A Neuromuscularly Enriched Coronin-like Protein, ClipinC, Is a Novel Candidate for an Actin Cytoskeleton-Cortical Membrane-linking Protein*

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Brain-enriched human FC96 protein shows a close sequence similarity to the Dictyostelium actin-binding protein coronin, which has been implicated in cell motility, cytokinesis, and phagocytosis. A phylogenetic tree analysis revealed that FC96 and two other mammalian molecules (p57 and IR10) form a new protein family, the coronin-like protein (Clipin) family; thus hereafter we refer to FC96 as ClipinC. A WD domain and a succeeding α-helical region are conserved among coronin and Clipin family members. ClipinC is predominantly expressed in the brain, and discrete areas in the mouse brain were intensely labeled with anti-ClipinC antibodies. ClipinC was also shown to bind directly to F-actin in vitro. Immunocytochemical analysis revealed that ClipinC accumulated at focal adhesions as well as at neurite tips and stress fibers. Furthermore, ClipinC was associated with vinculin, which is a major component of focal contacts. These results indicate that ClipinC is a component of the cross-bridge between the actin cytoskeleton and the plasma membrane. These findings and the previously reported function of coronin suggest that ClipinC may play specific roles in the reorganization of neuronal actin structure, a change that has been implicated in both cell motility and growth cone advance.

Actin filaments in neuronal cells form a cortical framework that helps to localize membrane proteins, and F-actin dynamics has been implicated in directing neuronal outgrowth. Rearrangement of the actin cytoskeleton occurs in response to various stimuli such as soluble factors or attachment to a substratum (1, 2). The regulation of F-actin patterns involves actin polymerization and actin cross-linking. Factors regulating these processes communicate with the small G proteins of the Rho family (3) and the phosphatidylinositol metabolism system (4), both of which are triggered by extracellular cues through a variety of receptors.

The Dictyostelium actin-binding protein coronin was first purified from an actin-myosin complex and was hypothesized to transmit signals from the membrane receptors to the cortical cytoskeleton (5). Coronin accumulates at the leading edges of moving cells and in crown-shaped extensions on the dorsal cell surface. The involvement of coronin in cell motility, cytokinesis, and phagocytosis, all of which depend on cytoskeletal rearrangement, has been demonstrated by use of a gene replacement mutant. In a mutant that lacks coronin, cell motility is reduced to less than half of the normal speed, and cytoplasmic cleavage in cytokinesis is impaired (6). Further, in the coronin null (cor−) mutant, the rate of yeast uptake is reduced by about 70% (7). However, the distribution of actin filaments in coronin− cells is similar to that in the wild-type ones (8).

In this study, we found a novel candidate for an actin cytoskeleton-cortical membrane linking protein; this protein, ClipinC, is also the third member of a family of mammalian homologs of Dictyostelium coronin. The ClipinC transcript was predominantly expressed in the nervous system. The association of ClipinC with F-actin was demonstrated in vitro. Immunocytochemical analysis of neuronal cells showed that ClipinC accumulated at neurite tips and focal adhesions and along stress fibers. Immunoprecipitation experiments demonstrated that ClipinC was associated with vinculin, which is a cytoskeletal protein implicated in the control of adhesion or motility (8) and is a major constituent of focal adhesions (9). Together with the recent report on the phenotype of a coronin null mutant, the present study indicates that ClipinC may play specific roles in the reorganization of neuronal actin structure, a change that has been implicated in both cell motility during neuronal development and growth cone advance leading to synapse formation.

