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Functional analysis and the molecular dissection of zinc-fingers and homeoboxes 1 (ZHX1)

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Abstract

Zinc-fingers and homeoboxes 1 (ZHX1) is a protein that interacts with the activation domain of the A subunit of nuclear factor-Y. The function of ZHX1, as a transcription factor, was characterized and their domains were mapped. To determine the nuclear localization signal, expression vectors, in which various truncated forms of ZHX1 were fused to the C-terminal of green fluorescence protein (GFP), were transfected into human embryonic kidney (HEK) 293 cells. All GFP–ZHX1 fusion proteins including an arginine-rich region that corresponds to the amino acid sequence between 734 and 768 were localized in the nuclei. A dimerization domain of the ZHX1 was also mapped using protein–protein interaction assays. The homeodomain (HD) 1 consisting of the amino acid sequence between 272 and 432 of ZHX1 was necessary and sufficient for dimerization. Lastly, the transcriptional activity of ZHX1 was examined using a mammalian one-hybrid system. ZHX1, fused to the C-terminal of the GAL4 DNA-binding domain, was co-transfected with luciferase reporter plasmids with or without five copies of the GAL4-binding site into HEK293 cells. The luciferase activity was decreased in both concentration- and GAL4-binding site-dependent manner. The acidic region corresponding to the amino acid sequence between 831 and 873 was a repressor domain and dimerization was prerequisited for full repressor activity. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: ZHX1; Transcriptional repressor; Nuclear localization signal; Dimerization; Zinc-finger; Homeodomain; Nuclear factor-YA-interacting protein

Human zinc-fingers and homeoboxes 1 (ZHX1) was cloned as a protein that interacts with the activation domain (AD) of the A subunit of nuclear factor-Y (NF-YA) [1]. In contrast, mouse ZHX1 was independently reported as an antigen that is recognized by the B92 monoclonal antibody produced by immunization with a cell lysate of 14F1.1 mouse endothelial–adipose stromal cells [2]. Recently, we reported on the cloning of rat ZHX1 as one of the ZHX1-interacting proteins and reported that ZHX1 is able to form a dimeric, quaternary structure [3]. Mammalian ZHX1 consists of 873 amino acid residues, contains two Cys_2–His_2-type zinc-finger (Znf) motifs and five homeodomains (HDs) [1–3], and belongs to the Znf class of the homeobox protein superfamily [4]. The amino acid sequence between 272 and 564 which contains the HD1 through the HD2 region of the human ZHX1 is required for its interaction with a glutamine-rich region in the AD of NF-YA [5].

ZHX1 mRNA expressed ubiquitously, although the level varies among tissues [1–3]. The level of ZHX1 mRNA was temporally increased within 6 h in mouse T cells that had been treated with interleukin-2 (IL-2) [6]. In addition, the stable expression of dominant negative NF-YA that contains the AD but is not able to bind to the cognate nucleotide sequence led to a retarded growth in mouse fibroblast cells [7]. The dominant negative form was still able to interact with ZHX1 [1].

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Although the biological function of ZHX1 remains unclear at present, currently available evidence suggests that ZHX1 may be involved in cell growth and differentiation.

The present study focused on the characterization of the ZHX1 protein as transcription factor. We clarified the transcriptional activity of ZHX1 and mapped the functional domains that are essential for nuclear localization, dimerization, and transcriptional repressor activity.

Materials and methods

Materials. ExTaq DNA polymerase was obtained from Takara Biomedicals (Kyoto, Japan). The pGEM-T Easy vector, pRL-CMV, and dual luciferase assay system were purchased from Promega (Madison, WI). The pEGFP-C1 vector and the yeast two-hybrid system were purchased from Clontech (Palo Alto, CA). The pGEX-5X-1, glutathione–Sepharose 4B, and [^{35}S]methionine (37 TBq/mmol) were purchased from Amersham Pharmacia Biotech (Cleveland, OH). The Big Dye Terminator FS Cycle Sequencing Kit was purchased from Applied Biosystems Japan (Tokyo, Japan). The Invisorb Plasmid Kit was purchased from Invitek (Berlin, Germany). The LIPOFECTAMINE PLUS was purchased from Invitrogen (Groningen, Netherlands). The QuikChange Site-directed Mutagenesis Kit and TOPP3 cells were obtained from Stratagene (La Jolla, CA).

