Microtubule Actin Cross-linking Factor (MACF): A Hybrid of Dystonin and Dystrophin that Can Interact with the Actin and Microtubule Cytoskeletons

Conrad L. Leung, Dongming Sun, Min Zheng, David R. Knowles, and Ronald K.H. Liem
Department of Pathology and Department of Anatomy and Cell Biology, Columbia University College of Physicians and Surgeons, New York, New York 10032

Abstract. We cloned and characterized a full-length cDNA of mouse actin cross-linking family 7 (mACF7) by sequential rapid amplification of cDNA ends–PCR. The completed mACF7 cDNA is 17 kb and codes for a 608-kD protein. The closest relative of mACF7 is the Drosophila protein Kakapo, which shares similar architecture with mACF7. mACF7 contains a putative actin-binding domain and a plakin-like domain that are highly homologous to dystonin (BPAG1-n) at its NH$_2$ terminus. However, unlike dystonin, mACF7 does not contain a coiled–coil rod domain; instead, the rod domain of mACF7 is made up of 23 dystrophin-like spectrin repeats. At its COOH terminus, mACF7 contains two putative EF-hand calcium-binding motifs and a segment homologous to the growth arrest–specific protein, Gas2. In this paper, we demonstrate that the NH$_2$-terminal actin-binding domain of mACF7 is functional both in vivo and in vitro. More importantly, we found that the COOH-terminal domain of mACF7 interacts with and stabilizes microtubules. In transfected cells full-length mACF7 can associate not only with actin but also with microtubules. Hence, we suggest a modified name: MACF (microtubule actin cross-linking factor). The properties of MACF are consistent with the observation that mutations in kakapo cause disorganization of microtubules in epidermal muscle attachment cells and some sensory neurons.

Key words: actin cross-linking family 7 (ACF7) • plakin • actin • microtubule • cytoskeleton

The cytoskeleton of most eukaryotic cells consists of three types of filamentous networks: microfilaments (MFs), intermediate filaments (IFs), and microtubules (MTs). The cytoskeleton provides mechanical strength to the cell and controls many other cellular events, such as cell division, intracellular trafficking, and locomotion. The organization of these filaments is strongly dependent on their associated proteins. Thus, the identification of proteins that could associate with different types of filaments is of great importance. Several proteins belonging to the plakin family have been shown to connect cytoskeletal elements to each other and to the junctional complexes at the plasma membrane (Uitto et al., 1996; Uhrberg and Watt, 1997; Wiche, 1998). So far, five plakin family members have been identified: desmoplakin, plectin, bullous pemphigoid antigen 1 (BPAG1), envoplakin, and periplakin (Green et al., 1992; Uhrberg and Watt, 1997; Uhrberg et al., 1997). A typical plakin exhibits a three-domain structure, consisting of a central $\alpha$-helical rod domain flanked by globular NH$_2$-terminal head and COOH-terminal tail domains (Uhrberg and Watt, 1997). The rod domain contains stretches of heptad repeats that mediate coiled–coil dimer formation. The NH$_2$-terminal head domain consists of six $\alpha$-helical segments (NN, Z, Y, X, W, and V) that are conserved among plakins (Green et al., 1990; Uhrberg et al., 1996; Uhrberg and Watt, 1997). These segments are organized into an antiparallel helical bundle and probably function as plaque to plate association domains. Except for periplakin, the COOH-terminal tail domains are made up of a variable number of homologous subdomains that interact directly with IFs (Green et al., 1990; Uhrberg et al., 1996, 1997). As a result, plakins are believed to play an important role in anchoring IFs to the junctional complexes.

Among the fully characterized plakins, only plectin and...
two of the neuronal isoforms of BPA G1 (BPA G1-n, also
known as dystonin) contain putative actin-binding domains
(A B D s) at their NH 2 terminals (Wiche et al., 1991; Brown et
al., 1995b). Plectin is an extremely large (>500 kD) cytoskeletal
protein and was originally purified as a major component
of IF extracts from a variety of cultured cells (Pytela and Wiche,
1980). A side from directly interacting with IFs, plectin also appears to associate with MFs and
MTs, making it the most versatile cytoskeletal linker
protein (for review see Wiche, 1998). Dystonin/BPA G1-n was
discovered from the analysis of BPA G1 knockout mice (Guo et
al., 1995; Yang et al., 1996) and was identified as the muta
ted gene product of natural mouse mutants with the neu-
rological disorder, dystonia musculorum (dt) (Brown et
al., 1995a). Mice carrying mutations in the BPA G1 gene suffer
from severe degeneration of primary sensory neurons and
exhibit abnormal accumulations of IFs in axons (Duchen
Gregory and Brown, 1998; Prokop et al., 1998; Strumpf
and Volk, 1998). Based on the morphological studies of the
Drosophila kakapo mutants, Kakapo was proposed to be
the putative actin binding domain associated with and stabilizes MTs. Furthermore,
transfected full-length ACF7 can associate with actin and
MTs, suggesting that ACF7 may be a novel cytoskeletal
linker protein.