EXPERIMENTAL PROCEDURES

Cell Culture—SH-SY5Y human neuroblastoma cells were maintained in RPMI medium containing 10% fetal bovine serum. PC12 rat pheochromocytoma cells were grown in RPMI medium containing 10% horse serum and 5% fetal bovine serum. COS-1 and NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Isolation of ClipinC cDNA—An equalized cDNA library was previously constructed from a human forebrain cortex (10). Individual clones from this library were sequenced and compared with sequences in the GenBank database, as described before (11). Thereby 100 unidentified clones were collected, and their tissue specificity was examined by RNA dot blot analysis (11). One brain-enriched clone, FC96, was selected for further study. To obtain the full-length clone of FC96, we screened a human frontal cortex-derived cDNA library (Stratagene) with the 1.0-kilobase EcoRI-Xhol fragment of clone FC96. Eight overlapping cDNAs were obtained, and the nucleotide sequences of these
cDNAs were determined to give rise to the complete coding sequence of FC96, i.e. of ClipinC.

**Northern Blot Analysis**—Human multiple tissue blots I and II (CLONTECH) were hybridized as described before (11) with DNA probes: ClipinA/p57 (nucleotides 489–930) (12), ClipinB/R10 (nucleotides 372–839) (13), and ClipinC (nucleotides 587–1602). The ClipinA and B probes used were the reverse transcription-polymerase chain reaction products from human brain poly(A)* RNA (CLONTECH).

**Antibody Production and Immunohistochemistry**—Rabbits were immunized with the purified recombinant ClipinC protein (amino acids 287–475) expressed as a histidine-tagged form. For immunohistochemistry, brains of mouse embryos and newborns were fixed in ice-cold 5% acetic acid in ethanol. Immunohistochemistry was performed on 8-μm thick microtome sections from paraffin-embedded brains. The sections were pretreated with 3% hydrogen peroxide, washed, and incubated with the polyclonal antibodies against ClipinC at a dilution of 1:5000. After having been washed, the sections were incubated with peroxidase-conjugated anti-rabbit IgG (MBL). The immunocomplexes were visualized in 0.05 μTris-HCl (pH 7.4), 0.1% diaminobenzidine tetrahydrochloride, and 0.1% hydrogen peroxide.

**Cosedimentation Assays**—For actin cosedimentation, skeletal muscle actin (Sigma) was resuspended in actin polymerization buffer (10 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.5 mM dithiothreitol, 0.2 mM ATP, 1 mM MgCl₂, 0.2 mM CaCl₂), [³⁵S]Methionine-labeled FLAG-tagged ClipinC and ClipinB protein (firefly luciferase) were synthesized by coupled transcription and translation by use of a TNT expression system (Promega). The FLAG-tagged [³⁵S]ClipinC was purified by means of anti-FLAG M2 affinity gel (Kodak). In tubes without actin, [³⁵S]ClipinC or luciferase TNT product was diluted in the actin polymerization buffer. In those with actin, the TNT product was added to the resuspended actin (1 mg/ml). Mixtures were incubated for 1 h at 25 °C and then centrifuged for 30 min at 4 °C and 100,000 × g. Supernatants and pellets were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

**Immunocytochemistry**—Cells were fixed in 1% formaldehyde and permeabilized with 0.1% Triton X-100. After having been soaked in phosphate-buffered saline containing 1% bovine serum albumin and 1% normal goat serum, the samples were incubated with the anti-ClipinC antibodies at a dilution of 1:2000 to 1:5000, washed with phosphate-buffered saline, and then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Tago). Staining the same cells for F-actin or vinculin was performed with rhodamine-phalloidin (Molecular Probes) or 5 μg/ml of anti-vinculin monoclonal antibody V284 (Cy- bus Biotechnology) and rhodamine-conjugated sheep anti-mouse IgG (Chemicon), respectively. The samples were then washed with phosphate-buffered saline and examined under a fluorescence microscope (Axiophoto2; Carl Zeiss). For antigen absorption experiments, anti-ClipinC antibody was incubated with the recombinant ClipinC-bound beads for 30 h before cell staining.