Oligonucleotides. Oligonucleotides, SG424B1mcs1, 5′-AATTC CCGGG ATCCG TCGAC GAGCT-3′, SG424B1mcs2, 5′-CTAGA GCTCG TCGAC GGATC CCGGG-3′, 565ZHX1, 5′-CCGGG GATCC CCTGA CTTTA CTCCC CAAAA G-3′, 272EcoZHX1, 5′-CCGGG AATTC TAATT TGATT CCCAA AG-3′, 272BamZHX1, 5′-CCGGG GATCC CCAAT TCTAA TTTGA TTCCC AAAG-3′, 432BamZHX1, 5′-CCGGG GATCC CTAAG GTACC TGACT TTTCT GTATA-3′, 564SalZHX1, 5′-CCGGG TCGAC CTAAA AAGGA TTCCA GGATT GCTTA G-3′, 873ZHX1, 5′-CCGGG TCGAC TCAGT CATCT GATTT AGACA GC-3′, 565EcoZHX1, 5′-CCGGG AATTC CCTGA CTTTA CTCCC CAAAA G-3′, 640EcoZHX1, 5′-CCGGG AATTC TCCAA AGAAG AAGCT GGAGA AAC-3′, 734EcoZHX1, 5′-CCGGG AATTC ATGAA TGGTC TGTCT TCCCT TAG-3′, 769EcoZHX1, 5′-CCGGG AATTC TGGGA CAGGG GACCA TCACT-3′, 831EcoZHX1, 5′-CCGGG AATTC TTATT TGAGG AAAAT GAGGA GGA-3′, 639BamZHX1, 5′-CCGGG GATCC TCAAC TACCT GCATT ACTTT CATCT-3′, 733BamZHX1, 5′-CCGGG GATCC TCAAC TACTT GAATT GGCGC TCTG-3′, 768BamZHX1, 5′-CCGGG GATCC TCAGT TGTTA ATTCT TTTGC TTCCT CT-3′, 830BamZHX1, 5′-CCGGG GATCC TCATT CTATA CCTAA TTCTG ATCTT CT-3′, 873BamZHX1, 5′-CCGGG GATCC TCAGT CATCT GATTT AGACA GCTTC-3′, E1, 5′-AATTC ATGGC AAGCC AG-3′, E1as, 5′-AATTC TGGCT TGCCA TG-3′, 734768S, 5′-GCCAA TTCAA GTAGT TGGGA CAGGG GACCA-3′, AS734768, 5′-TGGTC CCCTG TCCCA ACTAC TTGAA TTGGC-3′, 272432S, 5′-GTATC TGCTC AGCAG GCTGC TCAGC CTACT-3′, and AS272432, 5′-AGTAG GCTGA GCAGC CTGCT GAGCA GATAC-3′, were used in this study.

Plasmids. The pBSII-hZHX1E/X and pGEM-T Easy-ZHX1 (1–271) plasmids have been described previously [1,3]. Polymerase chain reactions (PCRs) were carried out using pBSII-hZHX1E/X as the template and combinations of 565ZHX1 and 873ZHX1, 272EcoZHX1 and 564SalZHX1, and 565EcoZHX1 and 873ZHX1 as primers. The PCR conditions have been described previously [3]. These products were subcloned into the pGEM-T Easy vector to produce pGEM-T Easy ZHX1 (565–873), pGEM-T Easy ZHX1ES (272–564), and pGEM-T Easy ZHX1ES (565–873).