Materials and Methods
cDNA Cloning
5' and 3' rapid amplification of cDNA ends (RACE)-PCR were
performed on adapter-ligated mouse (BALB/c) brain Marathon-Ready™
cDNA (Clontech), and long-range PCR was done on regular mouse
(BALB/c) brain QUICK-Clone™ cDNA (Clontech) using a dvantage®
cDNA PCR kit (Clontech). All procedures were carried out according to
the manufacturer's protocols PT1156-1, PT1150-1, and PT-1580-1 (Clon-
tech). In general, gene-specific 28-nucleotide primers were designed with
high guanine cytosine contents (50-70%) and melting temperature
>70°C. The following cycling parameters were employed for RACE-
PCR: an initial denaturing step of 94°C for 30 s, followed by 5 cycles of
94°C (5 s) and 72°C (4 min), 5 cycles of 94°C (5 s) and 70°C (4 min), 25
cycles of 94°C (5 s) and 68°C (4 min), and a final extension step of 68°C for 5
min. PCR products were cloned into pGEM-T vector (Promega) or
pcR 2.1-TOPO vector (Invitrogen) for sequencing. Most of the cDNA
clones were sequenced using the BigDye™ sequencing kit (Applied Bio-
systems) and processed by Perkin Elmer/A pplied Biosystems M odel A B I
377A Sequencers (D NA F acilities, Columbia U niversity, New Y ork). In
some cases, cDNA clones were also sequenced manually with SequeSe
2.0 (U.S. B iochemical Corp.).

Plasmid Construction
pTOPO-ACF7-5A, pTOPO-ACF7-5B, pTOPO-ACF7-red, and pTOPO-
ACF7-3A were generated by cloning the long-range PCR products into
pcR 2.1-TOPO vector or pcR-XL-TOPO vector (Invitrogen). To engineer
pFLAG-ABD, the 0.8-kb PCR fragment of pTOPO-ACF7-5A using
sense primer 5'-A AC CAG AAT TCT GTG CTTG A CCGT C-3' and
antisense primer 5'-T AA A G T CTC GGT T CCA A CCA G G-3' was
ligated into EcoRI/XhoI digested pcDNA-FLAG vector (Leung et al., 1999). Similarly, pGEM-mACF7-C was
cloned by generating the PCR fragment of pTOPO-ACF7-3A using sense
primer 5'-CA T G G A GA T T CCG C A G T G T A G-3' and
antisense primer 5'-T AT C G T T G G A C T GC T C G G G-3' into pGEM-T vec-
tor (Promega). The 1.3-kb EcoRI-NotI fragment of pGEM-mACF7-C was
then ligated into the EcoRI/NotI digested pcDNA-FLAG vector to
make pFLAG-mACF7-C. pGEM-GAR7 was created by cloning the PCR
fragment of pTOPO-ACF7-3A using sense primer 5'-C A A C A G A A T-
C C A T G C C A A C C -3' and antisense primer 5'-T T A T C G C T T G-
G A C T G C T C G G G-3' into pGEM-T vector (Promega). The 0.85-kb EcoRI-NotI fragment of pGEM-GAR7 was then cloned into
pcDNA-FLAG vector to generate pFLAG-GAR7. To construct pFLAG-
mACF7-mini, three-piece ligation was performed with 2.5-kb NotI col
fragment of pTOPO-ACF7-5A, 0.85-kb NcoI-hol fragment of pFLAG-
GAR7, and 5.4-kb NotI/hol-digested pcDNA 3 vector (Invitrogen). To
create a FLAG-tagged ACF7-3A clone, the stop codon on ACF7-3A was
first removed by PCR and the resulting fragment was ligated inframe to

A partial human actin cross-linking family 7 (ACF7) cDNA was originally isolated by screening for genes that
encode proteins with A B D s homologous to that of dystro-
phin (Byers et al., 1995). A analysis of a longer mouse ACF7
(mACF7) cDNA revealed that it shares more sequence homology and isoform diversity with dystonin/BPA G1-n
(Bernier et al., 1996), suggesting that mACF7 may repre-
sent a novel plakin/cytolinker. Three isoforms of ACF7
have been partially characterized in mice; two of them
contain putative A B D s (Bernier et al., 1996). Here, we
characterize a full-length mACF7 cDNA, and define its in-
teraction partners. Sequence analysis shows that ACF7 is a
hybrid of dystonin and dystrophin and is the mammalian homologue of Drosophila protein, Kakapo. Kakapo is a
cytoskeletal protein that is essential for neuronal growth and
adhesion between and within cell layers in Drosophila
(Gregory and Brown, 1998; Prokop et al., 1998; Strumpf
and Volk, 1998). Based on the morphological studies of the
Drosophila kakapo mutants, Kakapo was proposed to be
an MT actin organizer. In agreement with this notion,
we show that the A B D of ACF7 is functional in interact-
ing with actin filaments, whereas the COOH-terminal do-
main associates with and stabilizes MTs. Furthermore,
transfected full-length ACF7 can associate with actin and
MTs, suggesting that ACF7 may be a novel cytoskeletal
linker protein.
a FLAG-epitope tag coding sequence. Clones ACF-5A, ACF-rod, and FLAG-tagged ACF-3A were sequentially ligated together using the unique restriction endonuclease recognition sites KpnI and SalI at cDNA position 3665 and 11728, respectively, to generate a full-length FLAG-tagged ACF7 cDNA, which was subcloned into a multiple cloning site-modified eukaryotic expression vector pCI (Promega) to construct pFLAG-mACF7-fl (Fig. 1A).

### In Situ Hybridization

All studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Timed pregnant mouse C57BL/6 embryonic day 14.5 embryos were isolated, freshly frozen, and embedded directly in the OCT compound (Sakura Finetek, Inc.). Both sagittal and transverse cryostat sections were prepared. 35S-labeled cRNA transcripts were synthesized in vitro from pGEM-GARt using Riboprobe Gemini Systems (Promega). Both sense and antisense [35S]UTP-labeled cRNA were prepared. cRNA probes were purified on Sephadex G-50 columns (Boehringer Mannheim). In situ hybridization was performed as described previously (Zheng et al., 1998). A fter the nuclear emulsion autoradiography was performed, slides were examined in Leitz microscopes under both bright field and dark field illuminations. Hybridization with control (sense) probes yielded only low background staining in all cases.

