Identification of a Novel Human Zinc Finger Gene, ZNF438, with Transcription Inhibition Activity

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There were many different families of zinc finger proteins that contained multiple cysteine and/or histidine residues and used zinc to stabilize their folds. The classical C2H2 zinc finger proteins were the founding members of this superfamily and were among the most abundant proteins in eukaryotic genomes. C2H2 proteins typically contained several C2H2 fingers that made tandem contacts along the DNA. Here we reported a novel C2H2 type zinc finger gene, ZNF438, which encoded 828 amino acids that formed five zinc finger domains. Bioinformatics analysis revealed that the ZNF438 was mapped to human chromosome 10p11.2 and shared 62% identity with rat and mouse homologues. RT-PCR analysis indicated that it was ubiquitously expressed in 18 human adult tissues. With immunofluorescence assay, it was shown that the exogenous Flag-tagged ZNF438 was located in nucleus of COS-7 cells. To further explore the function of ZNF438, we examined the transcriptional activity of ZNF438 protein by transfecting recombinant pM-ZNF438 into mammalian cells. The subsequent analysis based on the duel luciferase assay system showed that ZNF438 was a transcriptional repressor.

Keywords: C2H2, Transcription inhibition, Transcriptional repressor, Zinc finger, ZNF438

Introduction

The zinc finger domain was first recognized as a repeated zinc-binding domain that contained conserved cysteine and histidine residues in Xenopus laevis transcription factor IIIA (TFIIIA) (Brown et al., 1985; Miller et al., 1985). Ever since then, numerous other zinc-binding domains had been identified and designated as zinc fingers. Zinc fingers constituted one of the most common structural domains in eukaryotes. According to their structural diversities, they were classified into eight groups: C2H2-like finger, Gag knuckle, treble clef finger, zinc ribbon, Zn2/Cys6-like finger, TAZ2 domain-like, short zinc binding loops and metallothioneins (Krishna et al., 2003). Zinc fingers were not only structurally diverse, but they also had distinct functions. They were involved in nucleic acid and lipid binding as well as in protein-protein interactions (Grishin, 2001). By performing these functions, zinc fingers played an important role in many fundamental cellular processes, such as replication and repair, transcription and translation, metabolism and signaling, cell proliferation and apoptosis.

The C2H2 zinc finger family belonged to the C2H2-like finger group according to Krishna’s classifying method, and represented the classical type of zinc fingers. All C2H2 zinc fingers typically contained a repeated 30 amino acid sequence, which folded in the presence of zinc to form a compact ββα domain (Wolfe et al., 2000). The zinc was coordinated between two cysteines at one end of the β-sheet and two histidines in the C-terminal portion of the α-helix.

Proteins containing C2H2 zinc fingers were quite common in eukaryotic organisms. In the human genome, approximately 700 C2H2 type zinc finger genes were found, making it the second most frequently occurring domain in the human proteome (Venter et al., 2001). These genes played important roles during differentiation and development through their involvement in DNA binding, causing the specific activation or repression of gene expression. The DNA binding activity...
of C2H2 zinc finger proteins had been studied in considerable detail. DNA recognition usually required 2 to 4 tandemly arranged zinc fingers; if only one or two finger were present, other secondary structure elements were generally used to augment DNA recognition. Some zinc finger proteins functioned independently as the major regulator of a set of genes, such as TFIIIA (Shasta, 1996) and NRSF/REST (Schoenherr and Anderson, 1995), while others worked cooperatively with other DNA binding proteins, such as the members of SP1 family (Lania et al., 1997). However, the zinc finger domain was used not only for protein-DNA but also for protein-RNA and protein-protein interactions (Lania, 1996; Mackay and Crossley, 1998).

