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ORIGINAL PAPER

Molecular cloning and characterization of a novel mouse actin-binding protein Zfp185

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Abstract Zinc finger protein (Zfp) 185 is a mouse protein containing a Lin-l1, Isl-1 and Mec-3 (LIM) domains at its C-terminus. It was recognized by comparing the genome sequence between humans and mice in 1997. In this study, we cloned the full-length *Zfp185* by means of RACE and RT-PCR. Zfp185 may be closely associated with F-actin in cells as determined by a confocal microscopy. With a series of deletants of Zfp185 and GST-pull-down assay, we determined that N-terminus region (1–144) but not the LIM domain at C-terminus of Zfp185 protein was essential and sufficient to bind to F-actin cytoskeleton. Thus, our data offered evidence for the association of mouse Zfp185 with F-actin, which supports the potential role of Zfp185 in cell fundamental activity.

Keywords Zfp185 · Clone · Actin-binding

Abbreviations

ZNF185/Zfp185	Zinc finger protein 185
LIM	Lin-l 1, Isl-1 and Mec-3
RACE	Rapid amplification of cDNA ends
GFP	Green fluorescent protein

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Introduction

The DXS52 locus on chromosome Xq28 is associated with many diseases including otopalato-digital syndrome (Biancala et al. 1991), Goeminne syndrome (Zuffardi and Fraccaro 1982), X-linked mental retardation (Gedeon et al 1991; Nordstrom et al. 1992), the X-linked dominant form of chondrodysplasia punctata (Traupe et al. 1992), dyskeratosis congenital (Arngrimsson et al. 1993; Connor et al. 1986), neuronal chronic idiopathic X-linked intestinal pseudoobstruction (Auricchio et al. 1996) and periventricular heterotopia (Eksioglu et al. 1996). Therefore, identification of genes in this region is of interest and is important for us to be able to understand the molecular basis for these syndromes.

Zinc finger protein (Zfp) 185 gene was originally discovered by a positional cloning approach in the DXS52 region (Heiss et al. 1997). In parallel, the orthologous murine transcript was isolated from the syntenic region (Heiss et al. 1997; Levin et al. 1996). The human and mouse cDNA have been designated as *ZNF185* and *Zfp185* respectively. Comparisons between human and mouse genomes have showed gene construction and gene sequence conservation between *ZNF185* and *Zfp185* (Levin et al. 1996). Both ZNF185 and Zfp185 have a highly conservative LIM domain which is a cysteine- and histidine-rich domain containing a double tandem zinc finger motif and is named after the three homeodomain proteins: Lin-1, Isl-1 and Mec-3 in which LIM domain was originally identified (Jurata and Gill 1998). Proteins containing LIM domain can combine with other proteins and are closely involved in cytoskeleton organization, development regulation, cell adherence, signal transduction and neuronal pathfinding (Dawid et al. 1998; Brown et al 2001; Bach 2000). Further research to detect the mRNA expression by Northern blot

showed that human *ZNF185* is expressed in many normal tissues including the testis, placenta, prostate, ovary, peripheral blood, skeletal muscle, lung, kidney and some infant tissues (Heiss et al. 1997). In recent years, several research groups discovered through gene chip screens that expression of *ZNF185* changed in amounts when comparing tumor and normal tissue (Vanaja et al. 2003; Wu et al. 2002; Medina et al. 2005; Gonzalez et al. 2003). Zhang et al. recently showed that ZNF185 can bind to F-actin directly (Zhang et al. 2006). All of these suggest that ZNF185 is important in basic cell functioning and perhaps it is associated with the systemic inherited diseases listed above. The genes of mouse *Zfp185* have not been cloned, however it is very important to study the function of Zfp185 in animal models.