### Transient Transfections and Indirect Immunofluorescence Microscopy

COS-7 cells were cultured at 37°C with 5% CO₂ in DMEM (Life Technologies, Inc.) supplemented with 10% FBS. Transient transfections for immunofluorescent analysis were performed on 18-mm coverslips using GenePORTER™ transfection reagents (Gene Therapy System) or LipofectAMINE PLUS reagents (Life Technologies, Inc.). 48 h after transfection, coverslips with adherent cells were fixed in cold methanol at −20°C or 4% paraformaldehyde in PBS at room temperature. To optimize stress fiber staining, fixed cells were permeabilized with 0.1% Triton X-100 for 5 min before further processing. A fter rinsing several times with PBS, cells on coverslips were blocked with 5% normal goat serum and incubated with primary antibodies at room temperature for 1 h. The primary antibody–treated cells were then washed with PBS and incubated with appropriate secondary antibodies or rhodamine-conjugated phalloidin (Sigma Chemical Co.) for 30 min. Subsequently, the coverslips were washed with PBS and mounted onto slides with a quambound (Lerner Laboratories) for indirect immunofluorescent microscopy. The following primary antibodies were used for immunostaining: mouse monoclonal anti-FLAG M2 antibody (IBI-Kodak); rabbit polyclonal anti-Glu- and anti-Tyr-tubulin antibodies; rat monoclonal anti-Tyr-tubulin Y12/2 antibodies (a gift from Dr. G Gregg G. Uden, Columbia University, New York, NY).

### In Vitro Binding Assays

The STP3 system (Novagen) was used to synthesize [35S]methionine-labeled proteins from the pcDNA-FLAG constructs that contain T7 RNA polymerase promoters. In vitro synthesized proteins were prespun before they were used for the binding assays. The actin-binding assays were performed with the Non-muscle Actin Binding Protein Spin-Down Biochem Kit (Cytoskeleton, Inc.). In each testing, 20 μl of crude [35S]labeled protein was incubated with 0.23 nmol of polymerized F-actin at room temperature for 30 min. After centrifugation for 90 min at 65,000 rpm, supernatants and pellets were collected for SDS-PAGE analysis. After centrifugation for 90 min at 65,000 rpm, supernatants and pellets were collected for SDS-PAGE analysis. After centrifugation for 90 min at 65,000 rpm, supernatants and pellets were collected for SDS-PAGE analysis. The following primary antibodies were used for immunodetection: mouse monoclonal anti-FLAG M2 antibody; rabbit polyclonal anti-Glu- and anti-Tyr-tubulin antibodies; rat monoclonal anti-Tyr-tubulin Y12/2 antibodies (a gift from Dr. G Gregg G. Uden, Columbia University, New York, NY).

### Results

Isolation of mACF7 cDNA Clones

The mRNA transcripts of mACF7 were estimated to be 14–18 kb, of which only 6 kb had been characterized previously (Bernier et al., 1996). Using a RACE-PCR-based method, we successfully isolated consecutively overlap-
ping clones of the remaining cDNA (Fig. 1 A). The 5' RACE product contained a putative start codon (ATG) that matched the Kozak consensus (Kozak, 1986). Moreover, an intramere stop codon was located 102 bp upstream of this ATG, making it the most likely translational start site. By sequentially performing 3' RACE-PCR with primers specific for the end sequence of the previous RACE products, we obtained 10 overlapping cDNA clones that spanned ~11 kb in length before reaching a polyadenylation signal followed by a polyA stretch. In addition, we also performed long-range PCR to obtain longer cDNA clones for more detailed sequence analysis. The composite cDNA was about 17.3 kb (sequence data available from EMBL/GenBank/DDBJ under accession no. A F 150755), and the longest open reading frame encoded a 5,327-amino acid polypeptide with a calculated molecular mass of 608 kD (Fig. 1 B).

Sequence Analysis of mACF7
The deduced amino acid sequence of mACF7 was used to search for homologous proteins in the GenBank database. The closest relative of mACF7 is the Drosophila protein Kakapo. mACF7 and Kakapo not only share homology in primary sequence but also the overall protein architecture, indicating that mACF7 is the mouse homologue of Kakapo. As described in the previous study (Bernier et al., 1996), mACF7 contains a dystonin/BPA1-G1 homologous NH2-terminal region, which includes a calponin homology ABD and a globular plakin-like domain. Interestingly, following the NH2-terminal domains, the sequences of mACF7 and dystonin start to diverge. Sequence comparison of mACF7 with dystrophin revealed that the central region of mACF7 would fold into 23 spectrin repeats (Fig. 1 B). By analogy to β-spectrin, this collection of spectrin repeats would also constitute the rod domain (not a coiled-coil rod) of mACF7. Following the rod domain, two calmodulin-like, EF-hand calcium-binding motifs were identified (Fig. 2 A). Together, these features suggest that mACF7 is a new member of the spectrin superfamily with plakin-like features.

A partial human brain cDNA clone, KIAA 0465 was found showing >80% sequence identity to mACF7 COOH terminus. A according to the UniGene database, the gene coded for KIAA 0465 is located on chromosome 1, between markers D1S2843 and D1S417. In mice, the mACF7 gene was mapped to chromosome 4, close to the marker D4mit11 (Bernier et al., 1996). Based on the chromosome synteny and the extensive sequence homology, the partial KIAA 0465 cDNA should encode the human orthologue of mACF7. In addition, a short stretch of the mACF7 COOH terminus displays significant homology to a portion of the recently identified protein, GA R22 (Gas2 related on chromosome 22) (Fig. 2 B). This part of GA R22 is also closely related to the Gas2 protein (growth arrest-specific 2 protein) (Zucman-Rossi et al., 1996; Collavin et al., 1998). Because of this similarity, we designated this region of mACF7 as the GAR region. A schematic structure of mACF7 is depicted in Fig. 2 C. To study the expression pattern of mACF7, mouse embryos at embryonic day 14.5 were hybridized with a mACF7 ribonucleotide probe. As illustrated in Fig. 3, mACF7 was ubiquitously expressed in all tissues, with higher levels in the nervous system, muscle, lung, heart, and adrenal glands.