In this report, we described the isolation of a novel human C2H2 zinc finger gene ZNF438. Genomic structure analysis indicated that this gene contained 7 exons and was localized to human chromosome 10p11.2. By RT-PCR it was found that ZNF438 was ubiquitously expressed in all human adult tissues. Subcellular localization revealed that ZNF438 was localized in nucleus when overexpressed in COS-7 cells. Our study also presented that ZNF438 protein possessed transcription inhibition activity and might play an important role as a transcription factor.

Materials and Methods

Cloning and sequencing of ZNF438 cDNA. According to the sequences flanking the predicted open reading frame (ORF), two pairs of primers ZNF-clone-F1/ZNF-clone-R1 and ZNF-clone-F2/ZNF-clone-R2 (Table 1) were designed to amplify ZNF438 from the brain cDNA library. The PCR reaction was performed as follows: initial 5 min at 94°C and then 35 cycles of 60 s at 94°C; 60 s at 53°C, 120 s at 72°C, and a final 5 min at 72°C. Two amplified fragments, F1R1 and F2R2, were obtained and subsequently integrated into a cDNA fragment with another PCR.

The final PCR product was then subcloned into the pMD-18-T vector (Takara) and was sequenced. The generated sequences had been deposited to GenBank Data Library under Accession No. AF428258.

Bioinformatics analyses. DNA and protein sequence comparisons were carried out by BLAST at NCBI Web server (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment was performed using the CLUSTALX and GENDOC program. Chromosomal localization and genomic organization prediction were carried out using the UCSC Genome Browser (http://www.genome.ucsc.edu/cgi-bin/hgBlat). Motif analysis was performed at SMART server (http://smart.embl-heidelberg.de/). SUMOplot (http://www.abgent.com/doc/sumoplot) was used to predict the probability for the SUMO consensus sequence to be engaged in SUMO attachment.

Plasmid construction. All inserted fragments were obtained by PCR amplification using the primers listed in Table 1. Full length ZNF438 was subcloned into the eukaryotic expression vector pFlag-CMV at HindIII and XbaI sites using primers pFlag-CMV-ZNF438-F and pFlag-CMV-ZNF438-R. The full-length of ZNF438 was subcloned into eukaryotic expression vector pM at BstXI and XbaI sites using primers pM-ZNF438-F and pM-ZNF438-R. After confirmation by sequencing, all eukaryotic expression plasmids were extracted and purified for transfection using Endofree Plasmid Maxi Kit (Qiagen).

RT-PCR. The human multiple tissue cDNA (MTC) panels (Clontech) were used as PCR templates according to the manufacture’s protocol. The specific RT-PCR primers of ZNF438 and β2-MG were listed in Table 1. 32 cycles for ZNF438 and 26 cycles for β2-MG of amplification (30 s at 94°C; 30 s at 54°C, 40 s at 72°C) were performed using DNA polymerase. The PCR products of ZNF438 and β2-MG were then electrophoresed on 2% agarose gel.

Cell culture, transfection and western blotting. COS-7 and H1299 cells were grown in RPMI 1640 medium (GIBCO BRL).