In this study, we have cloned the mouse *Zfp185* cDNA by means of 5′-RACE and RT-PCR. We also expressed the full-length mouse Zfp185 protein and its various deletions to determine the critical part of this protein for its actin-binding function. We found that the mouse Zfp185 is an actin-binding protein and its N-terminal region is an actin-binding region which has low conservation with other actin-binding domains. The present data indicate that the mouse Zfp185 is a cytoskeleton-associated protein that may be involved in modulating dynamics of actin filaments.

Materials and methods

Isolation of total RNA and 5′ RACE

Total RNA was extracted from mouse testes with Trizol™ according to the manufacturer's instructions. 5′-RACE was performed using the SMART™ RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. Briefly, 1 μg of total RNA was reverse-transcribed into 5′-RACE-ready cDNA using PowerScript reverse transcriptase, SMART II A oligonucleotide, and 5′-CDS primer. Then a PCR was performed with *Zfp185*-special primer 5′-TCGGTTGTCGAGCTGCTTAGGCCA GGA-3′ and UPM from the kit. The reaction conditions were as follows: first, a 2 min denaturation at 94°C, then 5 cycles of amplification (94°C for 30 s and 72°C for 3 min), another 5 cycles of amplification (94°C for 30 s, 70°C for 30 s and 72°C for 3 min), followed by 25 cycles of amplification (94°C for 30 s, 68°C for 30 s and 72°C for 3 min). The RACE products were ligated into pMD18-T vector (TaKaRa) and sequenced.

Plasmid constructs

The translation region of *Zfp185* was first cloned by PCR with primer pairs zfp185-F (5′-AGCTCACTGAATCC ACTAAT-3′) an dzfp185-R (5′-ACGCTGATACCTAGAC TTGT-3′). Then a nested PCR was performed with primer pairs zfp185-FN (5′-CTGAAAGATAGAATTCATGAGT ATTTCTTC-3′) and zfp185-RN (5′-CAGCTTCTGCTCG AGCTAGAAGAGCTTCTC-3′) to introduce an *Eco*RI/*Xho*I restriction site. The PCR product was digested with *Eco*RI/*Xho*I and cloned into pCDNA3, pEGFP-C2 and pGEX-4T-1 vectors digested at the same restriction sites. A series of deletants were also cloned by PCR with different primers and were constructed with the same strategy into a pEGFP-C2 vector. The reading frame of all constructs was identified by sequencing.

Generation of rabbit polyclclonal anti-Zfp185 antibody

Full-length Zfp185 protein was cloned into pGEX-4T-1 plasmid. Recombined GST-Zfp185 fusion protein was expressed in *E. coli* BL21(DE3) and purified by Glutathione Sepharose™ 4B beads (Amersham) according to the manufacturer's instructions. Immunization of rabbits was completed and serum was collected.

Cell culture and transfection

B16-F10 cells were cultured in a DMEM medium (Hyclone) supplemented with 10% FBS (Hyclone). GFP-Zfp185L, GFP-Zfp185LD, GFP-Zfp185SD, GFP-Zfp185D, GFP-Zfp185A, GFP-Zfp185S were expressed in vector pEGFP-C2 (Clontech). Full-length Zfp185 without tag was expressed in a vector pCDNA3 (Invitrogen). Transient transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Extraction of protein and immunoblot analysis

B16-F10 cells were digested by Trypsin-EDTA solution (0.25% Trypsin and 0.02% EDTA) and was washed three times with PBS. The cells were pelleted by centrifugation and lysed in icy RIPA solution (50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM PMSF and protease inhibitor cocktail [ROCHE]) by repetitive pipetting. Supernatant was collected by centrifugation and quantified by BCA assay. After electrophoresis and transferring to NC membrane (Pall), Zfp185 was detected with rabbit antiserum, as described previously.

Immunofluorescent staining and confocal microscope

B16-F10 cells were grown on cover slides overnight and then washed with PBS. Where indicated, cells were treated

with cytochalasin D (10 μg/ml) for 30 min before the staining process. The cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.2% TritonX-100 in PBS for 15 min at room temperature. Then the cells were blocked with 10% goat serum for 30 min. TRITC-conjugated phalloidin (Molecular Probes) was diluted into 5 U/μl and incubated with the cells for 30 min at room temperature. The slides were washed three times and mounted with antifade reagent (Zhongshan, Beijing). The cells were observed under a LSM510 laser confocal microscope with a 100× oil objective.

