Microtubule actin crosslinking factor 1b: a novel plakin that localizes to the Golgi complex

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Summary

MACF1 (microtubule actin crosslinking factor), also called ACF7 (actin crosslinking family 7) is a cytoskeletal linker protein that can associate with both actin filaments and microtubules. We have identified a novel alternatively spliced isoform of MACF1. We named this isoform MACF1b and renamed the original isoform MACF1a. MACF1b is identical to MACF1a, except that it has a region containing plakin (or plectin) repeats in the middle of the molecule. MACF1b is ubiquitously expressed in adult tissues with especially high levels in the lung. We studied the subcellular localization of MACF1b proteins in mammalian cell lines. In two lung cell lines, MACF1b was chiefly localized to the Golgi complex. Upon treatments that disrupt the Golgi complex, MACF1b redistributed into the cytosol, but remained co-localized with the dispersed Golgi ministacks. MACF1b proteins can be detected in the enriched Golgi fraction by western blotting. The domain of MACF1b that targets it to the Golgi was found at the N-terminal part of the region that contains the plakin repeats. Reducing the level of MACF1 proteins by small-interfering RNA resulted in the dispersal of the Golgi complex.

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Key words: plakin, cytoskeleton, microtubules, Golgi complex

Introduction

Eukaryotic cells contain a complex fibrous network of filaments: actin microfilaments, microtubules (MTs) and intermediate filaments (IFs). In addition, there are proteins that crosslink these filaments and anchor them onto various membrane structures. Some of these linker proteins belong to the plakin (or spectraplakin) family. To date, seven plakins have been identified in mammals: desmoplakin, plectin, bullous pemphigoid antigen 1 (BPAG1), envoplakin, periplakin, epiplakin and microtubule actin cross-linking factor (MACF1), also called ACF7 (actin cross-linking family 7) (reviewed by Leung et al., 2002; Jefferson et al., 2004). Each plakin is made up of combinations of different interacting domains. These domains include an actin-binding domain (ABD) that is composed of one or two calponin-homology domains; a plakin domain that associates with various adhesion and signaling molecules; a coiled-coil rod domain that mediates dimerization; a series of spectrin repeats that may render flexibility; an IF-binding region that consists of varying numbers of plakin (or plectin) repeats that can be organized into distinct domains (plakin repeat domains or PRDs) with a so-called linker (L) domain embedded between the PRDs; and a MT-binding domain (MTBD) that binds to and stabilizes MTs. The MTBD is composed of several subdomains including one that resembles the growth arrest specific 2 (Gas2) protein (Collavin et al., 1998; Zucman-Rossi et al., 1996).

Epithelial plakins have been known for more than two decades, but invertebrate plakins have only recently been isolated. *Kakapo* was first characterized in *Drosophila* during a screen for mutants with a wing blistering phenotype (Strumpf and Volk, 1998). It was later found to be allelic to *shortstop* (*shot*), a *Drosophila* mutation that affects axon elongation (Lee et al., 2000). *Shot* appears to be the *Drosophila* homologue of mammalian *MACF1*. *Shot* mutants exhibit diverse phenotypes related to defects in dendritic sprouting, axonal outgrowth, axonal guidance, arborization of axon terminals in neuromuscular junctions and tendon cell differentiation (reviewed by Roper et al., 2002). These multiple defects suggest that Shot is essential for many different kinds of cellular processes in various tissues. The *shot* locus contains many exons that can undergo alternative splicing. The original shortstop isoform contains an ABD, a plakin domain, a series of spectrin repeats and a Gas2-related MTBD (Gregory and Brown, 1998; Strumpf and Volk, 1998). More recently, a second isoform has been described that has a long stretch of plakin repeats inserted between the plakin domain and the spectrin repeats (Roper and Brown, 2003). Unlike mammalian plakins, the plakin repeats of this isoform of Shot are not organized into PRDs. The presence of plakin repeats in this isoform is surprising, because in other plakins, the region containing plakin repeats has been associated with binding to IFs. As there are no cytoplasmic IFs in *Drosophila*, the plakin repeats are expected to have other functions. Indeed, the long isoform of Shot localizes to adherens junctions of embryonic and follicular epithelia and plays a major role in maintaining the integrity of epithelia (Roper and Brown, 2003). The homologue of *shot* in *Caenorhabditis elegans* is called *vab10*.

There are at least two alternatively spliced Vab10 isoforms, Vab10A and Vab10B (Bosher et al., 2003). These two isoforms are expressed differentially in the epidermis and are suggested to perform different functions. Vab10A is believed to protect the epidermis from external forces, while Vab10B may protect epidermal cells against internal tension.

MACF1 is the mammalian homologue of Shot. Its partial cDNA was originally cloned on the basis of sequence homology to the ABD of dystrophin (Byers et al., 1995). After the complete cDNA was characterized, MACF1 was found to be structurally similar to Shot (Leung et al., 1999). In keratinocytes, MACF1 co-localizes with actin filaments and MTs near focal contacts at the cell periphery (Karakesisoglou et al., 2000). MACF1 null mice die at an early embryonic stage, but endodermal cells derived from the null embryos exhibit defects in MT dynamics: MTs no longer grow along polarized actin bundles and fail to response to external cues in polarized cells (Kodama et al., 2003). Like Shot and Vab10, there are multiple isoforms of MACF1. Three isoforms of murine MACF1 with different N-termini have so far been identified (Bernier et al., 1996). A large exon that encoded a series of plakin repeats was revealed in a study of the human MACF gene structure, and an isoform that contains these plakin repeats at its N terminus has been described (Gong et al., 2001). In this paper, we report that the mouse *Macf1* gene also contains a large exon encoding plakin repeats between the plakin domain and the spectrin repeats. We have cloned and characterized the cDNA of a novel isoform of MACF1, called MACF1b that contains these plakin repeats in addition to *all* the domains found in MACF1 (now called MACF1a). MACF1b is ubiquitously expressed with the highest expression levels in the lung. We also provide evidence that MACF1b is closely associated with the Golgi complex.

Materials and Methods

Molecular cloning of MACF1b

To isolate cDNAs of MACF1b, RT-PCR was carried out using the One-Step RT-PCR kit (Qiagen). All procedures were performed according to the manufacturer's protocols. Genomic PCR was conducted on purified mouse 129 SvJ genomic DNA using Pfu polymerase (Stratagene). PCR products were subcloned into pCR2.1-TOPO vector (Invitrogen) or pCR-Blunt II-TOPO vector (Invitrogen) for sequencing. The following cycling parameters were used for genomic PCR: an initial denaturing step of 95°C for 2 minutes, followed by 30-35 cycles of 95°C (20 seconds), 60-68°C (30 seconds), 72°C (4-6 minutes), and a final extension step of 72°C for 10 minutes. cDNA or genomic clones were sequenced using the BigDye™ sequencing kit (Applied Biosystems) and processed using an Applied Biosystems ABI 3100 analyzer (DNA Facilities, Columbia University, New York, USA).

Plasmid constructs

(1) pET-PRD-1990: cDNAs encompassing amino acids 1990-2551 of MACF1b (GenBank accession number: DQ067088) were amplified from mouse lung total RNAs by RT-PCR using a One-Step RT-PCR kit (Qiagen) with forward primer 5′-TATACCATGGATCAAGAGCT-GGTGGAGATGCTAAC-3′ and reverse primer 5′-GGTGCTCGAG-TATGGCTTCTGAGAAGGAAAGGGC-3′. Amplified products were gel purified using a Geneclean III kit (Qbiogene), digested with *Nco*I/*Xho*I, and cloned into the *Nco*I/*Xho*I sites of pET-21d (Novagen) to make pET-PRD-1990.

(2) pKH3-PRD-N: to isolate the N-terminal fragment (N, amino acids 1543-1966 of MACF1b) of the PRD region, PCR primers (forward, 5′-GCGAATTCAAGGGTTGCAGAGCAGTTGCTGGG-GTG-3′; reverse, 5′-CTCTCCATGGCTGGATCCTTCCTCTCTTG-GGCC-3′) were used to amplify the corresponding cDNAs from the mouse brain Marathon-ready cDNA library (BD Clontech, Palo Alto, CA, USA) with Pfu turbo DNA polymerase (Stratagene). PCR products were cloned into pCR-Blunt II-TOPO vector (Invitrogen) to obtain pCR-TOPO-N. The inserts from pCR-TOPO-N were digested with *Eco*RI and inserted into the *Eco*RI site of pKH3 (a kind gift from Ian Marcara, University of Virginia, Virginia, USA).