<p>| Table 1. Nucleotide sequence of oligonucleotides used for cloning of ZNF438 and construction of expression vectors |</p>
<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>cDNA cloning primers</td>
<td></td>
</tr>
<tr>
<td>ZNF-clone-F1</td>
<td>5'-TGCAGAAGGAAAGGGAAG-3'</td>
</tr>
<tr>
<td>ZNF-clone-R1</td>
<td>5'-TGTAGGGAACGGGAGGAG-3'</td>
</tr>
<tr>
<td>ZNF-clone-F2</td>
<td>5'-AAAGAGAAAAAGGAAGAAACG-3'</td>
</tr>
<tr>
<td>ZNF-clone-R2</td>
<td>5'-TGTTTGTCATTTCTAGCCTT-3'</td>
</tr>
<tr>
<td>RT-PCR primers</td>
<td></td>
</tr>
<tr>
<td>ZNF438-RF-F</td>
<td>5'-TGAATCTAACGTCACTCAGGAGGAC-3'</td>
</tr>
<tr>
<td>ZNF438-RF-R</td>
<td>5'-AAGGGATGTGTTGATCCACCTTCATC-3'</td>
</tr>
<tr>
<td>β2-MG-RF-F</td>
<td>5'-ATGAGATGGCGCTCCGTTTGAC-3'</td>
</tr>
<tr>
<td>β2-MG-RF-R</td>
<td>5'-TGTTGAGCGAACCTCTGCTAG-3'</td>
</tr>
<tr>
<td>Gene expression primers</td>
<td></td>
</tr>
<tr>
<td>pFlag-CMV-ZNF438-F</td>
<td>5'-TTCAAGAGCTTAACGAGATTCTGATATGCAGTAC-3'</td>
</tr>
<tr>
<td>pFlag-CMV-ZNF438-R</td>
<td>5'-AACTCTAGATCTTCTACCCGTCCTG-3'</td>
</tr>
<tr>
<td>pM-ZNF438-F</td>
<td>5'-AAAGATCTTCAGAGATTCTGATATGCAGTAC-3'</td>
</tr>
<tr>
<td>pM-ZNF438-R</td>
<td>5'-AAGTCTAGATCTTCTACCCGTCCTG-3'</td>
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with 10% fetal calf serum (GIBCO BRL); HEK293T cells were grown in DMEM medium (GIBCO BRL) supplemented with 10% fetal calf serum. The cultured HEK293T or COS-7 cells were seeded. After overnight growth the cells were transfected with expression vectors using Lipofectamine™ (Invitrogen) according to the manufacturer's protocol. Protein samples separated by SDS-PAGE were electro-transferred onto nitrocellulose membrane. The membrane was blocked at room temperature for 1 h with PBS containing 5% (w/v) BSA, and then washed with PBS containing 0.2% Tween-20 (Sigma; PBST). After blocking, the membrane was then incubated overnight at 4°C with primary antibody diluted with PBS containing 3% (w/v) BSA. The membrane was washed 3 times with PBST then incubated with HRP-conjugated secondary antibody at room temperature for 1 h. The membrane was washed 3 times again with PBST and then developed with ECL system (Santa Cruz Biotechnology).

Transcriptional function assay of ZNF438. The Gal4 fusion plasmid pM-ZNF438 was co-transfected with 100 ng of reporter plasmid pGal45tkLUC into 1 × 10⁶ HEK293T or H1299 cells on 24-well plates (Falcon), and luciferase activity was measured with Lumat LB9507 (Berthold). Moreover, 20 ng of plasmid pRL-SV40 (Promega) encoding Renilla luciferase was used as an internal control in each transfection. The plasmids pM3-VP16 and pM-ZNF438 were then used as activation and repression controls. The assay system of dual-luciferase reporter (Promega) was used to assay the luciferase activity according to the manufacturer's protocol. Transfection of each construct was repeated four times in one assay and each assay was repeated at least three times. The relative luciferase activity represented the value of the firefly luciferase versus that of Renilla luciferase, and all data were analyzed by statistics method (t-test).

Immunofluorescence analysis of ZNF438 subcellular localization. COS-7 cells were plated on cover slips and transfected with 900 ng of respective expression vectors pFlag-CMV-ZNF438 with Lipofectamine (Invitrogen) according to the manufacturer's protocol. 36 h after transfection, the cells were fixed in 4% paraformaldehyde for 10 min. The cells were washed once with TBS [50 mM Tris-HCl (pH 7.4), 150 mM NaCl], then permeabilized with 0.2% Triton X-100 for 5 min; washed three times in TBS followed by incubated in fresh 0.1% sodium borohydride in TBS for 5 min to quench endogenous fluorescence. After blocked in blocking buffer (1% horse serum, 1% BSA, 1 × PBST) for 1 h, the cells were incubated with Flag-antibody overnight at 4°C then washed three times in TBS and incubate with goat anti-mouse IgG Fluorescein-conjugate antibody (Upstate Biotechnology) for 30 min in dark. After washed two times in TBS, the slips were incubated with DAPI (4, 6-diamidino-2-phenylindole) for 15 min and wash another two times in TBS and drained. Microscopy observation was carried out under fluorescent light at relevant wavelengths.