Pull-down assay

GST-Zfp185 fusion protein was expressed in *E. coli* BL21(DE3), as mentioned above. The fusion protein in supernatant was collected with Glutathione Sepharose™ 4B beads and then washed three times with PBS. The beads were incubated with B16-F10 lysate for 2 h at 4°C and the beads were washed three times with PBS. The beads were lysed with a 6× loading buffer and we performed an SDS-PAGE and an Immunoblot assay detected by Coomassie brilliant blue staining and anti-actin monoclonal antibody (Sigma).

Results

Cloning and sequence analysis of mouse Zfp185

The genetic structure of Zfp185 is similar to that of human ZNF185 which has been proved to have no special splicing in its 3′ untranslated region. In addition, studies using PT-PCR with a series of primers, targeted different sites in the 3′ untranslated region and showed a single predicted band. According to the preliminary results, we considered that there was no special splicing in the 3′ untranslated region of the mouse Zfp185 gene. However, by means of 5′ RACE, we identified the putative full-length sequence of *Zfp185* as described in the Materials and methods section. Through RT-PCR from mouse testis RNA with the primers zfp185-F and zfp185-R as well as another nested PCR with the primers zfp185-FN and zfp185-RN, the full length of the Zfp185 gene was obtained (GenBank access No: EF494744; Fig. 1A). Compared with the predicted sequence (NM_009549) (Mallon et al. 2000; Davies et al. 2005), there is an additional sequence of 144 amino acids ahead of its N terminus as described in Fig. 1 and marked by a dashed line. An in-frame stop codon was found at −15 upstream of the putative translation start site. Thus, this cDNA sequence appears to contain the full-length protein-coding region of the *Zfp185* gene. Analysis of the putative translated sequence showed that there was a conserved LIM domain at C terminus of Zfp185 and no other significant homology to other proteins.

Analysis of the conversation of Zfp185 and the LIM domain

Although Zfp185 had little similarity with other proteins except the LIM domain, it shared a 46.5% identity with human ZNF185 in protein sequence as shown in Fig. 1B. The alignment showed that the protein sequences of ZNF185 and Zfp185 were highly conservative at the N- and C-terminal regions. In comparison with human ZNF185, mouse Zfp185 deleted nearly 200 amino acids in the middle of the protein sequence. The sequence of N-terminal 135 amino acids, which had been characterized to be an actin-binding region in human cell lines (Zhang et al. 2006), is 61% identical to the two proteins. The sequence of the LIM domain at C-terminal is 93% identical across species.

Zfp185 protein is associated with actin cytoskeleton structure

In order to determine the intracellular location and distribution of Zfp185, we generated GFP and His-tagged versions of Zfp185. The plasmid inserted with His-tagged Zfp185 expressed a protein of predicted size and was the same as the endogenous Zfp185 expression detected by the antibody against Zfp185 as shown in Fig. 2A. Then we examined the intracellular localization of GFP-Zfp185 in the B16-F10 cells by immunofluorescent (IF) staining and a confocal microscopy. As shown in Fig. 2B, GFP-Zfp185 as mainly expressed in the cytoplasm can target the actin cytoskeleton structure (Fig. 2B).

The amino acids 1–144 of Zfp185 are required for targeting the actin cytoskeleton structure

To identify which domain/region in Zfp185 is responsible for targeting the actin cytoskeleton, we generated a series of Zfp185 truncation constructs fused to GFP (Fig. 3A) and transfected them into B16-F10 cells. After the staining of F-actin by phalloidin, the expressions of Zfp185 and actin in the cells were detected by confocal microscopy. It appeared that only the full-length or N terminus of Zfp185 can target actin cytoskeleton (Fig. 3B a–f, j–f). Those constructs of Zfp185 without the 144 amino acids at N terminus can only localize dispersedly in cytoplasm (Fig. 3B j–i, m–r). The GFP-LIM domain fusion protein showed no specific localization in cytoplasm. All these results indicate that the N terminus 144 amino acids of Zfp185 are critical for its normal localization in cytoplasm.