(3) pKH3-PRD-C1: to isolate a C-terminal fragment of the MACF1b PRD region (C1, amino acids 1961-2800 of MACF1b), PCR primers (forward, 5′-GGCCCAAGAGAGGAAGGATCCAGCCA-TGGAG-3′; reverse, 5′-CGGAATTCTCACAGACTCCCAGCTT-GTAACACTGCCTC-3′) were used to amplify the corresponding cDNAs from the mouse brain Marathon-ready cDNA library (BD Clontech) with Pfu turbo DNA polymerase (Stratagene). PCR products were purified, digested with *Bam*HI/*Eco*RI, and cloned into *Bam*HI/*Eco*RI sites of pKH3.

(4) pKH3-FL-PRD: the pKH3-FL-PRD construct encoding the entire MACF1b-PRD region (amino acids 1543-2800) were obtained by three way ligation using *Eco*RI/*Bam*HI fragments of pCR-TOPO-N, the *Bam*HI/*Eco*RI fragments of pKH3-PRD-C1, and the *Eco*RI-linearized pKH3 plasmid. The sequence identity was confirmed by sequencing.

(5) pKH3-PRD-C2: to isolate a second C-terminal fragment of the MACF1b PRD region (C2, amino acids 2801-3569 of MACF1b), PCR primers (forward, 5′-GCGGATCCTGTGCTCCAGAAAAGGCTGG-CATAAGG-3′; reverse, 5′-CGGAATTCTCAGACCTGAACTTCT-TGCTGGACTTGCTG-3′) were used to amplify the corresponding cDNAs from the mouse brain Marathon-ready cDNA library (BD Clontech) with Pfu turbo DNA polymerase (Stratagene). PCR products were purified, digested with *Bam*HI/*Eco*RI, and cloned into *Bam*HI/*Eco*RI sites of pKH3.

(6) pFLAG-MACF1b-PRD: a cDNA clone coding for amino acids 2328-2797 of MACF1b was amplified from mouse lung total RNA using a one-step RT-PCR kit (Qiagen) with the following primer pairs (forward, 5′-CGGAATTCCCAGACCTGTGAGTCTTTGACGAC-TG-3′; reverse, 5′-CATGCTCGAGCTTGTAACACTGCCTCGTGT-TTCTCC-3′). The PCR products were gel-purified, digested with *Eco*RI/*Xho*I, and cloned into pcDNA-FLAG (Leung et al., 1999) to produce pFLAG-MACF1b-PRD.

(7) pET-PKN-832: a cDNA encompassing the last part of the plakin domain of MACF1b (amino acids 832-1282) were amplified and cloned into pET-21d (Novagen) to make pET-PKN-832.

(8) pFLAG-MACF1-plakin: this is the same as pFLAG-MACF-plakin described by Sun et al. (Sun et al., 2001).

Northern blot analysis

The probe, NB2, that was used for northern blotting corresponds to nucleotides (nts) 6137-7824 of MACF1b and is specific for MACF1b (Fig. 1B). The probe, NB1, nts 2665-4017 of MACF1b, is common to both MACF1a and MACF1b isoforms (Fig. 1B). Ready-to-use blot of mouse multiple tissues was purchased from Seegene Inc. (Seoul, Korea). Hybridizations were carried out at 60-62°C in hybridization buffer (6× SSC, 2× Denhardt's reagent, and 0.1% SDS) overnight. The membranes were washed twice with 1× SSC and 0.1% SDS at room temperature, and three times with 0.2× SSC and 0.1% SDS at 60-62°C. Membranes were blot-dried and exposed to Kodak X-AR films with intensifying screens at –80°C.

Antibody production

Plasmid pET-PRD-1990 was transformed into *E. coli* BL21 for protein production. Bacteria were resuspended in buffer containing 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, and 6 M urea. The

bacterial lysate was subjected to centrifugation at 17,210 ***g*** for 30 minutes. The supernatant was loaded onto $NiSO_4$ columns. Protein fractions were collected with elution buffer and each fraction was tested using SDS-PAGE. The purified protein fractions were pooled and dialyzed sequentially against PBS containing 6 M, 4 M, 2 M, 1 M urea and finally against PBS without urea. The protein concentrations were determined by Bradford assays using BSA as the standard. Purified proteins were used to immunize two New Zealand White rabbits to obtain antiserum (Covance). The antibody against the MACF1b PRD region was named rabbit polyclonal CU149 antibody (CU149). The anti-serum was further purified by a blot purification method (Gregory and Brown, 1998). The anti-MACF1 antibody (termed CU119) that recognizes both MACF1a and MACF1b was produced and purified as described above using pET-PKN-832. CU119 antibody has been tested against the equivalent region of the plakin domain of BPAG1a/b and no cross-reaction was found.

Cell culture

COS7 and HaCaT cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum (FBS; Life Technologies, Inc.). H460 (ATCC clone HTB-177) cells and A549 (ATCC clone CCL-185) cells were cultured in RPMI 1640 medium with 5% FBS. CAD cells were cultured in Dulbecco's Eagle's/Ham's F12 medium (DMEM/F12 medium; Life Technologies, Inc.) supplemented with 8% FBS. All cells were cultured in a humidified growth chamber with 5% CO_2 at 37°C. Cells were seeded onto 18×18 mm coverslips the day before drug treatment. Nocodazole (Sigma) was prepared as a stock solution of 10 mM in DMSO (Sigma) and applied to cells at 10 μM final concentration for 1 hour. Brefeldin A (Sigma) was used for 1 hour at 5 μg/ml final concentration from a stock solution of 10 mg/ml in methanol. Cytochalasin D (Sigma) was prepared in DMSO as a stock of 10 mM and applied to cells at 1 μM final concentration for 1 hour.

Transient transfections

COS7 cells, or H460 cells were seeded onto 10 cm Petri dishes or onto 18 mm coverslips the day before transfection. Cells were incubated in serum-free medium and transfected with DNA-lipid mixtures prepared from LipofectAmine PLUS reagents (Invitrogen) for COS7 cells or LipofectAmine 2000 reagents (Invitrogen) for H460 cells according to protocols provided by the manufacturer. Cells were incubated with the DNA-lipid mixtures for 3 hours (COS7 cells) or 6 hours (H460 cells) and then replenished with serum-containing medium. After 48 hours, cells were either harvested for western blot analysis or subjected to immunofluorescent staining. H460 cells that were transfected with MACF siRNA vector or control vector (see below) were incubated for 72 hours before fixation, staining or immunoblot analysis.

MACF siRNA vectors

To knockdown MACF1 expression in cells, nucleotides 407-427 of human *MACF1* (accession no.: NM 012090) were selected. This nucleotide sequence is not homologous to any other reported genes. The following primer pairs were designed, annealed and cloned into the linearized (*Bam*HI/*Hin*dIII-digested) vector pRNAT-U6.1/Neo (GeneScript), which contains a GFP expression cassette. phMACF1-siRNA: 5′-agcttaaaaagctgtggtcagagtcgctgattctcttgaaatcagcgactctgaccacagca-3′ and 5′-gatctgctgtggtcagagtcgctgatttcaagagaatcagcgactctgaccacagcttttt-3′. A construct with three mutated nucleotides, pControl: 5′-gatctccgtggtctgcgtcggtgatttcaagagaatcaccgacgcagaccacggcttttt-3′ and 5′-agctaaaaagccgtggtctgcgtcggtgattctcttgaaatcaccgacgcagaccacgga-3′ was used as a control. The three mutated nucleotides are underlined in both palindrome sequences, which are required for the formation of the hairpin-loop structure to generate the siRNA molecules.

Indirect immunofluorescence microscopy

Coverslips with adherent cells were fixed in cold methanol at –20°C for 10 minutes or in 4% paraformaldehyde in PBS at room temperature for 15 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes at room temperature. After 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 hour. The cells were rinsed three times with PBS and then incubated with the appropriate secondary antibodies (Molecular Probes) for 30 minutes. Subsequently, the coverslips were washed with PBS and mounted onto slides with Aquamount (Lerner Laboratories) for indirect immunofluorescent microscopy. The following primary antibodies were used: rabbit polyclonal CU149 antibody, rabbit polyclonal CU119 antibody, rabbit polyclonal anti-HA antibody (BD Clontech), rabbit polyclonal anti-p130 antibody (Covance), mouse monoclonal anti-HA antibody (Covance), mouse monoclonal anti-GM130 antibody (BD transduction), mouse monoclonal anti-GOSR1 antibody (Abcam), monoclonal anti-formiminotransferase cyclodeaminase antibody [FTCD, also called p58; a gift from Elizabeth Sztul, University of Alabama at Birmingham, AL, USA; (Gao et al., 1998)], mouse monoclonal anti-β-tubulin antibody (E7, culture supernatant), mouse monoclonal anti-GFP antibody (JL8; BD Biosciences) and mouse monoclonal anti-β-actin antibody (AC15; Sigma). Each antibody was titred not to bleed through into the other channel.