Association of mACF7 with MFs In Vivo
Full-length Gas2 was shown previously to be a component of the MF network, although the interaction domain had not been fully characterized (Brancolini et al., 1992; Collavin et al., 1998). Therefore, we examined possible interactions between the COOH-terminal domain of mACF7 (mACF7-C) and MFs by transient transfection assays. In addition, we also analyzed the actin-binding properties of...
the putative NH₂-terminal ABD of mACF7. To facilitate the detection of the truncated proteins, the NH₂ termini of mACF7-C and ABD were fused to FLAG epitope tags, which also provided the translational start codons. Transient transfections were performed on COS-7 cells. As shown in Fig. 4, A and B, overexpressed ABD protein colocalized with filamentous actin in stress fibers and membrane ruffles of transfected cells, confirming the interaction of this highly conserved domain with MFs. In contrast, the mA CF7-C protein, displayed a filamentous staining pattern that exhibited no significant correlation with the actin network (Fig. 4, C and D). Therefore, we compared this staining pattern with that of the other two cytoskeletal networks, MTs and IFs, and found that mACF7-C proteins codistributed with MTs (Fig. 5), but not with vimentin (data not shown).

Association of mACF7 with MTs In Vivo

Within each cell there are dynamic and stable MTs. Stable MTs are a small subset of dynamic MTs and are thought to be selectively generated from dynamic MTs. These stable long-lived MTs accumulate a posttranslationally modified form of tubulins known as detyrosinated or Glu-tubulins. These MTs are distinct from their dynamic counterparts that contain predominantly tyrosinated tubulin (Tyr-tubulin). In transfected cells, the overexpressed mA CF7-C proteins colocalized with many but not all Tyr-MTs (Fig. 5, A and B). Interestingly, mACF7-C proteins (Fig. 5, C and D) decorated all the Glu MTs. The dynamic Tyr MTs at the periphery of the cell did not appear to colocalize with mACF7, although it is possible that the bound mA CF7 was present in low amounts too scarce to be detected. As expected, the ABD of mA CF7 did not associate with MTs (Fig. 5, E and F). Since long MT-forming whorls were frequently observed in the transfected cells, we considered the possibility that mA CF7-C proteins might not only bind to, but also stabilize MTs. To explore this possibility, cells

Figure 3. Expression of ACF7 mRNAs in the mouse embryo. Sagittal sections (A–C) and transverse sections (D–G) of an embryonic day 14.5 mouse embryo were hybridized with mA CF7 antisense probe. Relative planes of sectioning for sections shown in D–G are illustrated on a schematic drawing of the embryo. In addition to ubiquitous expression of mA CF7 mRNA throughout the embryo, high levels of expression are observed in the brain and spinal cord. In the brain, both ependymal layer (B, arrow) and mantle layer are heavily labeled. In the peripheral tissue, an intermediate to high level of ACF7 expression is observed in the dorsal root ganglia, olfactory epithelium (B, arrowhead), intrinsic muscle of the tongue, bronchial epithelium, and mesenchyme of the lung (B, arrows), skeletal muscle of the trunk (E, arrow), myocardium (F, arrow), and adrenal glands (G, arrow). Bars: 1 mm (A, B, D–G); and 0.1 mm (C).

Figure 4. Association of ACF7 ABD with MFs. COS-7 cells from transient transfections of pFLAG-ABD (A and B) or pFLAG-mACF7-C (C and D) were double-labeled with monoclonal anti-FLAG M2 antibody (A and C) and rhodamine-conjugated phalloidin (B and D). The FLAG-tagged ABD proteins colocalized perfectly with filamentous actin in stress fibers and membrane ruffles of the transfected cells. A filamentous staining pattern was obtained for FLAG-tagged mA CF7-C proteins, yet it did not exhibit any correlation with actin structures. Bar, 20 μm.
transfected with mA CF7-C cDNA were treated with the MT depolymerization agent, nocodazole (10 μM) for 1.5 h before being fixed for immunofluorescence microscopy. These conditions have been shown to be sufficient to cause the complete depolymerization of the endogenous MTs (Khawaja et al., 1988). In contrast to cells without mA CF7-C protein, the MT networks of the transfected cells remained intact and were decorated with mA CF7-C proteins (Fig. 6, A-D), implying that the COOH-terminal domain of mA CF7 can associate with and stabilize MTs. In similar assays, the ABD of mA CF7 still only associated with the actin network (Fig. 6, E and F).

**Interaction of mA CF7 with MTs and MFs In Vitro**

In vitro spin-down binding assays were carried out to ascertain interactions between ABD and actin filaments, and between mA CF7-C and MTs. 35S-labeled proteins were synthesized in vitro and incubated with polymerized actin and tubulin before centrifugation. The bound proteins in the spin-down pellets were resolved on SDS-PAGE and visualized by autoradiography. As demonstrated in Fig. 7, the taxol stabilized MTs pull down mA CF7-C protein but not ABD protein, whereas polymerized actin filament pulls down a significant amount of ABD protein and a small amount of mA CF7-C protein. The interaction between mA CF7-C and polymerized tubulins is most likely direct, because no MT-associated proteins (MAPs) are present in the taxol stabilized MTs, al-
though proteins in the reticulocyte lysate system could enhance this binding. Since polymerized actin filaments were not able to pull down a partial BPAG1 COOH-terminal protein in similar assays (data not shown), the in vitro interaction between mA CF7-C protein and actin filaments may also be specific. However, no obvious colocalization of mA CF7-C and actin structures was observed in transfection studies; therefore, it is not clear that this interaction happens in vivo.