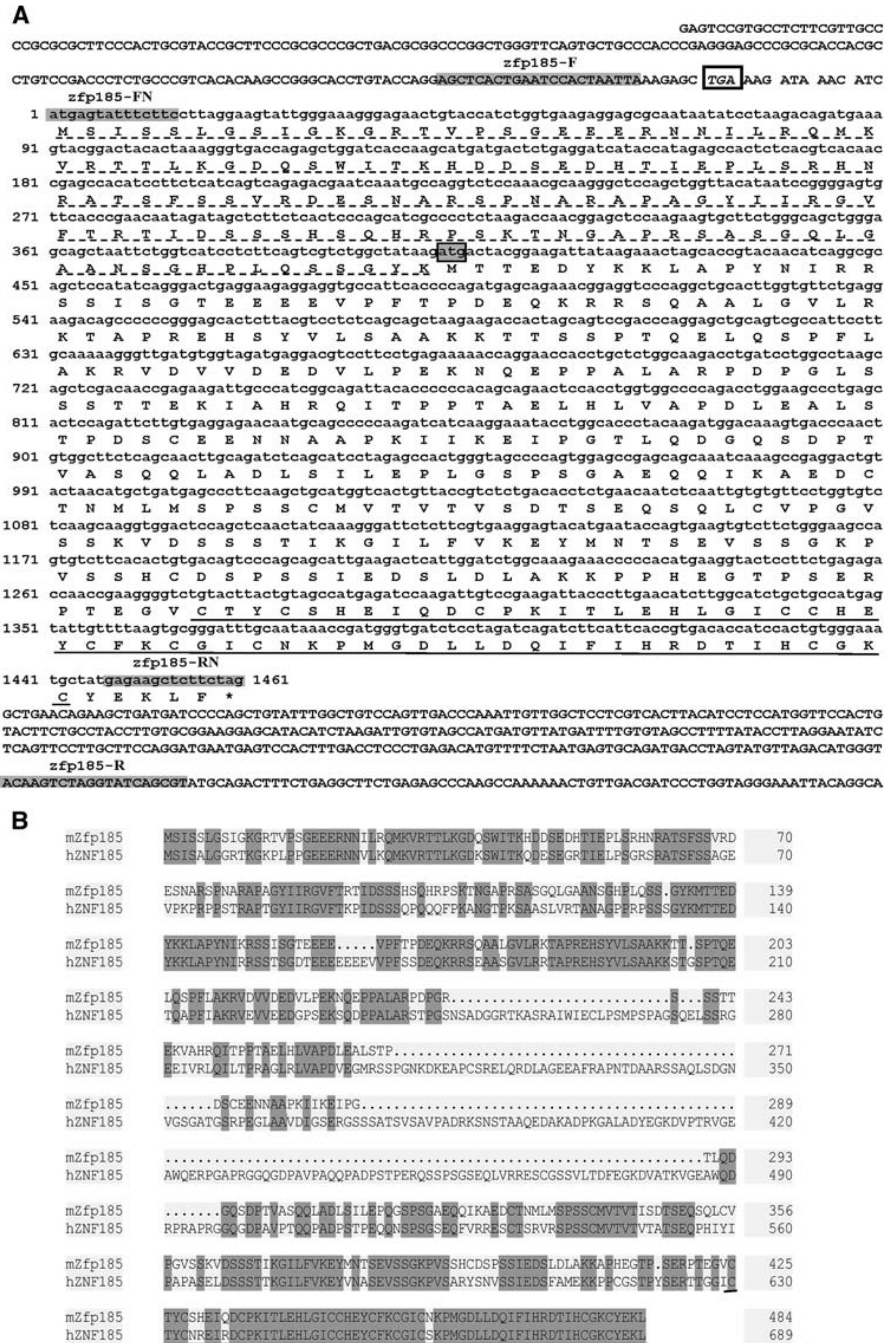

Fig. 1 Nuclear and amino acid sequences of mouse Zfp185 genes as well as protein sequence comparisons of human ZNF185 (hZNF185) and mouse Zfp185 (mZfp185). (**A**) The in-frame stop codon in the 5′ UTR region is boxed and the stop codon for the translated protein is indicated by an asterisk. The thick underline indicates the LIM domain in mouse Zfp185 and the dashed underline indicates the different region compared with the sequence predicted by Mallon et al. The predicted start codon is boxed and shaded. The in-frame stop codon at −15 upstream of the putative translation start site is boxed. The primers are shaded in gray. PCR was performed with primer pair Zfp185-F and Zfp185-R for the first time. Nested PCR was performed with primer pair Zf185-FN and Zfp185-RN for the Zfp185 gene ORF. (**B**) Protein sequence comparison of human ZNF185 (hZNF185) and mouse Zfp185 (mZfp185). Identical amino acids are shaded and LIM domain is underlined. For optimal alignment, gaps have been introduced as indicated by dots

Interaction of Zfp185 with actin filaments

To further confirm the F-actin binding property, B16-F10 cells transfected with full-length, Zfp185-A and Zfp185-SD expression plasmids were treated with cytochalasin B for 30 min. After staining the F-actin by phalloidin, the expressing patterns of F-actin and Zfp185 were observed by