Immunoblot analysis

Protein samples from cell lines were obtained as follows: the cells were suspended in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (v/v), and protease inhibitors (Roche). After centrifugation at 18,300 ***g***, proteins in the supernatants were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 hour with 5% non-fat milk in PBS containing 0.1% Tween 20 (PBST), incubated for 2 hours with primary antibody and 1 hour with HRP-conjugated secondary antibody (Sigma). The membranes were washed three times with PBST between each incubation step. Proteins were visualized using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL, USA). Antibodies used were: mouse monoclonal anti-FLAG antibody (M2; Sigma), monoclonal anti-β-tubulin antibody (Sigma), rabbit polyclonal CU149 antibody, and rabbit polyclonal CU119 antibody.

Isolation of enriched Golgi fractions by sucrose step gradient centrifugation

Enriched Golgi fractions were isolated from H460 cells by standard protocols (Gao et al., 1998; Sweeney, 2002). Briefly, H460 cells were suspended in 0.5 M sucrose in PMG (0.1 M potassium phosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol and protease inhibitors (Roche), pH 7.0), homogenized extensively with a Dounce homogenizer on ice and centrifuged at 1000 ***g*** for 10 minutes at 4°C to remove nuclei and cell debris. The nuclei-free supernatant was diluted to 0.25 M sucrose in PMG, loaded into Beckman SW28 ultracentrifugation tubes and underlayered with 0.5 M, 0.86 M and 1.3 M sucrose in PMG. The preparations were spun at 25,000 rpm using a SW28 rotor for 2 hours at 4°C. The interface between 0.5 M/0.86 M SPMG, enriched for Golgi proteins and the interface between 0.86 M/1.3 M SPMG, enriched for ER proteins were also isolated (Gao et al., 1998). The collected samples were mixed with SDS sample buffer, boiled for 5 minutes, resolved on 6% or 8% SDS-PAGE and transferred onto PVDF membranes. Western blot analyses were performed as

described above. Antibodies used were: rabbit polyclonal CU149 antibody, rabbit polyclonal anti-p115 antibody (a gift from Elizabeth Sztul), and mouse monoclonal anti-FTCD (p58) antibody.

Results

Analysis of mouse MACF1b transcripts

We analyzed the MACF1 gene sequence from the mouse genome project and found that the gene contains at least 100 exons (Fig. 1A). The structure of *Macf1* is similar to that of *shot* in *Drosophila* (Lee et al., 2000). Between the exons that code for the plakin domain and the spectrin repeats, we identified five alternatively spliced exons and we deduced that MACF1 could produce an isoform that resembles the longer isoform of Shot. One of the exons is 6,083 bp in length and encodes an amino acid sequence that shows homology to the PRDs of other plakin family members. By multiple rounds of RT-PCR using total RNAs from murine lung as template, we obtained overlapping cDNA fragments of this novel MACF1 isoform (Fig. 1B). We named this newly identified product MACF1b, and refer the previously described isoform to MACF1a. Lung RNAs were chosen for the RT-PCR experiments because a larger MACF1 transcript has been detected in the lung by northern blotting (Bernier et al., 1996). To confirm the nucleotide sequence of the cDNA fragments, we also sequenced mouse genomic clones. The composite cDNA of MACF1b is 23.4 kb in length and encodes a protein of 7,354 amino acids, with a calculated molecular mass of 809 kDa.

In order to define the boundaries of PRDs in MACF1b, a series of sequence homology searches were carried out using the Amino Acid Sequence Analysis Program (AASAP) software package, an in-house computer package to study protein structure from sequence data. After the plakin repeats had been identified in the ~6 kb exon, specific comparisons were made with the well-characterized A, B and C type PRDs of desmoplakin (Choi et al., 2002; Green et al., 1990). On the basis of the sequence identity, the appropriate A, B or C designations were given to each of the PRDs recognized in MACF1b. MACF1b contains two A type PRDs, one C type

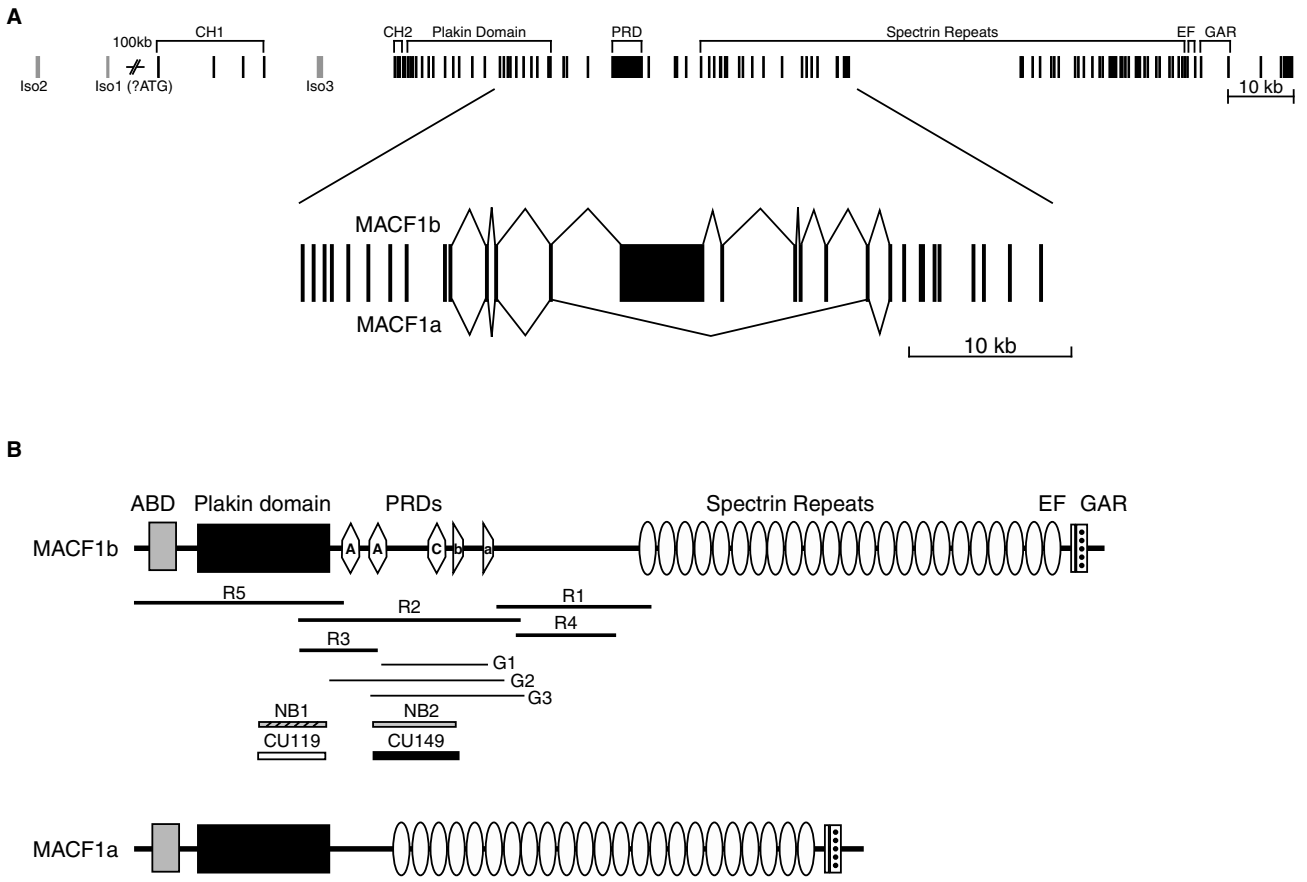


Fig. 1. MACF1 gene and protein isoforms. (A) Organization of mouse *Macf1* gene. Exons encoding the following domains are illustrated: CH domains, plakin domain, plakin repeats domain (PRD), spectrin repeats, EF hands (EF) and Gas2-related (GAR) domain. Alternatively spliced first exons that are responsible for generating isoform 1 (Iso1), isoform 2 (Iso2) and isoform 3 (Iso3) are in gray. The ATG start site of isoform 1 has not been identified. The region that undergoes alternative splicing to generate MACF1a and MACF1b is enlarged. (B) Domain organization of MACF1 isoforms. MACF1b contains two A PRDs (labeled A), one C PRD (labeled C), one incomplete B (labeled b) and one incomplete A (labeled a) PRD. Below the schematic drawing of MACF1b, bold lines indicate cDNA clones (R1, R2, R3, R4 and R5) and fine lines represent genomic PCR products (G1, G2 and G3). Clone names are shown above each line for cDNA clones and on the right for genomic products. The slashed bar (NB1) indicates the position of the probe for both MACF1a and MACF1b used for the northern blot; the gray bar (NB2), the position of the MACF1b specific probe used for the northern blot; the black bar, the region of MACF1b that was used for generating the CU149 antibody; white bar, the region that was used to generate the CU119 antibody (for all MACF1 isoforms).