**Connection of MFs and MTs by mA CF7 In Vivo**

To investigate whether mA CF7 might be able to cross-link MFs and MTs in vivo, a construct (pFLAG-mA CF7-mini) encoding for a chimeric protein, that contained the ABD and the COOH-terminal domain of mA CF7 connected by a FLAG epitope tag, was used for transient transfection studies. Triple-labeling with phalloidin, anti-FLAG, and antitubulin was performed and the results are shown in Fig. 8. Similar to cells expressing mA CF7-C protein, long MT-forming whorls and bundles were observed in cells transfected with pFLAG-mA CF7-mini. In contrast to the straight stress fibers and membrane ruffles stained by phalloidin in nontransfected cells, many curly actin fila-

---

**Figure 7.** Interaction of mA CF7 with polymerized actin and MTs in vitro. (A) Actin spin-down binding assays were performed with [35S]methionine ABD and mA CF7-C proteins. In vitro translated mA CF7-C protein appeared as a doublet in SDS-PAGE. These two different sized proteins might be the result of degradation or alternative initiation. In the presence of polymerized actin filaments (+MF), a significant portion of ABD was found in the pellet (p). Without actin (−MF), most of the ABD remained in the supernatant (s). A small portion of mA CF7-C protein was also found in the pellet, indicating weak interaction between mA CF7-C protein and actin filaments. (B) Microtubule spin-down binding assays were performed with [35S]methionine-labeled ABD and mA CF7-C proteins. In the presence of polymerized MTs (+MT), mA CF7-C but not ABD protein cosedimented with the MT pellet (p). Without MTs (−MT), both of the proteins remained in the supernatant (s).

---

**Figure 8.** mA CF7-mini cross-links MFs and MTs. pFLAG-mA CF7-mini, encoding a chimeric protein of ABD and the COOH-terminal domain of mA CF7, was transfected into COS-7 cells. Transfected cells were triple-labeled with rhodamine-conjugated phalloidin (A), mouse monoclonal anti-FLAG M2 antibody (B), and rat monoclonal anti-Tyr-tubulin antibody (C). Cells expressing mA CF7-mini contain more actin filaments that are morphologically distinct from stress fibers. These actin filaments colocalized with mA CF7-mini and MTs, demonstrating the MF-MT cross-linking property of mA CF7-mini. Bar, 20 μm.
ments were detected in cells expressing mA CF7-mini. These unusual actin filaments were perfectly colocalized with MTs and decorated with mA CF7-mini (Fig. 8). These results show that mA CF7-mini could enhance the formation of actin filaments and efficiently cross-link them to MTs.

To further characterize the function of mA CF7, a COOH-terminal FLAG-tagged full-length mA CF7 construct was prepared for transient transfections. In some transfected cells, we observed partial coalignment of mA CF7, MTs, and actin filaments (Fig. 9, A–C, arrows). However, in most cases, colocalization of mA CF7, MTs, and actin was only observed in patches (Fig. 9, insets). These differences appear to be due to the differential expression levels of mA CF7 in the transfected cells. Cells expressing lower levels of full-length mA CF7 had more stress fibers that colocalized with mA CF7. A subset of these stress fibers also colocalized with MTs (Fig. 9, A–C). In contrast, in most transfected cells there were few actin stress fibers (Fig. 9 D). In addition, mA CF7 also colocalized with actin at membrane ruffles (Fig. 9, D and E, arrows) and decorated all the MTs. These data show that mA CF7 can bind to both actin filaments and MTs, and may have the ability to cross-link these cytoskeletal elements. Since vimentin has also been shown to associate with MTs (Gurland and Gundersen, 1995), we studied the distribution of vimentin in mA CF7-overexpressing cells, but found no obvious colocalization of IFs with mA CF7 (data not shown).

Discussion

The plakin family is a group of sequence-related proteins that associate with IFs and localize to junctional complexes at the plasma membrane. Current interest in plakins has been spurred by the discoveries of their roles in cross-linking cytoskeletal elements, as implied by their other name, cytolinkers (Wiche, 1998). This functional feature is particularly prominent for plectin and dystonin.
(B P A G 1-n) as they contain both actin- and IF-binding domains. A thorough the precise functions played by each plakin are not fully understood, hereditary diseases and gene targeting experiments vividly demonstrate the detrimental results caused by their loss-of-functions. Effects of the plakin gene in humans cause the skin blistering disease epidermolysis bullosa simplex combined with muscular dystrophy (Gache et al., 1996; Smith et al., 1996; Mellero et al., 1997). Mutations in desmoplakins cause striate palmoplantar keratoderma in humans (Armstrong et al., 1996). The loss of mouse dystonin/BPA G 1 results in dystonia musculorum (Brown et al., 1995a; Y ang et al., 1996), whereas desmoplakin-null embryos cannot survive beyond the egg cylinder stage (Gallicano et al., 1998). Plectin knockout mice die shortly after birth and exhibit severe defects in skin, skeletal muscle, and heart (A ndra et al., 1997). Therefore, identification of new family members might help us gain further insights into the functions of this family of proteins and unexplained human diseases. In fact, more potential members of the plakin family had been identified, such as A CF7 (Bernier et al., 1996) and a 450-kD epidermal protein (F ujiwara et al., 1996).

