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Characterization of the GufA subfamily member SLC39A11/Zip11 as a zinc transporter

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Abstract

Cellular zinc influx and efflux are maintained by two major transporter families, the ZIP (SLC39A) and ZnT (SLC30A or CDF) molecules. The functions of one molecule in this class, ZIP11/SLC39A11, remain unclear. Bioinformatics analysis of the distribution and evolutionary relationships of different ZIP members in eukaryotes and prokaryotes indicated that Zip11, the sole member of gufA subfamily, is an ancient ZIP family member that might have originated in early eukaryotic ancestors. Murine *Zip11* mRNA is abundantly expressed in testes and the digestive system including stomach, ileum and cecum. Analysis of cellular zinc content, metallothionein levels, and cell viability under high or low zinc conditions in cells transfected with a murine Zip11 expression plasmid, suggest that Zip11 is a zinc importer. Further, cellular zinc concentrations and metallothionein levels decreased when Zip11 was knocked down. In mice supplemented with zinc, both mRNA and protein levels of Zip11 were slightly up-regulated in several tissues. The metal response element sequences (MREs) upstream of the first exon of *Zip11* responded to elevated extracellular zinc concentrations, as assessed by luciferase reporter assays. Mutagenic analysis showed that several of the MREs could regulate Zip11 promoter activity, and metal-responsive transcription factor-1 (MTF-1) was shown to be involved in this process. Collectively, these data suggest that Zip11 has unique protein sequence and structure features, it functions as a cellular zinc transporter, and its expression is at least partially regulated by zinc via hMTF-1 binding to MREs of the *Zip11* promoter.

Keywords: Zinc transporter; SLC39A11/ZIP11; ZIP family; MRE; MTF-1; Zinc homeostasis

1. Introduction

Zinc (Zn) is a trace element essential for life. More than 300 metalloenzymes of six major functional classes require zinc as a key structural component or as a cofactor. Moreover, zinc plays important roles in DNA synthesis, cellular signal recognition, and second messenger metabolism. Zinc deficiency impairs growth, immune activity and brain function; however, zinc can also be toxic if over-accumulated. Thus, sophisticated regulatory systems must exist to maintain zinc homeostasis at both the cellular and organismal levels [1–3]. In mammals, zinc balance is primarily regulated through intestinal absorption, renal reabsorption, fecal elimination of excess zinc and the loss of endogenous zinc in the intestine through both pancreatic and liver excretion.

Movement of zinc into and out of cells and subcellular organelles is mediated by zinc transporters [4]. Zinc transporters are largely assigned to two metal-transporter families: ZIP (ZRT/IRT-like protein, SLC39A) and ZnT (Cation Diffusion Facilitator, or SLC30A). The prevailing view is that members of the ZnT family transport zinc from within the cell, either out of the cell across the plasma membrane or into intracellular compartments, which reduces cytosolic concentrations. ZIP family members are believed to function in the opposite direction, by increasing cytosolic zinc concentrations via uptake across the plasma membrane or efflux from intracellular compartments [5–7]. The mammalian ZIP family consists of 14 members in 4 subfamilies: ZIP subfamily I (ZIP9), ZIP subfamily II (ZIP1, ZIP2 and ZIP3), LIV-1 family (ZIP4, ZIP5, ZIP6, ZIP7, ZIP8, ZIP10, ZIP12, ZIP13 and ZIP14) and gufA family (ZIP11). Previously, ZIP1-8, ZIP10, and ZIP14 have been shown to facilitate zinc uptake in cells [5,8–10].

Many reports document the regulation of gene expression by zinc, and identify zinc transporter genes as among those regulated, both

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positively (e.g., *ZnT1*) and negatively (e.g., *Zip4*), by increased zinc availability [11–13]. Transcriptional regulation of gene expression by zinc occurs through binding of the Metal-responsive transcription factor-1 (MTF-1), which is a six zinc-finger (Cys_2His_2) transcription factor, that functions as a sensor of intracellular zinc by binding to metal response elements (MREs). The presence of MREs in gene promoter regions is well defined for mouse metallothionein (MT) I and II genes, mouse *ZnT1*, *ZnT2* and *ZnT5* genes, and zebrafish *Zip10* gene [14–20].

Currently, the functions of Zip11, which is the sole member of the gufA subfamily of ZIP proteins, remain unknown. Here, we investigated both the regulation of *Zip11* gene expression by zinc and the cellular functions of the Zip11 protein. We identified several MRE sequences upstream of the first exon of Zip11, which appear to be involved in responses to elevated extracellular zinc concentrations. Moreover, through overexpression and knockdown studies we found evidence that Zip11 is a zinc transporter that increases intracellular zinc content and *MT* mRNA expression.