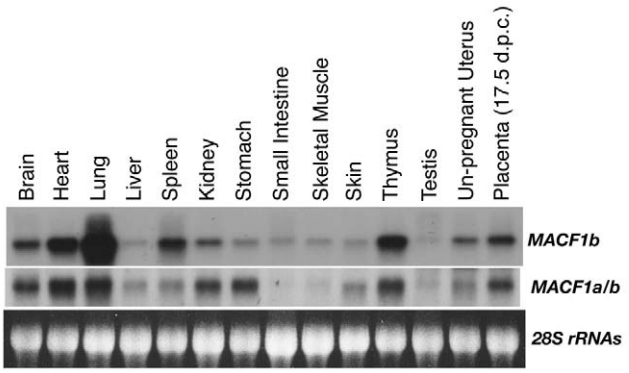


Fig. 2. Northern blot analysis of MACF1. Expression of MACF1 (MACF1a/b) in adult mouse tissues. A multiple tissue blot was hybridized with ^{32}P-labelled cDNA probes that recognized the plakin domain of both MACF1a and MACF1b (NB1 in Fig. 1B) or the PRD region of MACF1b (NB2 in Fig. 1B). Ethidium bromide stained 28S ribosomal RNAs were used as the loading control.

PRD, one incomplete B type PRD and one incomplete A type PRD (Fig. 1B). These PRDs displayed varying degrees of sequence identity to the PRDs of desmoplakin: 31% and 30% for the two A type PRDs, 26% for the C type PRD, 30% for the incomplete B type PRD and 25% for the incomplete A type PRD (Fig. S1 in supplementary material).

By northern blotting with a probe that recognized the plakin domain common to both MACF1a and MACF1b isoforms (NB1; Fig. 1B), MACF1a/b mRNA was found ubiquitously expressed in all mouse tissues, with especially high levels in lung, heart, brain, stomach, kidney, thymus and placenta (Fig. 2; middle panel) (Bernier et al., 1996; Bernier et al., 2000; Leung et al., 1999). We also analyzed the expression patterns of MACF1b mRNA in mice using a MACF1b-specific probe (NB2, Fig. 1B) and found that it was expressed in all adult tissues with high levels in lung, thymus, heart, spleen, brain and placenta (Fig. 2; upper panel).

MACF1 in mammalian cell lines

To study MACF1b protein, we prepared a rabbit polyclonal antibody against a portion of the PRD region of MACF1b (CU149; Fig. 1B). His-tagged MACF-PRD protein was produced in bacteria, purified with a nickel column and used as immunogen for antibody production. To characterize the CU149 antibody, we transiently transfected COS7 cells with the construct encoding the FLAG-tagged MACF1b PRD. Protein lysates were collected from transfected cells and analyzed by western blotting. Both the anti-FLAG M2 monoclonal antibody and CU149 antibody recognized the same FLAG-MACF1b-PRD protein that was not detected by the preimmune serum (Fig. 3A). CU149 antibody also recognized a single slow mobility band corresponding to MACF1b in HaCaT cells, a keratinocyte cell line (Fig. 3B). To confirm the specificity of CU149, we knocked down the expression of MACF1 proteins by siRNA in H460 cells, a lung cell line. There was a weak signal on the western blot of cells transfected with a MACF siRNA vector, in contrast to cells that were transfected with a control siRNA vector (Fig. 3C). These data confirmed that CU149 specifically recognized MACF1b.

By using a purified plakin domain protein for immunization, we also prepared an antibody that recognized both MACF1 isoforms (CU119; Fig. 1B). CU119 recognized the full-length MACF1 plakin domain protein ectopically expressed in COS7 cells (Fig. 4A), and also recognized endogenous MACF1 protein in CAD cells (lane 2 in Fig. 4B). This signal was abolished by pretreatment of CU119 with the plakin domain antigen (lane 1 in Fig. 4B). CU119 also recognized MACF1 in H460 lung cells. Although both MACF1a and MACF1b were expressed in H460 cells, CU119 only detected one single band on the western blot. This is probably because of the inability to separate the ~600 kDa MACF1a from ~800 kDa MACF1b by 6% SDS-PAGE. Nonetheless, this MACF1 signal was greatly reduced in H460 cells transfected with a MACF1-siRNA vector that reduced both MACF1a and MACF1b expression (Fig. 4C). Together, these data suggested that

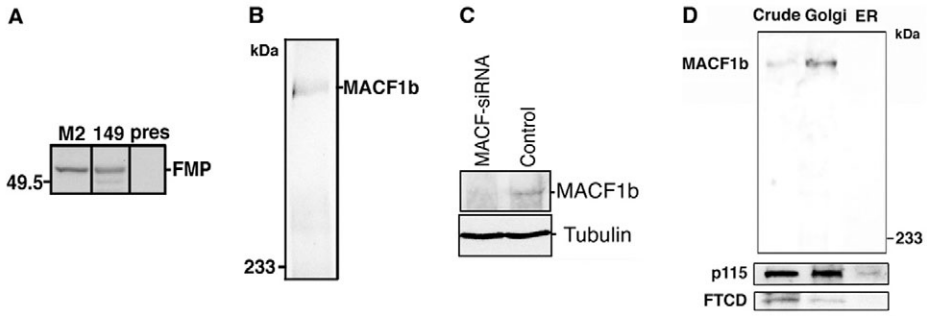


Fig. 3. Western blotting with anti-MACF1b antibody. (A) Analysis of rabbit polyclonal CU149 antibody. Cell lysate (80 μg) collected from pFLAG-MACF1b-PRD-transfected COS7 cells was subjected to western blotting with mouse monoclonal anti-FLAG antibody (M2), CU149 (149), and preimmune serum of the rabbit that generated CU149 (pres). CU149 and M2 both recognized FLAG-MACF1b-PRD protein (FMP) that was not detected by the preimmune serum. A molecular size standard (kDa) is indicated on the left. (B) Detection of MACF1b in HaCaT cells. HaCaT cell lysate was subjected to immunoblotting with CU149 (MACF1b). Only one slow mobility band was detected. (C) Specificity of CU149 antibody to MACF1b. H460 cells were transfected with MACF1 siRNA vector (MACF siRNA) that was designed to knockdown MACF1a/b, or control vector (Control) for 72 hours. Cell lysates were probed for either MACF1b (top panel) or β-tubulin (loading control; lower panel). MACF1b protein in cells transfected with MACF siRNA vector was reduced significantly compared to cells transfected with the control vector. (D) Detection of MACF1b proteins in the enriched Golgi fraction. H460 cell lysate was subjected to sucrose step gradient centrifugation, Golgi and ER fractions were isolated, resolved in SDS-PAGE, transferred to PVDF membranes, and probed with CU149 antibody (MACF1b; top panel), anti-p115 antibody (p115; middle panel), and anti-FTCD antibody (FTCD; lower panel). Crude: crude lysate. CU149 only recognizes MACF1b proteins in enriched Golgi fraction of H460 cells. A molecular size standard (233 kDa) is indicated.