Unfortunately, characterization of these new family members is very time consuming, because all plakins are large proteins encoded by tremendously long mRNA s. Therefore, rather than using the standard hybridization approach, we attempted to isolate the full-length mAC F7 cDNA by an efficient PCR-based method. By sequentially performing R A CE-PCR, we were able to obtain overlapping clones spanning 11 kb in a relatively short period of time. The success of this approach not only accelerates the characterization of plakins, but can also be applied to other large-sized proteins.

Because the partially characterized cDNA of mAC F7 displayed 38–76% identity to that of dystonin/ BPA G 1-n (Bernier et al., 1996), mAC F7 was initially speculated to be a new plakin. However, after careful examination of its complete primary sequence, we found that mAC F7 also represents a novel member of the spectrin superfamily. mAC F7 can be structurally divided into three domains: NH2-terminal head, central rod, and COOH-terminal tail domains. The head domain consists of an ABD followed by a plakin-like globular domain. This portion of mAC F7 displays the strongest homology to dystonin/ BPA G 1-n as described previously (Bernier et al., 1996). However, unlike plakins, the rod domain of mAC F7 does not conform to a coiled-coil; instead it is composed of 23 repetitive motifs that are more closely related to dystrophin’s spectrin repeats. The tail domain of mAC F7 contains two putative EF-hand calcium-binding motifs and a G a 2-like G A R region. Because mAC F7 is structurally related to spectrin, bearing the highly homologous ABD, spectrin repeats, and EF-hand motifs, mAC F7 also belongs to the spectrin superfamily.

As illustrated by in situ hybridization, mAC F7 is ubiquitously expressed in mouse embryos, with the highest expression level in the nervous system. In transfected cells, the transiently expressed mAC F7 COOH-terminal protein associated with the endogenous MT networks, and this interaction stabilized the MTs from disassembly by nocodazole. Interaction of the COOH-terminal domain of mAC F7 and MTs was also suggested by in vitro binding assays. The putative ABD at the NH2-terminus of mAC F7 was proven to be functional. In vivo, colocalization of overexpressed ABD and actin structures was observed. In vitro, polymerized actin filaments were able to pull down ABD, and to a lesser extent the COOH-terminal domain of mAC F7 in spin-down assays. To study the actin-MT cross-linking properties of mAC F7, we generated mAC F7-mini and COOH-terminal FLAG-tagged full-length mAC F7 constructs. Overexpression of mAC F7-mini caused coagulation of MTs and actin filaments. In contrast, colocalization of full-length mAC F7, MTs, and actin was observed mostly in patches. Most of the stress fibers were disrupted in the transfected cells. Colocalization of actin, MTs, and mAC F7 were only occasionally observed in the remaining stress fibers. These differences in the transient transfection results between mAC F7-mini and full-length mAC F7 are most likely due to the presence of the plakin-like domain and spectrin repeats in full-length mAC F7. The ABD and the MT-associated domains are separated by the flexible spectrin repeats in the rod domain, making full-length mAC F7 less likely to form a directly observable link between MFs and MTs. In addition, the plakin-like domain and the spectrin repeats may mediate association of mAC F7 with membrane proteins and induce dimerization of mAC F7. Transient transfections with full-length plectin, another known cytoskeletal linker protein with a homologous ABD as mAC F7, also showed that the actin stress fibers appeared considerably reduced in number and complexity. Full-length plectin led to the collapse of the vimentin network in transfected cells into perinuclear aggregates, which were immunoreactive with antivimentin and antiplectin, but colocalization of vimentin, plectin, and actin stress fibers (or MTs) was not readily evident (A ndra et al., 1998). The transfection results that we obtained with full-length mAC F7 are therefore consistent with those described for plectin.

The plakin-like domain is highly conserved among the plakin family members. In desmoplakin, this globular domain clusters desmosomal cadherin-plakoglobin complexes and binds directly to plakoglobin (K owalczyk et al., 1997). A similar domain in plectin binds integrin β4 both in vitro and in vivo, although the plectin-integrin β4 interaction is probably more complex, involving multiple interfaces of the proteins and even the ABD of plectin (R ezniczek et al., 1998; Geerts et al., 1999). Hence, mAC F7 might also interact with membrane proteins. Indeed, some of the Drosophila kakapo alleles were identified from a screen of mutations affecting processes requiring integrin adhesion, suggesting that the mAC F7 homologue Kakapo is closely associated with integrin functions (G regory and B rown, 1998; W alsch and B rown, 1998). By analogy to that of other spectrin superfamily members, the spectrin repeats of mAC F7 could confer flexibility to the molecule for adjusting to the curvature of the cell membrane and may also mediate dimerization of two antiparallel mAC F7 molecules. Accordingly, mAC F7 could also cross-link individual MF fibers and/or MT fibers in its dimerized form. Collectively, mAC F7 represents a potential cytoskeletal cross-linking protein; the NH2-terminal ABD binds to MFs, whereas the COOH-terminal tail domain associates with MTs. Because mAC F7 binds to MTs as well as actin, we suggest a modification of its name ACF7 (actin cross-
linking family 7) to MACF (microtubule actin cross-linking factor).