2. Materials and methods

2.1. Bioinformatics analysis of ZIP family

Twenty-five representative eukaryotic genomes from different eukaryotic taxa (mammals, vertebrates, insects, nematodes, fungi, plants and protozoa) with substantial sequence coverage generated by the Entrez Genome Project at the NCBI were used to analyze the distribution of organisms that express ZIP proteins. For analysis of the differential distribution of ZIP family members, the human ZIP1-14 protein sequences were used as initial queries to search for homologous sequences in these genomes using BLASTP and TBLASTN [21]. Orthologous proteins were defined on the basis of the bidirectional best hit test [22] and phylogenetic analysis. Representative archaeal and bacterial ZupT/gufA proteins (ZIP homologs) were also included. Multiple sequence alignments were performed using CLUSTALW [23] with default parameters. The phylogenetic tree was reconstructed with PHYLIP programs [24] and visualized with PhyloDraw [25].

2.2. Animal experiments

All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences and Chinese Academy of Sciences. Male C57BL/6 mice (28 days old) were purchased from Shanghai Laboratory Animal Center of CAS and fed a standard rodent laboratory diet (Shanghai Laboratory Animal Center of CAS) for 1 week. Then, these mice were fed an AIN76-based diet (Research Diets, Inc.) containing 50 mg Zn/kg diet (Zn-adequate, control) for 3 weeks. To examine the tissue expression of *Zip11*, six mice were killed, and the indicated tissues were harvested and put in TRIZOL for subsequent extraction of RNA and real-time PCR analysis. To examine the effects of Zn on *Zip11* expression, 8-week-old mice (six mice per group) were administered $ZnSO_4$ (35 mg Zn/kg body weight) intragastrically and euthanized 3 hours (Zn-3h) or 8 hours (Zn-8h) later. The control group was administered the same volume of saline as experimental mice. Control, Zn-3h, and Zn-8h groups were used in the Zn supplemental study. The indicated tissues were harvested and put in TRIZOL or snap-frozen in liquid nitrogen for real-time PCR analysis and western blot. Plasma samples were collected for assaying zinc content.

2.3. Cell culture

Human embryonic kidney 293T (HEK293T) cells, Raw 264.7 cells and Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) plus 0.45% glucose under 5% CO_2. All culture media contained 100 units/ml penicillin, 100 g/ml streptomycin, 2 mM L-glutamine, and 100 μM non-essential amino acids (Invitrogen) supplemented with 10% fetal bovine serum (PAA, Germany). Stably transfected MDCK cell lines were selected with 500 μg/ml G418 (Sigma) for 2 weeks after mZip11 transfection.

2.4. Plasmids constructs and site-directed mutagenesis

The open reading frame (ORF) of *hZIP5, mZip11, or mZip14* were cloned into the pCMV-3tag-3A vector (Stratagene). Plasmids were confirmed by sequencing and protein expression by western blot. *Mouse Zip11* promoter sequence was predicted using UCSC Genome Bioinformatics and online software (Promoter 2.0 Prediction Server, http://www.cbs.dtu.dk/services/Promoter/). The *Zip11* promoter construct (−2.04 kb to +278 bp relative to the start site of exon 1 of *Zip11*) was cloned from genomic DNA of C57BL/6 mouse, using PCR with primers containing the EcoR I restriction site. The PCR product was then subcloned into the pGemT–Easy plasmid using T/A cloning and further inserted into the pGL3 Basic plasmid (Promega) by using Sac I and Nhe I restriction sites. Plasmid constructs were confirmed by DNA sequencing. Then the other two *Zip11* promoters (−305 bp or −123 bp to +278 bp relative to the start site of exon 1 of *Zip11*) were cloned from the first promoter and inserted into the pGL3 Basic plasmid. The *Zip11* Luc mutant constructs containing the 583 bp promoter (−305 bp to +278 bp) were created using site-directed mutagenesis (Quick Change II, Stratagene). Further confirmation of mutations and integrity of promoter fragments was performed by DNA sequencing. MRE sequences were identified via the Genomatix and USCS Genome Bioinformatic databases. The oligonucleotides used to generate the *mZip11* plasmid, three promoters, and mutations of the MREs sites are listed in Supplementary Table 1. The analysis of the promoter activity and the MRE mutation used in this experiment were the same as a previously report [26]. Endotoxin-free preparations of these plasmids (EndoFree Plasmid Maxi kit, Qiagen) were used for transfections.