**Immunoprecipitation**—Cells were lysed on ice for 1 h in Nonidet P-40 lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (Complete, Roche Molecular Biochemicals). The lysates were then centrifuged for 30 min at 14,000 × g. Immunoprecipitation was done by incubation with the desired primary antibodies and anti-mouse or rabbit IgG-agarose beads (American Qualex) at 4 °C for 12 h. Immune complexes were washed three times with 1% Nonidet P-40 lysis buffer, eluted, and resolved on SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed as described previously (14). For exogenous expression of FLAG-tagged ClipinC, the human ClipinC cDNA with the FLAG peptide tag at the carboxyl terminus was subcloned in the pBS296 vector (15) and transfected into NIH3T3 cells by use of LipofectAMINE Plus (Life Technologies, Inc.).

**RESULTS**

**Isolation of Human ClipinC cDNA**—An equalized cDNA library was previously constructed from human forebrain cortex. Using this library, we obtained and analyzed individual clones to search for novel genes that showed regional expression in the adult brain. In one of this reverse transcriptase-cDNA libraries were collected based on partial DNA sequencing and comparison with a DNA data base, and then their tissue specificity was examined by RNA dot blot analysis. Among these clones, several genes were found to be abundantly expressed in the brain. The sequence of one of the brain-enriched cDNAs, clone FC96, had a remarkable similarity to that of coronin, an actin-binding protein in Dictyostelium discoideum (Fig. 1), and was selected for further investigation.

The full-length FC96 transcript was 3.6 kilobases in size (Fig. 2A) and contained one open reading frame of 1728 base pairs. The open reading frame encoded a putative protein of 475 amino acids with a predicted molecular mass of 54.0 kDa (Fig. 1A). Analysis of the FC96 protein sequence with a protein database disclosed an overall similarity to the Dictyostelium coronin (38.5%) and its mammalian homologs, i.e. p57 (43.9%) (12) and IR10 (61.0%) (13). An amino-terminal domain containing five WD repeats and a succeeding domain covering about 100 amino acids with a tendency to form an a-helical structure were well conserved among these molecules. A phylogenetic tree analysis revealed that p57, IR10, and FC96 form a new protein family, the Clipin (coronin-like protein) family (Fig. 1B); thus hereafter we refer to p57, IR10, and FC96 as Clpins A, B, and C, respectively.

**ClipinC Is Predominantly Expressed in Brain**—We examined the expression of ClipinC mRNA in various human adult tissues and compared this expression with that of Clpins A and B (Fig. 2A). The level of theClipinC mRNA was extremely high in the brain, moderate in heart and ovary, and very low or undetectable in the other tissues examined in this study. As previously reported (12), the ClipinA transcript was mainly detected in immune system tissues, i.e. spleen, thymus, and peripheral lymph nodes. A high level expression of ClipinB was restricted to some tissues, e.g. colon, prostate, and testis, in which ClipinA and C transcripts were nearly undetectable. It is interesting to note that the expression profiles of Clipin members were tissue-specific and almost mutually exclusive.

Northern blot analysis showed that ClipinC mRNA was preferentially expressed in brain tissue. We confirmed this point at
ClipinC is predominantly expressed in brain. A, tissue distribution of human Clipin family members. Northern blot analysis of ClipinA (top), ClipinB (middle), and ClipinC (bottom) mRNAs from various adult human tissues. B–D, immunohistochemical staining for ClipinC in parasagittal (B) and coronal (C) sections of P1 mouse brain and in a frontal coronal section of E16 mouse embryos (D). Ob, olfactory bulb; Ctx, cerebral cortex; Hp, hippocampus; Th, thalamus; Cb, cerebellum; Re, retina.

The protein level by using polyclonal antibodies against ClipinC (data not shown). The antibodies specifically detected ClipinC protein in human and rat brains and neuronal cell lines of peripheral origin, such as SH-SY5Y and PC12 cells. ClipinC protein was undetected in the other tissues and non-neuronal cells examined.