confocal microscopy. The results show that, similar to the full-length Zfp185, Zfp185-A can colocalize with phalloidin staining under cytoskeleton disruption (Fig. 4A a–l). However, Zfp185-SD dispersedly localized in cytoplasm under both treated and untreated conditions (Fig. 4A m–r).

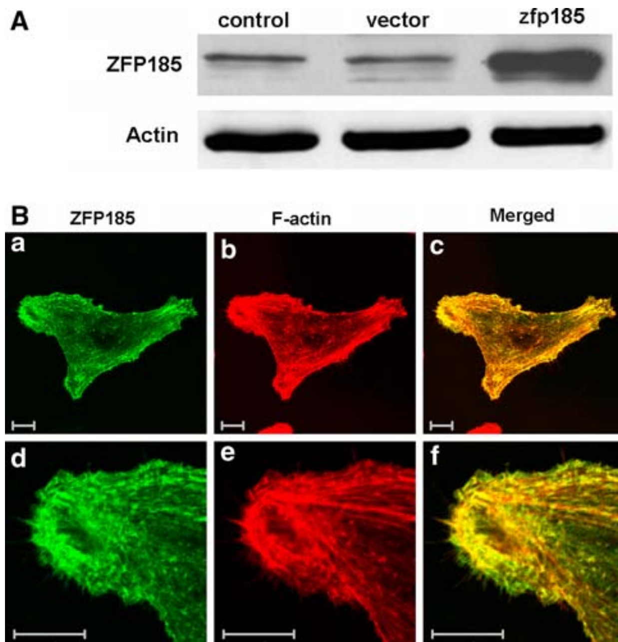

Fig. 2 Expression and localization of Zfp185 in B16-F10 cells. (**A**) Expression of Zfp185 (top) and β-actin (bottom) in B16-F10 cell line detected by Western Blot. Control, untreated; vector, transfected with pCDNA3 control plasmid; Zfp185, transfected with pCDNA3-Zfp185 plasmid. (**B**) B16-F10 cells were transfected with full-length Zfp185 fused with GFP and detected by confocal microscopy. F-actin was stained with phalloidine-TRITC. The size bar is 10 μm for all images. One representative of three experiments is shown

To further confirm the interaction between Zfp185 and actin, a pull-down assay was performed with prokaryotic expressed GST-Zfp185 in B16-F10 cells. As shown in Fig. 4B, Zfp185-A can bind actin (Fig. 4B, lane 5) similar to the full-length of Zfp185 (Fig. 4B, lane 3). But Zfp185-SD can not bind to actin (Fig. 4B, lane 4) similar to the control GST (Fig. 4B, lane 2). Taken together, these results showed that the 144 amino acids at N terminus of Zfp185 are necessary to target the full-length protein to actin cytoskeleton structure.