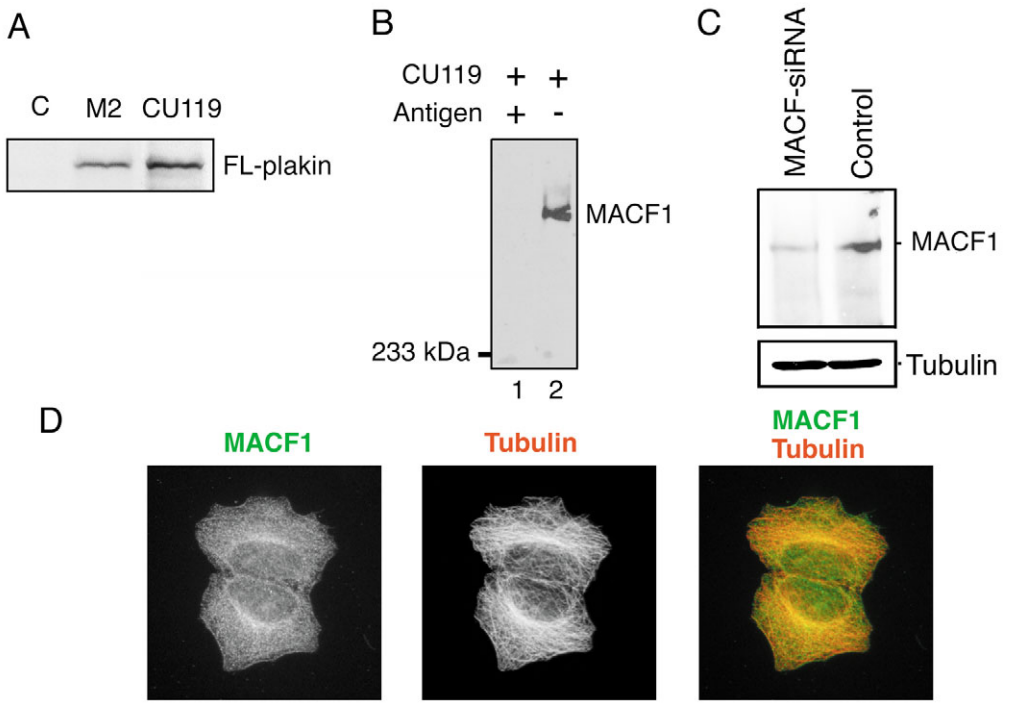


Fig. 4. Characterization of anti-MACF1 (CU119) antibody. (A) Detection of the full-length plakin domain of MACF1 proteins by CU119 antibody. COS7 cells were transfected with plasmid pFLAG-MACF1-plakin and cell lysate was resolved by SDS-PAGE. The transblot was then probed with mouse monoclonal anti-FLAG antibody (M2) and anti-MACF1-plakin antibody (CU119). The expressed full-length MACF1-Plakin protein (FL-plakin) can be detected by both M2 and CU119 antibodies, but not in mock-transfected control (C). (B) Detection of endogenous MACF1 proteins by CU119 antibody in CAD cells. Neuronal CAD cells were lysed and resolved by SDS-PAGE, and the transblot was probed with CU119 antibody. MACF1 proteins can be detected in CAD cells (lane 2), and this signal can be completely abolished by pretreatment of CU119 antibody with antigen (lane 1). (C) Specificity of CU119 antibody revealed by the detection of MACF1 proteins in siRNA-treated cells. H460 cells were transfected with MACF siRNA vector (MACF-siRNA) that was designed to knockdown MACF1, or control vector (Control) for 72 hours. After SDS-PAGE, the transblot was probed for either MACF1 (top panel) or β-tubulin (loading control; lower panel). MACF1 proteins in cells transfected with MACF siRNA vector (MACF siRNA) were reduced compared to cells transfected with the control vector (Control). (D) Localization of MACF1 in H460 cells. H460 cells were stained for MACF1 (left panel; CU119 antibody that recognizes all isoforms; green) and β-tubulin (middle panel; red). MACF1 protein was found around the nuclei, dotted in the cytoplasm and decorating microtubules (right panel; merged picture).

CU119 antibody specifically recognized both MACF1 isoforms in cells.

MACF1b localizes to the Golgi complex

Since we found that MACF1b mRNA is abundant in the lung (Fig. 2), we studied the subcellular localization of MACF1b in H460 and A549 lung cell lines. H460 cells were derived from the pleural fluid of a human patient with a large lung cell carcinoma and A549 cells were originally established as an explant culture from a lung carcinoma. Immunostaining on H460 cells using CU119, the antibody that recognizes both MACF1a and MACF1b showed cytoplasmic staining that appeared to decorate MTs, as well as staining in the perinuclear region (Fig. 4D), consistent with studies reported previously using immunostaining with other anti-MACF1 antibodies (Karakesisoglou et al., 2000; Kodama et al., 2003) and transfections of full-length MACF1a (Leung et al., 1999). By contrast, CU149 antibody showed strong labeling predominantly in the perinuclear region of both lung cell lines (Fig. 5A,C). To investigate whether this perinuclear staining by CU149 antibody was at the Golgi complex, we co-stained the cells with an antibody against Golgi protein GM130 (Fig. 5B,D). GM130 protein was originally purified from Golgi membranes (Nakamura et al., 1995) and identified in the stacked cisternae of the Golgi complex (Slusarewicz et al., 1994). The MACF1b antibody staining near the nucleus co-localized with that of GM130 (compare A with B and C with D of Fig. 5), suggesting that MACF1b resides near or at the Golgi complex. Similar co-localization patterns were also observed in H460 cells that were co-stained with CU149 antibody and antibodies to the Golgi-associated protein formiminotransferase cyclodeaminase (FTCD; data not shown), and GOSR1 (GOS-28, a Golgi SNARE; data not shown) (Hay et al., 1997; Subramaniam et al., 1997; Subramaniam et al., 1996). The punctate cytoplasmic staining of MACF1b appeared to partially decorate MTs (Fig. 6A,B), but not MFs (Fig. 6C,D) in H460 cells. Furthermore, treatment of H460 cells with cytochalasin D for 1 hour did not change the localization of MACF1b (Fig. 7E), although the actin filaments were disrupted (not shown). We also performed staining in HaCaT cells and found that the CU149 staining co-localized with markers for the Golgi complex (not shown).

We also isolated enriched Golgi and endoplasmic reticulum

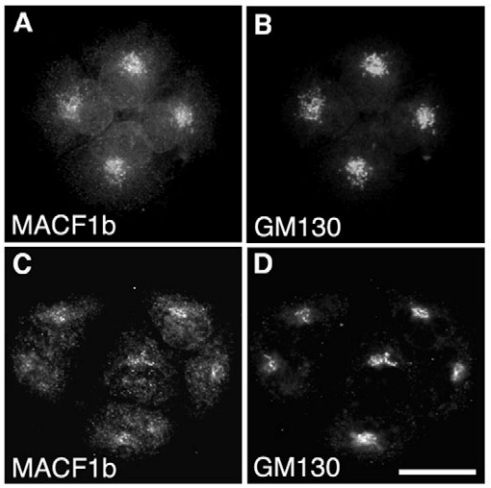


Fig. 5. Localization of MACF1b to the Golgi complex in lung cell lines. H460 (A,B) and A549 (C,D) cells were stained with antibodies against MACF1b (A,C) and GM130 (B,D). MACF1b staining was of a widespread vesicular pattern with strong labeling near the Golgi complex. Bar, 20 μm.

(ER) fractions from H460 cells using sucrose step gradients and analyzed them by western blots. p115 and FTCD were used as markers for the Golgi complex. p115 is a peripherally associated Golgi protein that is found in lesser amounts in the ER (Waters et al., 1992). FTCD is a 58 kDa Golgi-associated protein that can bind to MTs and IFs (Bashour and Bloom, 1998; Gao et al., 1998; Hennig et al., 1998). MACF1b was found to be present chiefly in the Golgi- and not the ER-enriched fractions, suggesting that MACF1b associated with the Golgi complex in H460 cells (Fig. 3D). CU149 recognized a single high molecular mass band, indicating that CU149 does not cross-react with other Golgi proteins.

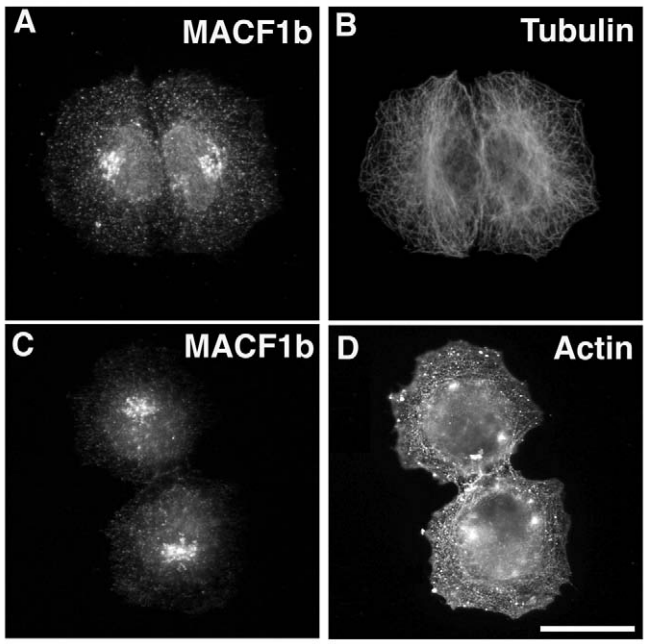


Fig. 6. Localization of MACF1b proteins, microtubule elements and actin microfilaments in H460 cells. H460 cells were stained for MACF1b (A,C), β-tubulin (B) or actin (D). In addition to Golgi staining, staining of MACF1b proteins was strong around the nucleus and a dotted pattern in the cytoplasm that partially decorated the microtubule network. Bar, 20 μm.

