2.1-kb introns, these isoforms must be generated by alternative splicing.

Cortactin was originally designated as p80/85 because the human, mouse, and chicken cortactin proteins represent two bands with molecular masses of 80 and 85 kDa on SDS-PAGE, while their calculated molecular masses are 62.2, 61.0, and 63.3 kDa, respectively (Schuuring *et al*., 1993; Wu *et al*., 1991; Zhan *et al*., 1993). Recently, it was shown that p80 is converted to p85 by a post-translational modification, most probably a protein phosphorylation (van Damme *et al*., 1997). Western blot analysis using the anti-chicken cortactin antibody showed that *X*cortactin has more varied forms than p80/85 (Fig. 5). Most of them showed much larger molecular masses on SDS-PAGE than the calculated molecular masses of the *X*cortactin isoforms with 6.5 or 5.5 cortactin repeats (59.8 and 55.6 kDa, respectively). The profiles of the multiple forms of *X*cortactin differ from tissue to tissue. Relative expression levels of the transcripts for two *X*cortactin isoforms also varied among the tissues examined (Fig. 3). It is likely that the multiple forms of *X*cortactin are due to the post-translational modification of the protein as well as to the alternative splicing of the transcript.

A recent study on rat cortactin isoforms revealed that the isoform with 6.5 cortactin repeats has F-actin cross-linking activity but the isoforms with 5.5 or 4.5 cortactin repeats do not (Ohoka and Takai, 1998). On the other hand, several lines of evidence have shown that the property of cortactin is regulated by phosphorylation. The conversion of p80 to p85, most probably by phosphorylation, is accompanied by a redistribution of cortactin from cytoplasm to cell-matrix contact sites (van Damme *et al*., 1997). The F-actin cross-linking activity of cortactin is down-regulated by Src mediated tyrosine phosphorylation (Huang *et al*., 1997). Substitutions of the tyrosine residues responsible for Src mediated phosphorylation impair cortactin to enhance cell migration, when it is ectopically expressed in an endothelial cell line (Huang *et al*., 1998). We found that these tyrosine residues are conserved in *X*cortactin (represented by asterisks in Fig. 1). Thus, it should be possible that each of the multiple *X*cortactin forms has a different functional property in the tissue specific cytoskeletal regulation.

The dynamic reorganization of the cytoskeleton is essential for the active proliferation and migration of cells during ontogenesis. Especially in the cortical actin network, cortactin should play an important role. The involvement of cortactin has been suggested in osteoclast and megakaryocyte differentiation (Hiura *et al*., 1995; Zhan *et al*., 1997). Investigation of *X*cortactin in embryos utilizing microinjection of synthetic mRNA is currently under progress, and will provide further information on the role of cortactin in development.

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