Results and Discussion

Molecular cloning and characterization of human ZNF438. When searching human EST databases for sequences with homology to the conserved Cys2/His2 zinc finger motif, we obtained a series of human ESTs and assembled them into a contig of 3151 nucleotides. This contig contained an open reading frame (ORF) of 2487 nucleotides. To verify the contig, PCR primers (ZNF-clone-F1/ZNF-clone-R1, ZNF-clone-F2/ZNF-clone-R2) (Table 1) were designed to perform PCR in a human brain cDNA library. Two amplified fragments, F1R1 and F2R2, were obtained and subsequently integrated into a cDNA fragment. The final PCR product was then subcloned into the pMD-18-T vector (Takara) and was sequenced. The sequencing result verified the contig sequence, and this sequence was subsequently deposited in GenBank with the GenBank Accession No. AF428258 and was later named ZNF438 by the Human Gene Nomenclature Committee. As shown in Fig. 1, the cDNA sequence had an intact ORF extending from nucleotide 331 to 2817, and it encoded a putative protein of 828 amino acids. An in-frame stop codon upstream of the initiator, and a typical polyadenylation signal, AATAAA, was found at two distinct sites: n. 2974-2979 and 3068-3073.

The molecular weight of the putative protein was predicted to be 91.8 kDa. After searching the SMART database (http://smart.embl-heidelberg.de/), we found that the protein contained five C2H2 zinc finger domains at the C-terminus (Fig. 1). The first two domains belonged to the Krüppel type; the other three were non-Krüppel type. ZNF438 lacked some N-terminal domains that were present in other zinc finger proteins such as POZ, KRAB, SCAN, FARM and ZIN (Knochel et al., 1989; Bellefroid et al., 1993; Nuttall et al., 1993; Bardwell and Treisman, 1994; Zollman et al., 1994; Williams et al., 1999). These domains seemed to be involved in transcriptional regulation and protein-protein interactions. However, some zinc finger proteins, such as the GC box binding transcription factor Sp3 protein, lacked those conserved domain, but still displayed transcriptional activity (Hagen et al., 1994; De Luca et al., 1996; Liang et al., 1996; Kumar and Butler, 1997; Noti, 1997; Suske, 1999; Ross et al., 2002).

The multiple sequence alignments indicated that the putative human ZNF438 protein exhibited 98.4% identity to chimpanzee (XP_001138402), 96.4% identity to orangutan (CAH0705), and 62% identity to the mouse (NP_848373) and rat (XP_001056328) homologous protein, respectively. And the alignments showed us that human ZNF438 protein had the highest similarity to chimpanzee and orangutan homologous protein, while the mouse homologous protein was closest to the rat homologous protein (Fig. 2A). We analyzed subsequently the evolutionary relationship among different zinc finger proteins with cladogenesis tree analysis (Fig. 2B), which showed the relationship among the ZNF438 homologous proteins more clearly. Furthermore, the N-terminal sequence of ZNF438 was more conserved than the middle sequence when compared to the mouse and rat homologues. This suggested that there might be another conserved domain at the N-terminal that remained to be identified.
Further sequence analysis indicated that the putative human ZNF438 had seven highly probable SUMO binding sites (Table 2). SUMO was a small ubiquitin-related modifier that got attached to lysine residues in target proteins via an isopeptide linkage in a process that is analogous to ubiquitination (Melchior, 2000). SUMO modification was a dynamic, reversible reaction, and distinct enzymes were responsible for adding or removing it from target proteins. Several transcription factors, such as the androgen receptor, c-jun, p53, NF-κB, and Sp3, had been found to be modified by sumoylation (Gostissa et al., 1999; Poukka et al., 1999; Rodriguez et al., 1999; Muller et al., 2000; Poukka et al., 2000; Rodriguez et al., 2001; Rois et al., 2002), which causes the repression of their transcriptional activation. We speculated that ZNF438 would also undergo such post-translational modification based on the presence of the SUMO sites.