Discussion

In the present study, the full length of mouse Zfp185 was cloned. Zfp185 cDNA sequence appears to contain the full-length protein-coding region of the Zfp185 gene. The sequence of N-terminal 135 amino acids, which had been characterized to be an actin-binding region in human cell lines (Zhang et al. 2006), is 61% identical to mouse proteins. The sequence of LIM domain at C-terminal reveals a 93% identity across species.

After over-expressing GFP-Zfp185 into B16-F10 cells, only the full-length or N terminus of Zfp185 can target the actin cytoskeleton as determined by a confocal microscope.

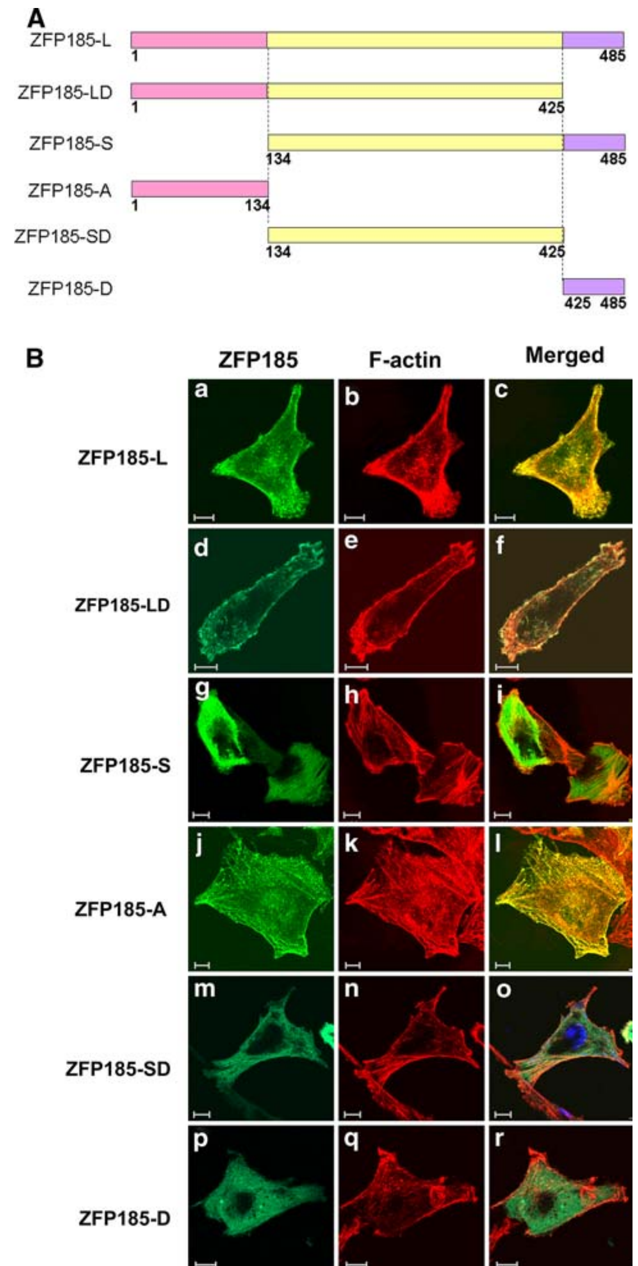

Fig. 3 N-terminal region, but not C-terminal region of Zfp185 is required for actin-cytoskeleton targeting. (**A**) Schematic representation of Zfp185 constructs which fused to GFP at N-terminal. (**B**) The association of GFP-Zfp185 with F-actin. B16-F10 cells were transfected with various constructs expressing GFP-fused wild-type and mutant Zfp185 protein. F-actin was stained with phalloidine-TRITC. The size bar is 10 μm for all images. One representative of two experiments is shown