So far, very few proteins have been reported to be able to bind both MFs and MTs. The yeast protein coronin promotes the rapid assembly and cross-linking of actin filaments and contains sequences homologous to the MT-binding region of MAP1B (Goode et al., 1999). However, the actin- and MT-binding domains are contiguous, and the region homologous to MAP1B is unique to yeast coronin. None of the mammalian homologues of coronin was found to possess this putative MT-binding domain (Goode et al., 1999). MAP1B is a 320-kD MAP that was originally copurified with MTs from mammalian brain. Microtubule-binding domains were found in both of the polypeptides, heavy chain and light chain, that constitute MAP1B (Noble et al., 1989; Zauner et al., 1992). Recently, transient transfection studies illustrated that a COOH-terminal fragment of the light chain was also able to associate with actin stress fibers (Togel et al., 1998). However, the overexpressed native light chain colocalized only with MTs in transfected cells, raising the possibility that the ABD of MAP1B is functional only under certain unknown circumstances. The other protein that has been reported to associate with MFs and MTs is plectin. Plectin contains a functional ABD near its NH₂ terminus and a defined IF-binding domain at the COOH-terminal tail domain (Nikolic et al., 1996; Andra et al., 1997). Although plectin has been reported to bind MTs, the association appears to be indirectly through other MAPs in neurons (Herrmann and Wiche, 1987). Nevertheless, plectin can directly associate with MTs in nonneuronal cells as observed by EM (Svitkina et al., 1996), although no specific MT-binding region on plectin has been defined. We have been able to show that the COOH terminus of MACF binds directly to MTs. The MT-binding region of MACF has no obvious sequence similarities to the MT-binding domains of other MAPs, such as the MT-binding repeats of tau, MAP2, and MAP4, or the MT-binding domains of MAP1B.

Recently, the Drosophila gene encoding for the MACF homologue, kakapo, was cloned and characterized in three studies by examinations of mutant flies with blistered wings or with a paralytic phenotype, and by library screenings with an antibody that stained the epidermal muscle attachment (EMA) cells in a unique pattern (Gregory and Brown, 1998; Prokop et al., 1998; Strumpf and Volk, 1998). Similar to MACF, Kakapo also contains an ABD, a plakin-like domain, a rod domain composed of spectrin repeats, and a GAR-region containing COOH-terminal tail domain. These studies indicate that Kakapo expression is restricted to ectodermally derived cells and that mutations of the Kakapo gene cause defects in the muscle-dependent tendon cell differentiation and the local development of neuronal processes (Prokop et al., 1998). In EMA cells, Kakapo is localized to the termini of MT bundles (Strumpf and Volk, 1998) and the absence of Kakapo causes detachment of these MT bundles from the basal membrane (Gregory and Brown, 1998). In addition, disorganization of MTs was also observed in the scolopidial sensory neurons of kakapo mutants (Prokop et al., 1998). Therefore, Kakapo was deduced to mediate the connection between the actin network, MTs, and membrane-associated proteins. Since we have shown that the COOH-terminal region of MACF can associate with MTs, we can therefore infer that Kakapo could also associate with MTs. The ubiquitous expression of MACF implies that it could also function as a linker protein connecting MFs, MTs, and membrane-associated proteins in a variety of tissues. The high level of expression of MACF mRNA that we observe in the nervous system could also indicate that it may have an important function in neurons.

The architecture of MACF clearly demonstrates the intricacies of gene evolution. MACF is a union of different kinds of structural domains that have been conserved throughout evolution. Although some of these structural domains have been well-studied in other proteins, together they make MACF bear very unique properties that have not been described previously for other cytoskeletal linker proteins. It has a clearly identified ABD as well as an MT-binding domain that are spatially well-separated. These domains may connect both MFs and MTs. The importance of this kind of connection is indicated by some of the lethal alleles obtained in the kakapo locus of Drosophila. A through the functional significance of MACF in mice as well as in human remains to be studied, it is certain that the ABD of MACF is functional and that its COOH-terminal domain functions like a MAP, i.e., it interacts with and stabilizes MTs.

We thank Dr. Gregg Gundersen for helpful discussions and Ms. Beth Rosen for technical assistance.

This work was supported by National Institutes of Health grant NS15182.
D. Sun and C.L. Leung were supported in part by training grant AG 00189.

Submitted: 27 May 1999
Revised: 28 October 1999
Accepted: 29 October 1999

References

al-Ali, S.Y., and A.G. al-Zuhaier. 1989. Fine structural study of the spinal cord and spinal ganglia in mice afflicted with a hereditary sensory neuropathy, dystonia musculorum. J. Submicrosc. Cytol. Pathol. 21:737–748.
Andra, K., H. Lassmann, R. Bittner, S. Shorny, R. Fassler, F. Propst, and G. Wiche. 1997. Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture. Genes Dev. 11:3143–3156.
Armstrong, D.K.B., K.E. McKenna, P.E. Purkis, K.J. Green, R.A.J. Eady, I.M. Leigh, and A.E. Hughes. 1999. Haploinsufficiency of desmoplakin causes a striate subtype of palmoplantar keratoderma. Hum. Mol. Genet. 8:143–148.
Bernier, G., M. Mathieu, Y. De Repentigny, S.M. Vidal, and R. Kohlery, 1996. Cloning and characterization of mouse ACF7, a novel member of the dystonin subfamily of actin binding proteins. Genomics. 38:19–29.
Braconni, C., S. Bottega, and C. Schneider. 1992. Gas2, a growth arrest-specific protein, is a component of the microfilament network system. J. Cell Biol. 117:1251–1261.
Brown, A., G. Bernier, M. Mathieu, J. Rossant, and R. Kohlery. 1995a. The mouse dystonia musculorum gene is a neural isoform of bullous pemphigoid antigen I. Nat. Genet. 10:301–306.
Brown, A., G. Dalpe, M. Mathieu, and R. Kohlery. 1995b. Cloning and characterization of the neural isoforms of human dystonin. Genomics. 29:777–780.
Byers, T.J., A.H. Beggs, E.M. McNally, and L.M. Kunkel. 1995. Novel actin crosslinker superfamily member identified by a two step degenerate PCR procedure. FEBS Lett. 368:500–504.
Collavin, L., M. Buzzi, E. Saconn, L. Bernard, C. Federico, G. Delia^alla, C. Brancolini, and C. Schneider. 1998. CDNA characterization and chromosome mapping of the human GA S2 gene. Genomics. 48:265–269.
Dubreuil, R.R., E. Brandin, J.H. Reisberg, L.S. Goldstein, and D. Branton. 1991. Structure, calmodulin-binding, and calcium-binding properties of reconstituent alpha spectrin polypeptides. J. Biol. Chem. 266:7189–7193.
Duchen, L.W., and S.J. Strich. 1964. Clinical and pathological studies of an hereditary neuropathy in mice (Drosophila musculorum). Brain. 87:367–378.
Fujiiara, S., K. Kohno, A. Iwamatsu, I. Naito, and H. Shinkai. 1996. Identification of a 450 kD a human epidural autotransplant as a new member of the
plectin family. J. Invest. Dermatol. 106:1125–1130.