We next examined the distribution of ClipinC protein in various brain regions by using the ClipinC-selective antibodies (Fig. 2, B–D). ClipinC immunostaining was detected in discrete areas in the mouse brain. In the P1 brain, immunoreactivity was observed in the cerebral cortex, hippocampus, thalamus, olfactory bulb, and cerebellum (Fig. 2, B and C). In the cerebellum, the Purkinje cell layer was intensely labeled; but no immunoreactivity was detected in the molecular layer or granule cell layer (Fig. 2B). Intense immunoreactivity was also observed in the inner nuclear (neuroblastastic) layer in the retina and in the olfactory bulb of the mouse embryo (Fig. 2D).

In Vitro Binding of ClipinC to F-actin—The close sequence similarity between ClipinC and coronin (Fig. 1) suggested that ClipinC is also an actin-binding protein; thus binding of ClipinC to actin filaments was investigated by spin-down experiments. Full-length ClipinC was prepared in vitro, added to the actin polymerization buffer, and incubated in the absence or presence of actin. Thermally, macroaggregates were isolated by centrifugation. Fig. 3A showed that ClipinC cosedimented with F-actin. In control experiments, we also examined the cosedimentation property of firefly luciferase protein, which was in the soluble fraction even in the presence of actin (Fig. 3B). This confirmed that the ClipinC interaction with actin macroaggregates is selective.

Immunofluorescence Localization of ClipinC in Focal Adhesions, Stress Fibers, and Neurite Tips in Neuronal Cells—The anti-ClipinC antibodies were used for immunocytochemical studies of neuronal cells to assess the subcellular localization of ClipinC. Shown in Fig. 4 A and C are various shapes of SH-SY5Y human neuroblastoma cells stained for ClipinC. The site of localization of ClipinC in flattened SH-SY5Y cells was clarified by double-labeling of ClipinC (Fig. 4A) and F-actin (Fig. 4B). Staining for both ClipinC and F-actin was most intense at the focal contacts (arrowhead) and stress fibers (arrows). The accumulation of ClipinC at the focal adhesion, i.e., cross-bridge between the actin cytoskeleton and the substrate-adherent plasma membrane, was confirmed by double-staining for ClipinC (Fig. 4C) and vinculin, a major constituent of focal adhesive complexes (Fig. 4D). The data suggest that ClipinC is a component of the cross-bridge between actin filaments and the cortical membrane. Nuclear staining in SH-SY5Y cells stained for ClipinC (Fig. 4, A and C) was shown to be a nonspecific artifact by antigen absorption experiments (Fig. 4F). In Fig. 4 (E and F), nerve growth factor-treated PC12 cells showing neurite outgrowth were stained for ClipinC. ClipinC remarkably accumulated at the tips of the neurites (arrow). Note that ClipinC was also abundant at the protrusions in the cellular periphery (arrowheads in Fig. 4E). In addition to the apparent accumulation of ClipinC at neurite tips, a considerable amount of ClipinC was dispersed in the cell body (Fig. 4G); this cytoplasmic distribution of ClipinC overlapped with that of F-actin (Fig. 4H). These data fit well with the previous observation that Dictyostelium coronin is reversibly recruited from the cytoplasm and is incorporated into the actin network of leading edges of the slime mold (16).

Physiological Interaction between ClipinC and Vinculin—The accumulation of ClipinC at focal adhesions was an unexpected result, and thus we tried to identify a focal adhesive protein(s) that specifically binds to ClipinC. [35S]Methionine-labeled SH-SY5Y cells were lysed and subjected to immunoprecipitation with the anti-ClipinC antibodies. We observed the
binding of several proteins to ClipinC; one of them had an approximate molecular mass of 120 kDa (data not shown). Vinculin, which was used as a marker of focal contacts in Fig. 4D, is a cytoskeletal protein of 117 kDa; thus we examined whether ClipinC could interact with vinculin in a physiological complex. In Fig. 5A, the ClipinC-selective antibodies coprecipitated vinculin from SH-SY5Y cells. The specificity of the immunoprecipitation was demonstrated by an antigen absorption experiment: the amount of vinculin coprecipitated with the antigen-absorbed anti-ClipinC antibodies (Fig. 5A, lane 2) was significantly reduced from that of the vinculin coprecipitated with the untreated antibodies (Fig. 5A, lane 1). Conversely, the anti-vinculin antibody coprecipitated ClipinC from SH-SY5Y cells (Fig. 5B, lane 1), and the corresponding band of about 54 kDa was absent from the anti-vinculin immunoprecipitate of the COS-1 lysate containing no ClipinC protein (Fig. 5B, lane 2). The FLAG-tagged ClipinC exogenously expressed in NIH3T3 cells (Fig. 5C, lane 1) was also coprecipitated with the anti-vinculin antibody (Fig. 5C, lane 2). The specificity was confirmed by a similar experiment in mock-transfected cells (Fig. 5C, lane 3). In NIH3T3 cells, the exogenously expressed ClipinC accumulated at focal adhesions in addition to being associated with stress fibers (data not shown), as it did in SH-SY5Y cells (Fig. 4, A–D). These results indicate that some amount of ClipinC was present in complexes with vinculin in focal adhesive structures.