2.5. RNA extraction and quantitative real-Time PCR (qRT-PCR)

HEK293T cells were grown in 6-well plates and transiently transfected with empty vector or plasmids expressing *hZIP5*, *mZip11*, or *mZip14* using FuGene 6 (Roch) for 36 h. Transfections were performed following the manufacturer's instructions. Media were replaced with 2 ml of serum-free medium containing 100 μM Zn ($ZnCl_2$) for 24 h.

Raw264.7 cells were grown in 12-well plates and transiently transfected with negative control and mZip11 siRNA (Dharmacon, Thermo) using DharmaFECT siRNA transfection reagents (Thermo) for 24 h. Transfections were performed following the manufacturer's instructions. Media was replaced with serum-free medium containing 0 or 100 μM Zn ($ZnCl_2$) for 24 h.

Total RNA was isolated from tissues or cells using Trizol (Invitrogen) according to the manufacturer's instructions and treated with DNase I (Promega). RNA concentration and purity were assessed by spectrophotometry. RNA (2.0 μg) was reverse-transcribed with M-MLV reverse transcriptase (Promega) and oligo (dT) 18 primers (Takara) as recommended. qRT-PCR was performed using a CFX96™ Real-Time System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad) as described by the manufacturer. Raw data were normalized to the internal control, *GAPDH,* and presented as relative expression level calculated by $2^{\Delta\Delta}$Ct method. All primers for qRT-PCR are described in Supplementary Table 2. All experiments were performed in triplicate.

2.6. Western blot and immunofluorescence

HEK293T cells were grown in 6-well plates and transiently transfected with empty vector or plasmids expressing *hZIP5-Flag*, *mZip11-Flag*, and *mZip14-Flag* for 48 h. Cell extracts were then prepared for western blot. Raw264.7 or MDCK cell lyses were used to detect mZip11 protein expression.

Samples from lysed cells or tissues (30 μg total protein) were resolved on 12% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes and probed with rabbit anti-mouse-Zip11 (1:200 dilution, Abcam), rabbit anti-Flag (1:1000 dilution, Cell Signaling) or monoclonal antibody anti-β-actin (1:2000 dilution, Sigma), followed by either anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase at a dilution of 1:4000 (Protein Tech Group, Inc) and detection with the ECL System (Pierce).

HEK293T cells were grown in 24-well plates and transiently transfected with the empty vector, mZip11-Flag, or mZip14-Flag plasmid. After 36 h, the transfected cells were incubated with 5 μM FluoZin-3^{AM} (Molecular Probes, Gibco), a cell-permeable zinc fluorophore, in medium without FBS for 30 min. The cells were then stimulated with Zn^{2+} (80 μM) to measure intracellular Zn^{2+} accumulation by fluorescence microscopy. Raw264.7 cells were transiently transfected with negative control or mZip11 siRNA for 24 h, then treated as HEK293T cells to measure zinc fluorescence.

2.7. Zinc measurement with inductively-coupled plasma mass spectrometry (ICP-MS)

The study of using ICP-MS to measure zinc content in cells has been reported previously [27]. HEK293T cells were grown in six-well plates and transiently transfected with the empty vector, *mZip11-Flag* or *mZip14-Flag* plasmids for 36 h. Then the cells were treated with or without 100 μM Zn for 24 h then scraped off plates using 5 ml of PBS ultra pure (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Solutions were centrifuged and the cell precipitate was prepared for ICP-MS detection. Protein was extracted from duplicate samples and measured by Bradford in SpecTRA MAX 190 microplate reader (Sunnyvale). Raw264.7 cells were grown in 6-well plates and transiently transfected following the protocol described above. MDCK cells stably expressed mZip11 or empty vector for 24 h, then media were replaced with serum-free medium containing 100 μM Zn ($ZnCl_2$), Cu ($CuCl_2$), Fe (FAC), Mg ($MgCl_2$) or Mn ($MnCl_2$) for 24 h. Cells were then prepared for ICP-MS and protein detection. The pre-treatment method of cell and plasma for ICP-MS detection has been described previously [28].

For analysis of metal concentrations, ICP-MS was performed using Agilent 7500cx ICP/MS system (Agilent Technologies) equipped with a G3160B I-AS integrated autosampler. The G3148B ISIS system was used to reduce the detection time and volume of each sample. Ni sample cone and skimmer cone were used with an orifice diameter of 1.0 and 0.4 mm, respectively. Sample introduction was performed with a micromist nebulizer combined with a Scott-type double pass spray chamber (Agilent