Although it was a special kind of zinc finger structure that has always been identified to mediate protein–protein interaction, the LIM domain, the only noticeable conserved domain in Zfp185 protein, had no special localization in cells and diffused in both the nucleus and cytoplasm which was similar to its analogues ZNF185 as described by Zhang

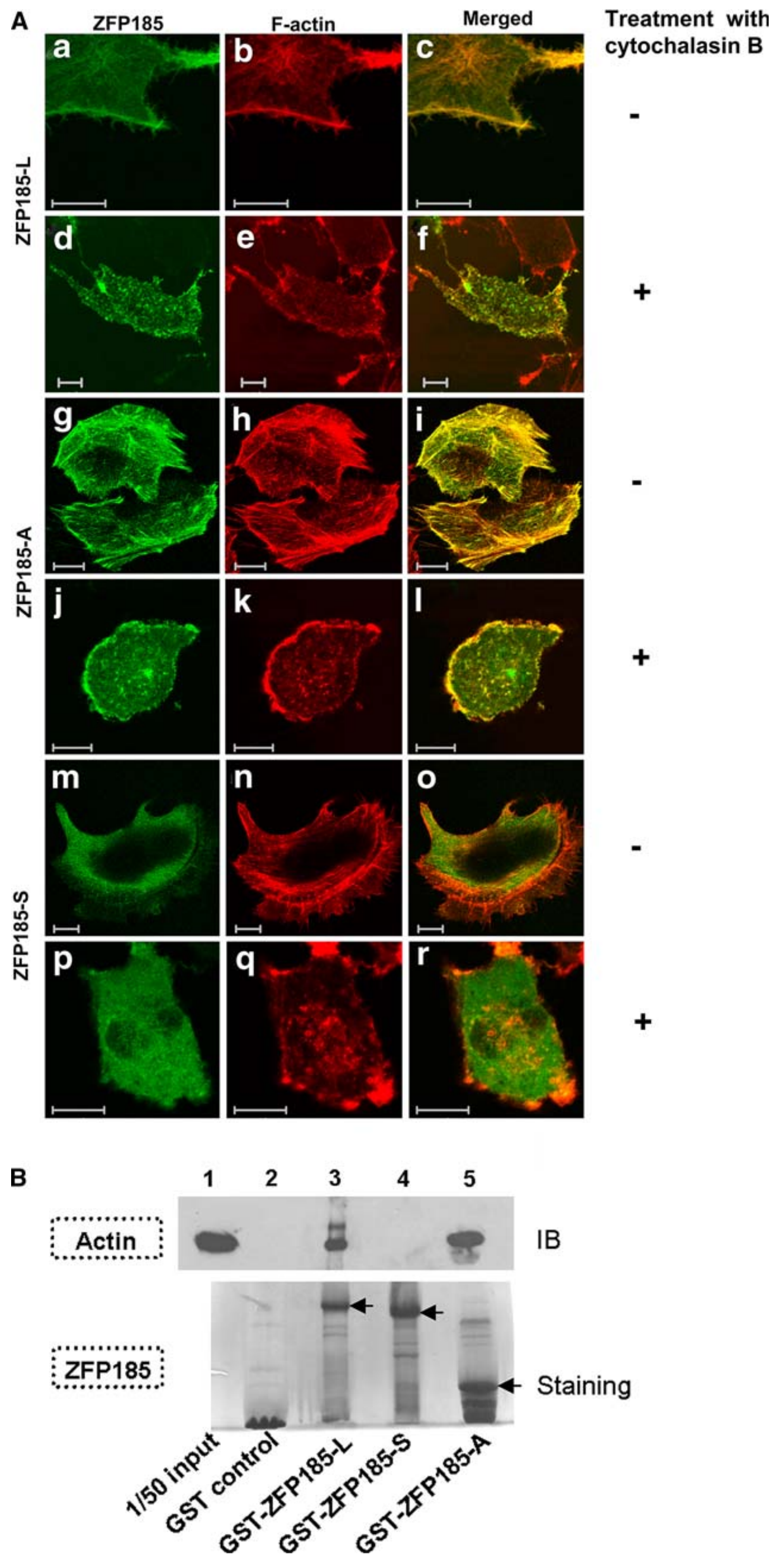

Fig. 4 N-terminal region of Zfp185 mediates F-actin binding and actin-cytoskeleton targeting. (**A**) B16-F10 cells were transfected with various constructs expressing GFP-fused wild-type and mutant Zfp185 protein, and 24 h post-transfection, cells were treated with cytochalasin B for 30 min before being processed for IF staining with TRITC-phalloidin and confocal microscopy. F-actin was stained with phalloidine-TRITC. The size bar is 10 μm for all images. (**B**) Co-immunoprecipitation of GST-Zfp185 with β-actin. Actin was detected by Western blot analysis with anti-actin antibody (top panel). The GST-Zfp185 band is indicated by the arrow in the panel and was detected by staining with Coomassie-blue in a SDS-PAGE gel. Lane 1 was 1/50 input of B16-F10 supernatant and Lane 2 was GST control. Lanes 3–5 were GST-Zfp185-L, GST-Zfp185-S and GST-Zfp185-A, respectively. One representative of three experiments is shown

et al. (2006; Krupp et al. 2006). The middle part of Zfp185 diffused in cytoplasm but showed no detectable function according to our data. The direct interaction between Zfp185 and actin was further supported by the pull-down assay showing that only the full-length or N terminus but not other parts of Zfp185 can bind to actin. In addition, the Zfp185-transfected adherent cells appeared to be flatter with more extended lamellipodia (data not shown). The cells transfected with GFP-Zfp185LD (deletant without LIM domain but within an actin-binding region in N-terminus) extended more dendrites than wild cells (data not shown). The function of Zfp185 in actin cytoskeleton organization remains elusive at present.