Dispersion of MACF1b after treatments with nocodazole and brefeldin A

MTs are important for positioning of the Golgi complex. After nocodazole treatment, Golgi stacks disperse into the cytoplasm (Ho et al., 1989; Minin, 1997; Rogalski and Singer, 1984; Thyberg and Moskalewski, 1999). To determine whether the Golgi localization of MACF1b is dependent on MTs, we disassembled the MTs by treating H460 cells with 10 μM of

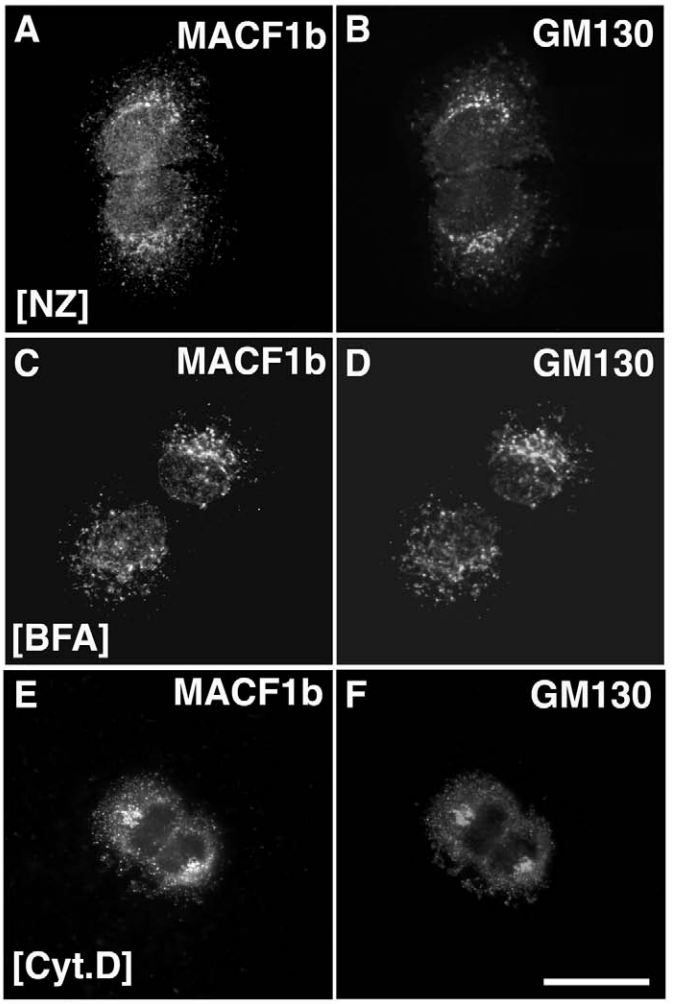
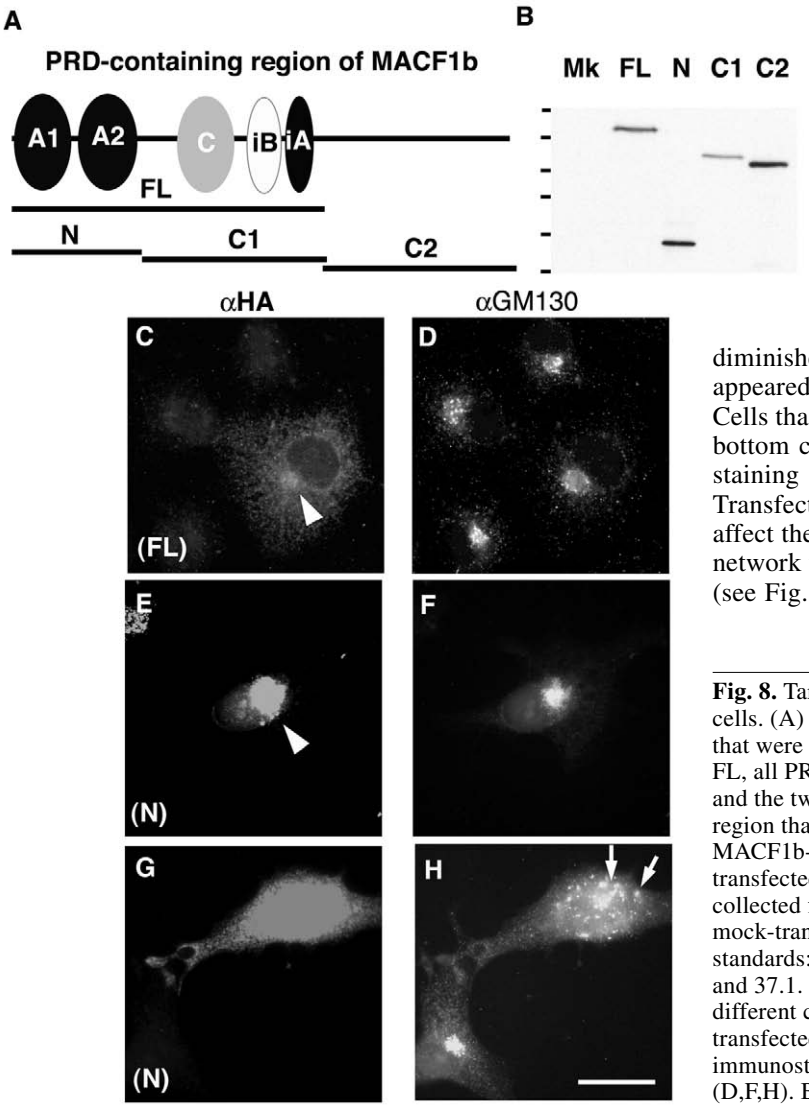


Fig. 7. Displacement of MACF1b in H460 cells treated with drugs that disrupt microtubules, Golgi or actin. (A-D) MACF1b colocalizes with the displaced Golgi ministacks in H460 cells treated with nocodazole and brefeldin A. H460 cells were treated with nocodazole [NZ] for 1 hour (A,B) or brefeldin A [BFA] for 1hour (C,D) and stained for MACF1b (A,C) or GM130 (B,D). MACF1b proteins colocalized with the dispersed Golgi ministacks in the cytoplasm after nocodazole or brefeldin A treatments.
(E,F) Localization of MACF1b in H460 cells after cytochalasin D treatment [Cyt.D] for 1 hour. Cells were then stained for MACF1b (E) or GM130 (F). MACF1b remained on the Golgi. Bar, 20 μm.

nocodazole for 1 hour. Under these conditions, MACF1b redistributed throughout the cytosol with a vesicular staining pattern that coincided with the anti-GM130 staining, suggesting that MACF1b redistributed with the Golgi ministacks after depolymerization of MTs (Fig. 7A,B). The same result was observed with an antibody to FTCD (data not shown). Brefeldin A has been shown to disrupt the Golgi complex by integrating into the lipid membrane and creating channels that disturb the stability of the Golgi causing it to disintegrate. MTs are not disrupted by this treatment (not shown). MACF1b was also redistributed into the cytosol after brefeldin A treatment with an immunofluorescent staining pattern similar to that of GM130 (Fig. 7C,D). Carrier reagents (DMSO and methanol) did not affect the morphology and staining of H460 cells (not shown).

Targeting of MACF1b to the Golgi complex

To confirm that the PRD region is responsible for targeting MACF1b to the Golgi complex, we HA-tagged the entire PRD region of MACF1b (FL; Fig. 8A) and expressed the truncated proteins in COS7 cells. The ectopically expressed HA-PRD proteins localized to the Golgi complex (Fig. 8C, arrowhead). To delineate the Golgi-interaction domain, we transfected COS7 cells with smaller deletion constructs, N, C1 and C2 (Fig. 8A) and assayed the localization of the tagged truncated proteins. The results showed that the N-terminal portion of the PRD region, which harbors two consecutive A-type subdomains, was sufficient to target the protein to the Golgi (Fig. 8E,F; arrowhead in E), while C1 and C2 protein at the C-terminal portions of the PRD region were not able to do so (see Fig. S2 in supplementary material). The Golgi complex was disrupted in cells that expressed high levels of N-terminal PRD proteins (Fig. 8G,H, arrows). Immunoblot analysis indicated that the transfected proteins had the correct sizes (Fig. 8B). These data suggest that MACF1b interacted with the Golgi via the N-terminal portion of the PRD region.