$\text{FIG. 5.} \quad \text{Physiological interaction between ClipinC and vinculin.} \ A, \ \text{SH-SY5Y cells were immunoprecipitated with the untreated (lane 1) or the antigen-absorbed (lane 2) anti-ClipinC antibodies. The immunoprecipitates were then immunoblotted with the anti-vinculin monoclonal antibody.} \ B, \ \text{SH-SY5Y cells (lane 1) and COS-1 cells (lane 2) were immunoprecipitated by the anti-vinculin antibody. The immunoprecipitates were probed with the anti-ClipinC antibodies.} \ C, \ \text{NIH3T3 cells transfected with FLAG-tagged ClipinC cDNA (lane 2) or the empty vector (lane 3) were immunoprecipitated with the anti-vinculin antibody, followed by the immunoblot analysis with the anti-FLAG M2 monoclonal antibody. The whole lysate of the FLAG-tagged ClipinC-transfected cells was immunoblotted with the anti-FLAG antibody (lane 1).}$
G proteins has been considered. Mutant is an intriguing possibility. Axonal growth in PC12 cells (8). The involvement of ClipinC in shown to cause loss of growth cone stability and to reduce extracellular cues. In particular, the reduction of vinculin was indicated vinculin, talin, and paxillin (8, 20) were observed following regulation of some components of focal adhesive complexes including vinculin, a cytoskeletal protein that is a major component of focal contacts (9) and is implicated in the control of growth cone motility (8). These data suggest that ClipinC may play specific roles in the reorganization of neuronal actin structure.

In coronin null mutants, cell locomotion, chemotaxis, and phagocytosis are slowed down, and cytokinesis is impaired (6, 7); these defects of cor− mutants strongly suggest that coronin plays a regulatory role in actin reorganization. We expect that Clipin members potentially share this regulatory role with coronin based on the close conservation of their structure (Fig. 1) and shared capability of actin binding (Fig. 3 and Refs. 5, 12).

A recent study on Dictyostelium cells (16) has provided two important clues for clarifying how coronin plays a regulatory role in F-actin dynamics: (i) In chemoattractant-stimulated cells, the temporal relationship between the coronin-green fluorescent protein accumulation and the appearance of a protrusion at a cell front (i.e., local actin rearrangement) was examined. Although the local accumulation of coronin-green fluorescent protein was seen 7 s after a protrusion became detectable on average, coronin-green fluorescent protein accumulation could precede the protrusion by 5 s at most (16). Thus coronin accumulation is not merely controlled by binding to a newly polymerized actin but may be regulated itself, at least in part. (ii) In coronin null mutants, the extended organelle-free zone, which appeared as a hyaline area, was formed at the front region, and more importantly, treatment with cytochalasin A, an actin-depolymerizing agent, partially rescued the wild-type phenotype (16).