In 2005 Medina et al. reported that the recruitment of BRG1, a chromatin remodeling enzyme, can increase expression level of ZNF185 in lung cancer cell lines (Medina et al. 2005). Similarly with ZNF185, the expression of BRG1 also appeared to down-regulate in some cancer tissues such as lung cancer and prostate cancer (Wong et al. 2000; Reisman et al. 2003; Valdman et al. 2003). So BRG1 may be one of the up-stream genes of ZNF185. BRG1 had been characterized to function in actin cytoskeleton organization (Rosson et al. 2005), cancer suppression (Gunduz et al. 2005; Medina et al. 2004; Hendricks et al. 2004; Strobeck et al. 2002), neuron division (Seo et al. 2005; Matsumoto et al. 2006) and T lymphocyte activation (Gebuhr et al. 2003). These data suggest that ZNF185 may be involved in the biological processes of the cells mentioned above. This needs to be determined by further research.

Data from gene screens showed that the level of *ZNF185* mRNA was down-regulated in lung cancer (Medina et al. 2005), prostate cancer (Vanaja et al. 2003; Zhang et al. 2006) and head and neck squamous cell carcinoma (Gonzalez et al. 2003) as compared to normal tissues. Over-expression of ZNF185 in human prostate cancer cell lines can significantly inhibit cancer cell proliferation (Zhang et al. 2006). All of these data support the conclusion that ZNF185 may be a tumor suppressor. Also we have found that protein expression of Zfp185 increased during T lymphocyte activation (data not shown).

In summary, the full-length of Zfp185 was cloned. Only the full-length or N terminus (1–144) but not other parts of Zfp185 can bind to actin, indicating the potential role of Zfp185 in cell cytoskeleton regulation. Further studies are required to determine the function of this novel gene.

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