Reduction of MACF1b using small-interfering RNA (siRNA) in H460 cells

Two siRNA vectors that were specifically designed to knockdown MACF1b failed to reduce the expression of MACF1b in cells. We therefore prepared an siRNA vector that targeted both MACF1 isoforms (phMACF1-siRNA), and used a three-nucleotide mutated vector as a control (pControl). A GFP cassette on the siRNA vector allowed us to identify the transfected cells. Immunoblot analyses of cells expressing the MACF1 siRNA vector showed a significant reduction in the levels of both MACF1a and MACF1b (Fig. 3C and Fig. 4C), while cells expressing the control vector did not. In cells that expressed MACF1 siRNA (as shown by the GFP labeling), the staining of MACF1b was diminished (compare Fig. 9B,D), and the Golgi complex appeared to be dispersed into the cytosol (Fig. 9D,H, stars). Cells that were not transfected (see arrowhead in Fig. 9D and bottom cell in Fig. 9H) as indicated by the absence of GFP staining did not show the dispersed Golgi complex. Transfection with the control siRNA (Fig. 9B,F) also did not affect the Golgi complex. Finally, the morphology of the MT network was not affected by the expression of MACF siRNA (see Fig. S3 in supplementary material).

Fig. 8. Targeting of MACF1b-PRD to the Golgi complex in COS7 cells. (A) Illustration showing MACF1b PRD and cDNA clones that were used to generate HA-tagged MACF1b PRD constructs. FL, all PRD subdomains; N, two A sundomains; C1, C subdomains and the two incomplete B and A subdomains; C2, C-terminal region that did not include any PRDs. (B) Expression of HA-tagged MACF1b-PRD proteins in COS7 cells. COS7 cells that were transfected with various cDNA constructs (FL, N, C1, C2) were collected for western blot analysis with anti-HA antibody. Mk, mock-transfected cells. Bars on left indicate molecular mass standards: from top to bottom (kDa), 181.8, 115.5, 82.2, 64.2, 48.4 and 37.1. (C-H) Immunostaining of cells that were transfected with different cDNA clones of MACF1b-PRD. COS7 cells were transfected with cDNA constructs FL (C,D), N (E-H), and immunostained with antibodies against HA-tag (C,E,G) or GM130 (D,F,H). Bar, 20 μm.

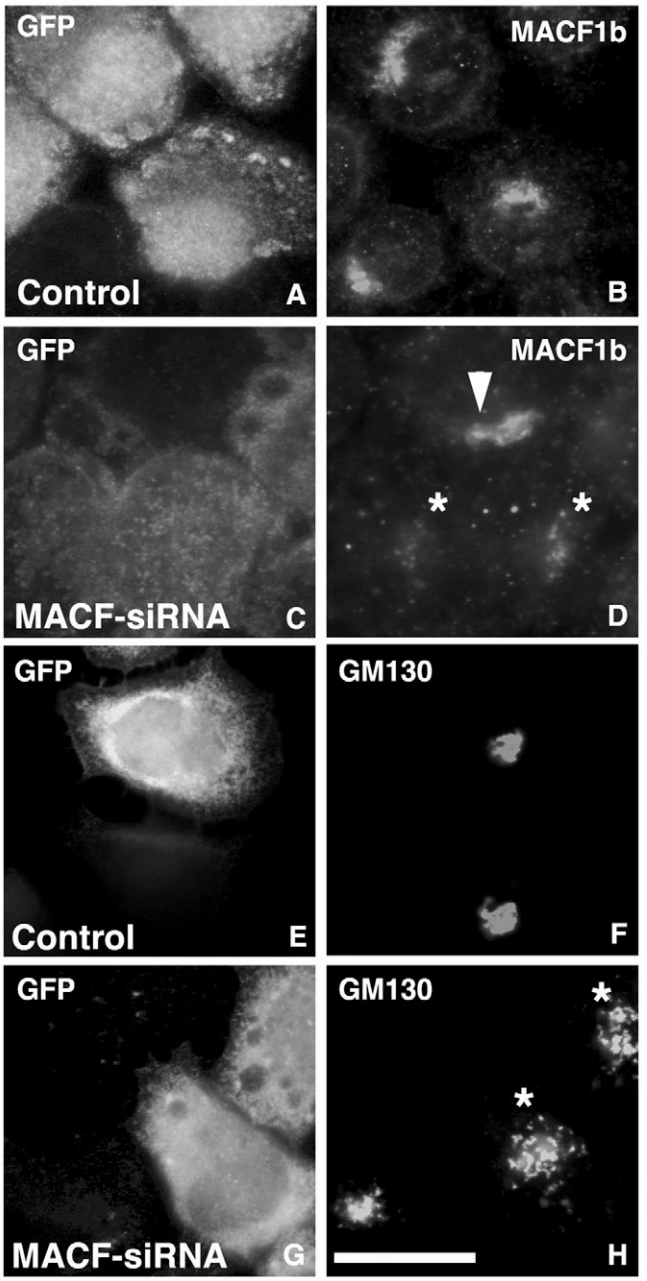


Fig. 9. Knockdown of MACF1b resulted in the disassembly and dispersion of the Golgi complex in H460 cells. H460 cells transfected with control vector (Control; A and B, E and F) or MACF-siRNA vector (MACF-siRNA; C and D, G and H) were fixed and stained for MACF1b (B,D), GM130 (F,H), and GFP (A,C,E,G). The siRNA plasmid contained a GFP cassette to identify the transfected cells. Note that MACF1b staining in cells treated with MACF1-siRNA (GFP positive) was greatly reduced (compare D with B) and the Golgi complex was disassembled (asterisks in D and H). Arrowhead in D indicates a cell that was not transfected with the MACF-siRNA vector (GFP negative) and displayed an undisrupted Golgi pattern. Bar, 20 μm.

Discussion

We have characterized a novel isoform of MACF1 called MACF1b. MACF1b harbors extra plakin repeats between the plakin domain and the spectrin repeats of MACF1a, the MACF1 isoform that we previously described (Leung et al., 1999). MACF1b is a gigantic protein of about 800 kDa. MACF1b transcripts are expressed throughout the development of the mouse embryo and in all adult tissues that were examined. Higher expression levels were found in the lung, the heart, the thymus, the spleen and the brain (Fig. 2). We have detected MACF1b protein in human cell lines, H460, A549 and HaCaT cells. In these cells, MACF1b is closely associated with the Golgi complex, perturbation of the Golgi complex caused MACF1b to redistribute with the Golgi fragments (Fig. 7), and the Golgi complex was disassembled when the levels of MACF1a and MACF1b proteins were reduced (Fig. 9). In addition, the N-terminal part of the PRD region contained the domain required for the localization of MACF1b at the Golgi complex (Fig. 8).

Genomic organization of MACF1

The mouse *Macf1* gene is made up of at least 100 exons. The human *MACF1* gene has also been reported to contain more than 100 exons (Gong et al., 2001). The first CH domain is encoded by four exons and the second CH domain is encoded by three. So far, three alternatively spliced first exons have been reported (Bernier et al., 1996). One of them is located between exons encoding the two CH domains (Fig. 1A). Alternative splicing of these three exons generates N-terminal MACF1 isoforms. Isoform 3 contains only the second CH domain, while isoforms 1 and 2 contain both CH domains. There is a 100-kb uncharacterized genomic sequence between the first exon of MACF1 isoform 1 and the exons encoding the first CH domain; this region could contain additional alternatively spliced first exons (Fig. 1A). An EST clone with a *MACF1* exon that is located in this region and is about 20 kb downstream of the first exon of isoform 1 has been reported in the GenBank database (acc. no. BY749754). Similar organization of multiple variable first exons has also been described in other genes (Zhang et al., 2004), including that of plectin where eleven variable first exons are organized in a tandem array (Fuchs et al., 1999). This organization of multiple first exons has been suggested as a genetic mechanism for directing distinct cell-specific patterns of gene expression (Bernier et al., 2000). Whether this phenomenon is also true for *MACF1* remains to be elucidated. It is not yet known whether there is a preferred first exon for MACF1a and/or MACF1b. We suggest naming the three MACF1a N-terminal isoforms: MACF1a1, MACF1a2 and MACF1a3. Similar nomenclature should also be applied to MACF1b.