**Chromosomal localization and genomic organization of human ZNF438.** With the completion of the draft sequence of the human genome, it was possible to localize a gene with known sequence to a specific site on a chromosome. By searching the UCSC genomic database (http://genome.ucsc.edu), the human ZNF438 was localized to 10p11.2. Numbers of well-known gene, such as PAPD1, MAP3K8, LYZL2, and TCF8, were localized near ZNF438. Genomic structure analysis of this gene revealed that it contained 7 exons, and the exon/intron boundaries were all consistent with the AG/GT rule (Fig. 3).

**Tissue distribution of human ZNF438.** With using the human MTC (multiple-tissue cDNA) panels (Clontech), the tissue distribution of ZNF438 transcription was examined by cycle-limited reversed transcription polymerase chain reaction (Fig. 1).
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As shown in Fig. 4, ZNF438 was expressed ubiquitously in the eighteen tissues examined. However, it was strongly expressed in adult uterus, bladder, colon, ovary, testis, skeletal muscle, heart and brain, while weakly expressed in bone marrow, kidney, liver and stomach.

Table 2. Putative SUMO acceptor sites of ZNF438

<table>
<thead>
<tr>
<th>No.</th>
<th>Position</th>
<th>SUMO acceptor sites</th>
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<tbody>
<tr>
<td>1</td>
<td>K309</td>
<td>MEVYK IKSD ANIAG</td>
</tr>
<tr>
<td>2</td>
<td>K416</td>
<td>DGKER VKND PQEFQR</td>
</tr>
<tr>
<td>3</td>
<td>K503</td>
<td>NGFSG IKKP WHRC</td>
</tr>
<tr>
<td>4</td>
<td>K118</td>
<td>SLPEN LKPL IPYQ</td>
</tr>
<tr>
<td>5</td>
<td>K230</td>
<td>TPEEP AKOD LTALS</td>
</tr>
<tr>
<td>6</td>
<td>K648</td>
<td>EVKLO IKCG RCQYT</td>
</tr>
<tr>
<td>7</td>
<td>K709</td>
<td>RHPER GKPE KVHSS</td>
</tr>
</tbody>
</table>

Fig. 2. The alignment of the deduced amino acid sequences of human ZNF438 (AAP97298), chimpanzee homologue (XP_001138402), orangutan homologue (CAH90705), mouse homologue (NP_848837) and rat homologue (XP_001056328). (A) Identity was indicated by black shading and the similarity was indicated by gray shading. Dashes and spaces introduced to optimize the alignment; dots omitted amino acid residues outside of the domain. The sequences were aligned by ClustalX software and viewed by GENEDOC software. (B) Unrooted phylogenetic tree of the five members were constructed by comparing the whole sequences using the ClustalW software.

Transcriptional function analysis of human ZNF438

Several kinds of protein motifs that are important to DNA recognition and binding have been identified in transcription factors, such as zinc finger, helix-loop-helix, helix-turn-helix, and leucine zipper (Busch and Sassone-Corsi, 1990).

Multiple papers showed that the zinc finger motifs existed in numerous transcription factors. Lots of zinc finger transcription factors, such as ZNF191 (Han et al., 1999), ZNF396 and ZNF397 (Wu et al., 2003), FOXP3 (Lopes et al.,
functioned as transcriptional repressors with the putative zinc finger domain providing DNA-binding specificity. Accordingly, it was reasonable to postulate that the ZNF438 might possess transcription-regulating activity because of its zinc finger motif. With the experiment based on dual luciferase assay system to assay the transcription function, the ORF of ZNF438 was inserted into mammalian cell expression plasmid pM-vector, and recombinant plasmid pM-ZNF438 encoding the fusion protein Gal4-ZNF438 was cotransfected into different cell lines with reporter plasmid pGal4tkLuc encoding firefly luciferase which was correlated with the transcription-regulating activity of ZNF438, and the plasmid pRL-SV40 encoding Renilla luciferase which provided an internal control to serve as the baseline response, respectively.