Gache, Y., S. Chavanas, J. P. Lacour, G. Wiche, K. Owaribe, G. M eneuzui, and J. P. Ortonne. 1996. Defective expression of plectin/HD 1 in epidermolysis bullosa simplex with muscular dystrophy. J. Clin. Invest. 97:2289–2298.

Gallicano, G. J., P. Koukis, C. Baurer, M. Y in, V. Vashoukhin, L. Degenstein, and E. Fuchs. 1998. Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. J. Cell Biol. 143:2009–2022.

Geerts, D., L. Fontao, M. G. Nevers, R. J. Q. Schaapveld, P. E. Purkis, G. N. Wheeler, E. B. Lane, I. M. Leigh, and A. Sonnenberg. 1999. Binding of integrin α 6β 4 to plectin prevents plectin association with F-actin but does not interfere with intermediate filament binding. J. Cell Biol. 147:417–434.

Goode, B. L., J. J. Wong, A. C. Butty, M. Peter, A. L. McCormack, J. R. Yates, D. G. Drubin, and G. Barnes. 1999. Coronin promotes the rapid assembly and cross-linking of actin filaments and may link the actin and microtubule cytoskeletons in yeast. J. Cell Biol. 143:84–98.

Green, K. J., D. A. Parry, P. M. Steiner, M. L. Virata, D. A. Parry, B. Perman, and E. Fuchs. 1995. Stable, detyrosinated microtubules are a novel component of cornified envelopes and desmosomes that belongs to the plakin family and forms complexes with envoplakin. J. Cell Biol. 139:1835–1849.

Smith, F. J., R. A. Eady, I. M. Leigh, I. R. McLellan, E. L. Rugg, D. P. Kelsell, S. P. Bryant, N. K. Spurr, J. F. Geddes, G. Kirtschig, et al. 1996. Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. Nat. Genet. 13:450–457.

Sotelo, C., and J. L. Guenet. 1988. Pathologic changes in the CNS of dystonia musculorum mutant mouse: an animal model for human spinocerebellar ataxia. Neuroscience. 27:403–424.

Speicher, D. W., and V. T. Marais. 1984. Erythrocye spectrin is comprised of many homologous triple helical segments. Nature. 311:177–180.

Strumpf, D. T., and T. Volk. 1998. Kapako, a novel cytoskeletal-associated protein is essential for the restricted localization of the neuregulin-like factor, vein, at the muscle-tendon junction site. J. Cell Biol. 143:2129–2170.

Svitkina, T. M., A. B. Verkhovsky, and G. G. Borisy. 1996. Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. J. Cell Biol. 135:991–1007.

Tegel, M., G. Wiche, and F. Propst. 1998. Novel features of the light chain of microtubule-associated protein MA P1B: microtubule stabilization, self interaction, actin filament binding, and regulation by the heavy chain. J. Cell Biol. 143:695–707.

Uitto, J., L. Pulkkinen, F. J. Smith, and W. H. M. Mclean. 1996. Plectin and human genetic disorders of the skin and muscle. The paradigm of epidermolysis bullosa with muscular dystrophy. Exper. Dermatol. 5:237–246.

Walsh, E. P., and N. H. Brown. 1998. A screen to identify Drosophila genes required for integrin-mediated adhesion. Genetics. 150:791–805.

Wiche, G. 1998. Role of plectin in cytoskeleton organization and dynamics. J. Cell Sci. 111:2477–2486.

Wiche, G., B. Beiker, K. Uber, G. Weitzer, M. J. Castanon, R. Hauptsam, C. Stratowa, and M. Stewart. 1991. Cloning and sequencing of rat plectin indicates a 466-kD polypeptide chain with a three-domain structure based on a central alpha-helical coiled coil. J. Cell Biol. 114:83–99.

Yang, Y., E. W. W fitz, A. Viel, T. Cronin, S. C. Harrison, and D. Branton. 1993. Crystal structure of the repetitive segments of spectrin. Science. 262:2027–2030.

Yang, Y., J. D. Dowling, Q. C. Y u, P. Koukis, D. W. Cleveland, and E. Fuchs. 1996. An essential cytoskeletal linker protein connecting actin microfilaments to intermediate filaments. Cell. 86:655–665.

Yang, Y., C. Bauer, G. Strasser, R. Wollman, J. P. Julien, and E. Fuchs. 1999. Integrators of the cytoskeleton that stabilize microtubules. Cell. 98:229–238.

Zauner, W., J. J. K ratz, J. Staunton, P. Feick, and G. Wiche. 1992. Identification of two distinct microtubule binding domains on recombinant rat MAP 1B. Eur. J. Cell Biol. 57:66–74.

Zheng, M., C. L. Leung, and R. K. Liem. 1998. R gene-specific expression of cyclin-dependent kinase 5 (cdk5) and its activators, p35 and p39, in the developing and adult rat central nervous system. J. Neurobiol. 35:141–159.

Zucman-Rossi, J., P. Legpix, and G. Thomas. 1996. Identification of new members of the GAS2 and Ras families in the 22q12 chromosome region. Genomics. 38:247–254.