Between exons encoding the plakin domain and the spectrin repeats, we identified five alternatively spliced exons; one of them was approximately 6 kb in length and encodes three complete and two incomplete PRDs. Interestingly, plakin repeat-containing regions are always encoded by one exon. This is even true in human and mouse epiplakin, which consist of only a series of PRDs (Fujiwara et al., 2001; Spazierer et al., 2003; Takeo et al., 2003). PRDs are evolutionarily conserved in the plakin family and have been divided into three types, designated A, B and C (Green et al., 1992; Kowalczyk et al., 1997; Kowalczyk et al., 1999; Ruhrberg and Watt, 1997; Sawamura et al., 1991; Wiche et al., 1991). Different plakin

members harbor PRD(s) located at a different region of the protein and the number of PRDs range from 13 B type PRDs in epiplakin (Fujiwara et al., 2001) to no PRDs in periplakin (Ruhrberg and Watt, 1997). Desmoplakin, plectin and BPAG1e, also have a linker (L-subdomain) between the B and C type PRDs. Envoplakin has one L subdomain before the C type PRD and periplakin has only an L subdomain at its C terminus. The L subdomain has been shown to be important in mediating interactions of plakins to IFs (Fontao et al., 2003; Karashima and Watt, 2002; Kazerounian et al., 2002). MACF1b has two A, one C, one incomplete B and one incomplete A type PRDs (Fig. 1 and Fig. S1 in supplementary material). No L-subdomain is found in MACF1b. The subdomain structure arrangement of MACF1b is unique among plakins (Leung et al., 2002). A human cDNA called MACF1-4, which has the PRD region at its N terminus, has been cloned from heart and pituitary libraries (Gong et al., 2001). The resulting protein would be smaller than MACF1b and is encoded by a 19 kb mRNA. This predicted protein has not yet been identified. Other isoforms of MACF1, with or without PRDs are also possible, but have not yet been described.

The overall gene structure of *MACF1* is similar to that of *shot* in *Drosophila*. *Shot* produces two isoforms that share similar domain organization of MACF1a and MACF1b, respectively. However, the plakin repeat-containing region of Shot is not organized into PRDs. Each PRD is made up of four and a half plakin repeats and the long isoform of Shot only contains stretches of plakin repeats. The presence of two incomplete PRDs in MACF1b may represent the transition of organization of plakin repeats into PRDs during evolution. The functions of plakin repeats remain elusive but it clearly involves more than binding IFs, since no cytoplasmic IFs have been found in *Drosophila*. The localization of MACF1b at the Golgi complex of the lung cell lines and the fact that reduction of MACF1b resulted in dispersion of the Golgi in H460 cells suggest that plakin repeats may also be important in maintaining the normal structure and/or functions of the Golgi complex in these cells.

MACF1b is localized at the Golgi complex in mammalian cells

By in vitro binding assays, transfection studies and immunocytochemical analyses, MACF1 has been shown to bind both actin filaments and MTs via the N-terminal ABD and the C-terminal MTBD (Karakesisoglou et al., 2000; Leung et al., 1999; Sun et al., 2001). In this report, we provide evidence that MACF1b localized to the Golgi complex in two human cell lines. In H460 and A549 cells, MACF1b co-localized with the Golgi complex near the cell center and was found in enriched Golgi preparations. Colocalization of MACF1b with the Golgi complex was also observed in HaCaT cells (not shown). Dispersion of the Golgi complex by nocodazole or brefeldin A caused MACF1b proteins to redistribute to the cytosol where MACF1b still associated with the dispersed Golgi remnants (Fig. 7). In addition, the N-terminal part of the PRD region of MACF1b localized to the Golgi complex in transient transfections (Fig. 8). We also found that high levels of over-expression of the construct encoding the N-terminal part of the PRD region resulted in the disruption and dispersion of Golgi into the cytosol. This effect could be the result of the dominant-negative disruption of the over-expressed protein on the endogenous localization of MACF1b at the Golgi. Furthermore, the structure of the Golgi complex was affected when MACF1a and MACF1b levels were reduced (Fig. 9). Our data are consistent with the notion that MACF1b localizes to the Golgi, although we have not yet determined whether MACF1b interacts directly with a resident Golgi protein, or if it interacts with a protein that interacts specifically with the Golgi complex.

An interaction between the MACF1 C-terminal region with the trans-Golgi network protein, p230 (golgin-245) has been reported in HeLa cells (Kakinuma et al., 2004). The region of MACF1 that was reported to interact with p230 is present in both MACF1a and MACF1b, although we do not know if MACF1b is expressed in HeLa cells. Disruption of the interaction of MACF1 with p230 could affect the transport of glycosyl phosphatidylinositol-anchored proteins from the trans-Golgi network to the cell surface. The interaction between the PRD of MACF1b and the Golgi complex that we report here may also be important in mediating the transport of Golgi proteins to various cellular compartments. In addition, it is also possible that MACF1b plays a structural role in maintaining the architecture of the Golgi complex.

Various cytoskeletal proteins have been reported to associate with the Golgi complex, including motor proteins, MT-associated proteins and IF-associated proteins. Both MT-based and actin-based motors have been implicated in maintaining the proper structure and function of the Golgi complex (Allan et al., 2002). These motor proteins are important in transporting membrane components to and from the Golgi complex. It has long been shown that disruption of MTs will cause the Golgi complex to disperse (Ho et al., 1989; Minin, 1997; Rogalski and Singer, 1984; Thyberg and Moskalewski, 1999). Hence, it is not surprising to find that many MT-associated proteins are also connected to the Golgi. These MT-associated proteins include members and association partners of cytoplasmic linker proteins (CLIPs) and Hook families (Kramer and Phistry, 1996; Kramer and Phistry, 1999; Schroer, 2000). GMAP-210 is a CLIP that links the cis-Golgi network to the minus ends of MTs and this interaction seems to be essential for ensuring the proper morphology and size of the Golgi complex (Infante et al., 1999). Hook proteins link membrane compartments to MTs. Hook 3 participates in defining the structure and localization of the Golgi complex (Walenta et al., 2001). Association of IFs with the Golgi complex mediated through FTCD has been described (Bashour and Bloom, 1998; Gao et al., 1998; Hennig et al., 1998). FTCD was originally identified as a novel MT-binding protein that can associate with both the Golgi complex and MTs (Bloom and Brashear, 1989), but was later found to bind vimentin and stimulate vimentin filaments formation. FTCD may therefore also serve as an anchor protein that integrates the Golgi compartment with the IF cytoskeleton (Gao and Sztul, 2001).

Potential additional functions of MACF1b: insights from Shot and MACF null cells

In *Drosophila*, Shot is essential for maintaining the stability of the epidermal cell layer (Gregory and Brown, 1998). Shot is concentrated at the apical and basal surfaces of epidermal muscle attachment cells, at the termini of MT bundles near

position-specific integrin adhesion complexes. Recently, another isoform of Shot that contains plakin repeats was described (Roper and Brown, 2003). Like MACF1b, the plakin repeats of this isoform of Shot are inserted between the ABD and the spectrin repeats and this isoform was localized to adherens junctions of embryonic and follicular epithelia. Loss of *shot* in the mutant flies disrupted adherens junction integrity. Fusion proteins of green fluorescence protein and the N-terminal plakin repeats of Shot localized to junctional areas, suggesting that this portion of plakin repeats can target Shot to the junctions (Roper and Brown, 2003). It is possible that MACF1b plakin repeats can also target to junctional complex and possess additional functions (i.e. linking MFs, MTs and/or IFs to junctions) besides its association with the Golgi complex.

MACF1 null endodermal cells have been derived from MACF1 knockout mice (Kodama et al., 2003). These cells exhibit defects in MT dynamics and the MTs are bent and parallel to the plasma membrane near the cell periphery. This phenomenon is probably due to the reduction in MT catastrophe frequency and continuous growth of MTs. Moreover, near the cortex of cells without MACF1, MTs fail to track along actin cables, suggesting that MACF1 is important for guiding MTs on actin filaments. When a confluent layer of cells is wounded, cells at the wound edge become polarized and MTs become stabilized and oriented towards the wound edge (Kodama et al., 2003). Reorientation is also observed for the Golgi complex and the MT organizing center. MACF1 null cells do not show any of these rearrangements. It is not known which MACF1 isoforms are expressed in the endodermal cells. Although the Golgi of MACF1 null endodermal cells failed to re-orient under external stimuli, it did not appear to be disrupted, in contrast to our siRNA results. This discrepancy may be due to the differences in the cell lines that were used in each study, since it is not known whether MACF1b is expressed in the endodermal cells. We also did not see any gross changes in the MT network as a result of the MACF siRNA treatment, but we do not know if there was an effect on microtubule dynamics.

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