One negative control, mock pM-vector, and two positive control, pM3-VP16 and pM-ZNF191, were introduced in this system. Among them, VP16 served as transcriptional activator and ZNF191 served as transcriptional repressor (Han et al., 1999). The luciferase activities of samples were determined by Dual-luciferase reporter assay system (Promega, Madison, USA). In the Dual-luciferase reporter assay system, the activities of firefly and Renilla luciferase were measured sequentially from a single sample. The relative luciferase activity that normalized the activity of firefly luciferase to the activity of Renilla luciferase was to minimize experimental variability caused by differences in cell viability or transfection efficiency, and to eliminate effectively other sources of variability, such as differences in pipetting volumes, cell lysis efficiency and assay efficiency. As shown in Fig. 5, the pM-ZNF438 could significantly inhibit the expression of the firefly luciferase reporter. Compared with that of the negative control, the firefly luciferase activity were down-regulated about 3 and 2 folds in HEK293T and H1299 cells, respectively ($p < 0.001$, $n = 4$).

Furthermre, we explored whether transcriptional repression of the reporter gene was proportioned to the used amount of ZNF438. In this experiment, we applied a fixed amount of pGal4tkLuc reporter gene with varying amounts of ZNF438 to transfect HEK293T cells. By t-test statistical analysis, it was shown that ZNF438 had dose-dependent expression inhibition of the reporter gene (Fig. 5D). Therefore, it seemed that ZNF438 might play its role as transcriptional repressor in mammalian cells.

Subcellular localization of human ZNF438. After we indicated that the ZNF438 had a transcription repression function, the chromosomal localization and genomic organization of human ZNF438 were indicated. Near this gene, a number of well-known genes and the STS markers were indicated. ZNF438 consisted of 7 exons. They were drawn to scale, numbered, and represented by rectangles. The triangle and asterisk indicated the positions of the start and stop codon, respectively. The sequences flanking the exon/intron boundaries were given; intron and exon nucleotide sequences were shown in lowercase and uppercase letter, respectively.

![Fig. 3. The chromosomal localization and genomic organization of human ZNF438.](image)

![Fig. 4. Expression analysis of ZNF438 in 18 human tissues by RT-PCR.](image)
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function, we further explore the subcellular localization of ZNF438. We hypothesize that, if ZNF438 was a transcription factor, it might be localized in nucleus. In order to test our assumption, indirect immunofluorescence was used to explore the subcellular localization. COS-7 cells were transfected with the pFlag-CMV-ZNF438 constructs. Indirect immunofluorescence assay with anti-Flag antibody showed that the Flag-ZNF438 was localized in nucleus (Fig. 5). Thus, the nuclear accumulation experiments of ZNF438 proved our hypothesis.

In conclusion, we had cloned, characterized, and mapped a novel human zinc finger gene ZNF438 which was widely expressed ubiquitously in 18 human tissues. ZNF438 was localized primarily in the nuclei. Overexpression of pM-ZNF438 in cultured cells could significantly inhibit the expression of the reporter gene in a dose-dependent manner, which suggested ZNF438 might serve a role as transcriptional repressor. In addition, we also performed Dual-luciferase reporter assay system to investigate the potential role of ZNF438 on signaling pathways, including AP1, CRE, E2F, GRE, Myc, NfκB, Rb and SRE pathway. However, it showed that ZNF438 failed to have any effect on the eight signaling pathways (data not shown). Further study would focus on whether ZNF438 would be modified by sumoylation and whether its activity was regulated by such modification.

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References