A third clue for the function of coronin/Clipsins is provided by our present finding that ClipinC becomes accumulated at focal contacts. Furthermore, ClipinC was shown to bind to vinculin, which occurs in multimolecular complexes at focal adhesions. In collaboration with other cytoskeleton-membrane linking proteins, the accumulated Clipsins/coronin at the cell front may possibly construct the molecular machinery that enables the movement of organelles into the front region. A similar mechanism elicited by ClipinC could be considered at neurite tips and focal contacts and along stress fibers. ClipinC was shown to interact with vinculin, a cytoskeletal protein that is a major component of focal contacts (9) and is implicated in the control of growth cone motility (8). These data suggest that ClipinC may play specific roles in the reorganization of neuronal actin structure.

In coronin null mutants, cell locomotion, chemotaxis, and phagocytosis are slowed down, and cytokinesis is impaired (6, 7); these defects of cor− mutants strongly suggest that coronin plays a regulatory role in actin reorganization. We expect that Clipin members potentially share this regulatory role with coronin based on the close conservation of their structure (Fig. 1) and shared capability of actin binding (Fig. 3 and Refs. 5, 12, and 13). The WD repeat motif is thought to be capable of undergoing pairwise or multimeric interactions (27). The β subunits of G proteins, the best known proteins with WD repeats, act in signal transduction by forming multiprotein complexes through such repeats (28–30). Therefore, Gerisch et al. (16) suggested that coronin binds not only to actin but also to other proteins and in this way couples regulatory proteins to the actin-myosin system; the same suggestion applies to the Clipin family molecules. The presumed α-helical domain was suggested to be important for actin binding (5). In a ClipinC deletion mutant containing a WD domain only, in vitro F-actin binding was reduced to one-third of that by the intact ClipinC (data not shown). This may indicate the importance of ClipinC’s internal α-helical domain for actin-binding, although the residual WD repeats can weakly associate with F-actin.

Attention should be paid to the tissue-specific expressions of Clipin members, for their expression profiles were almost mutually exclusive. In marked contrast, the majority of cytoskeleton-membrane linking proteins, e.g., vinculin, paxillin, and ERMs (ezrin/radixin/moesin) family proteins, are ubiquitously expressed (9, 14). Further, the expressions of the three members of the ERM family overlap nearly completely. It is an interesting possibility that the differential expressions of Clipin members may reflect a member-specific function in addition to the shared role in regulating actin organization. A recently reported interaction between p57/ClipinA and p40phox, a cytosolic component of the NADPH oxidase that generates microbialid superoxide in phagocytes, may be related to the ClipinA-specific function (31).

Another exceptional characteristic of ClipinC as an actin-binding protein at the periphery of neuronal cells is that the protein was also detected along stress fibers in flattened SH-SY5Y cells (Fig. 4A), because GAP-43 (32), MARCKS (33), and ERM family proteins (34), typical actin-regulating proteins in the growth cone, are known to be found at nerve terminals only. If ClipinC at the cell front functions as a connector between the actin cytoskeleton and the cortical membrane, ClipinC along stress fibers is unlikely to have the same function. Thus we may have to consider another role for ClipinC (or other Clipin members) residing along stress fibers. In fact, a homolog of coronin in yeast was recently reported to modulate actin filament assembly (35).

Guided neuronal migration in development and growth cone motility leading to neuronal plasticity are both controlled by cytoskeletal dynamics in neurons (36, 37). In this respect, functional linkage between the intracellular cytoskeleton and extracellular substrates is a particularly important theme. As is important in other tissues (9), cytoskeleton-cortical membrane linking complexes play a fundamental role in this process at the periphery of neurons. In the developing neocortical inter-
mediate zone, the distribution of tangentially migrating neurons overlapped with that of intense immunoreactivity of ClipinC in neocortex (Fig. 2C); this may mean the involvement of ClipinC in the tangential cell migration in this area. Furthermore, we found that the overexpressed ClipinC had an effect on the cell attachment to the substrate. Our results provide a strong indication that ClipinC, a possible candidate for a cytoskeleton-membrane connector, is implicated in the control of cell adhesions and cell movements in neuronal cells.

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