The pSG424, pSG424 (N/R), pEGFP-C1E1, pEGFP-ZHX (1–873), and pZHX1 (272–564) plasmids have been described previously [3,5,8–10]. A 1.9-kb *Eco*RI/*Bam*HI fragment was isolated from the pBSII-hZHX1E/X and subcloned into the *Eco*RI/*Bam*HI sites of the pSG424 (N/R) and pEGFP-C1 to give pSG424-hZHX1EB and pEGFP-ZHX1 (272–873), respectively. E1 and E1as oligonucleotides were annealed, phosphorylated, and subcloned into the *Eco*RI site of the pSG424-hZHX1EB to give pGAL4-ZHX1 (272–873). A 0.9-kb *Eco*RI fragment from the pGEM-T ZHX1 (1–271) was subcloned into the *Eco*RI site of the pSG424 (N/R), pSG424-hZHX1EB, and pEGFP-C1E1 to give pGAL4-ZHX1 (1–271), pGAL4-ZHX1 (1–873), and pEGFP-ZHX1 (1–271), respectively. SG424B1mcs1 and SG424B1mcs2 oligonucleotides were annealed, phosphorylated, and subcloned into the *Eco*RI/*Xba*I sites of the pSG424 to give the pSG424B1 [11]. A 0.9-kb *Bam*HI/*Sal*I fragment of the pGEM-T Easy ZHX1 (565–873) was subcloned into the *Bam*HI/*Sal*I sites of the pSG424B1 and pEGFP-C1 to produce pGAL4-ZHX1 (565–873) and pEGFP-ZHX1 (565–873), respectively. A 0.9-kb *Bam*HI fragment of the pZHX1 (272–564) was subcloned into the *Bam*HI site of the pSG424B1 and pEGFP-C1 to give pGAL4-ZHX1 (272–564) and pEGFP-ZHX1 (272–564), respectively.

PCRs were also performed using pBSII-hZHX1E/X as a template and combinations of primers; 565EcoZHX1 and 639BamZHX1, 640EcoZHX1 and 733BamZHX1, 734EcoZHX1 and 830BamZHX1, 734EcoZHX1 and 768BamZHX1, 769EcoZHX1 and 830BamZHX1, and 831EcoZHX1 and 873BamZHX1. After digestion with *Eco*RI and *Bam*HI, these fragments were subcloned into the *Eco*RI/*Bam*HI sites of the pEGFP-C1E1 and pSG424 (N/R) to produce pEGFP-ZHX1 (565–639), pEGFP-ZHX1 (640–733), pEGFP-ZHX1 (734–830), pEGFP-ZHX1 (734–768), pEGFP-ZHX1 (769–830), pEGFP-ZHX1 (831–873), pGAL4-ZHX1 (565–639), pGAL4-ZHX1 (640–733), pGAL4-ZHX1 (734–768), pGAL4-ZHX1 (769–830), and pGAL4-ZHX1 (831–873), respectively.

The pGBKT7-ZHX1 (1–873) expresses an entire coding region of the human ZHX1 fused to DNA-binding domain (DBD) of the yeast GAL4 transcription factor [3].The pZHX1 (142–873) (previously referred to as the pACT2-#111), pZHX1 (272–873), pZHX1 (565–873), pZHX1 (272–432), pZHX1 (430–564), and pZHX1 (345–463) plasmids have been described previously [5]. PCR was then carried out using the pBSII-hZHX1E/X as a template and 272BamZHX1 and 432BamZHX1 as primers. After digestion with *Bam*HI, the product was subcloned into the *Bam*HI site of the pGEX-5X-1 to give pGST-ZHX1 (272–432).

Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit. The pGAL4-ZHX1 (1–873) and 734768S, AS734768, 272432S, and AS272432 oligonucleotides were used as a template DNA and mutagenesis primers, respectively. After confirmation of the nucleotide sequence, these *Eco*RI/*Xba*I fragments were subcloned into the *Eco*RI/*Xba*I sites of the pSG424 (N/R) to produce pGAL4-ZHX1 (1–873)ΔNLS and pGAL4-ZHX1 (1–873)ΔDD, respectively.

All plasmid structures were confirmed by nucleotide sequencing.

Cell culture and DNA transfections. HEK293 cells, a human embryonic kidney cell line, were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

DNA transfections were carried out using the LIPOFECTAMINE PLUS reagent. All plasmids used for transfection were prepared using a Invisorb plasmid kit, followed by CsCl gradient ultracentrifugation. Cells (5×10^4) per well were inoculated in a 24-well plate on the day prior to transfection. For observation of GFP-fusion protein, 300 ng indicated GFP plasmid was used. For determination of transcriptional activity of ZHX1, 100 ng reporter plasmid and 2 ng pRL-CMV, the indicated amount of GAL4 DBD/ZHX1 fusion protein expression plasmid, were used. The total amount of plasmid DNA (202 ng) was adjusted by the addition of pSG424, if necessary. Three hours after transfection,

the medium was changed. After 48 h, the cells were subjected to a luciferase assay or observed with an Olympus IX-70 fluorescence microscope (Tokyo, Japan). Firefly and sea pansy luciferase assays were performed according to manufacturer's recommended protocol. Luciferase activities were determined using a Berthold Lumat model LB 9501 (Wildbad, Germany). Firefly luciferase activities (relative light units) were normalized by sea pansy luciferase activities.

Yeast two-hybrid system and liquid β-galactosidase assays. The yeast strain SFY526 that contains a quantifiable *lacZ* reporter was transformed with the pGBKT7 or pGBKT7-ZHX1 (1–873) plasmid using a TE/LiAc-based high efficiency transformation method [12]. These yeast strains were designated as yDBD and yDBD-ZHX1 (1–873), respectively. To analyze the dimerization domain of ZHX1, these reporter yeast strains were transformed with plasmids, which express various truncated forms of ZHX1 fused to the GAL4 AD or the parent vector, pACT2, and their β-galactosidase activities were subsequently determined. Quantitative β-galactosidase assays, using *o*-nitrophenyl-β-D-galactoside, were carried out on permeabilized cells, as described previously [1,5,13].

Glutathione-S-transferase (GST) pull-down assays. TOPP3 cells were transformed with the pGEX-5X-1 or pGST-ZHX1 (272–432) fusion protein expression plasmid. The preparation of GST-fusion protein, the ^{35}S-labeling of in vitro-translated ZHX1, and pull-down analysis have been previously described [1,3]. Finally, the beads were resuspended in an equal volume of 2× SDS sample buffer and each supernatant was loaded on a 10% SDS–PAGE gel, along with a prestained molecular weight marker. The gel was dried and exposed to a FUJIX imaging plate (Kanagawa, Japan). Interaction signals were detected with the FUJIX BAS-2000 image analyzing system. The relative purity and amounts of each fusion protein were determined by gel-staining with Coomassie brilliant blue R-250.

Results

Determination of the nuclear localization signal (NLS) of human ZHX1

Three putative NLSs are present in the amino acid sequences 469–473, 741–765, and 861–867 of human ZHX1. To examine the issue of whether either or all of the sequence is required for nuclear localization, we prepared various GFP-ZHX1 fusion protein expression plasmids. When pEGFP-C1E1 encoding GFP alone was transfected into HEK293 cells, the protein was observed in the whole cell (Fig. 1A). In contrast, when the pGFP-ZHX1 (1–873) plasmid in which an entire coding sequence of ZHX1 was fused to the C-terminal of GFP, was transfected, the fusion protein was observed in the nuclei (Fig. 1B).

Various truncated forms of ZHX1 were then fused to the C-terminal of GFP to map the minimal NLS (Figs. 1C–L). The pGFP-ZHX1 (1–271) and pGFP-ZHX1 (272–873), in which the N-terminal Znf domains (amino acid sequence between 1 and 271) and C-terminal HDs (amino acid sequence between 272 and 873) of ZHX1 were fused to the GFP, respectively, were transfected. While GFP fused to the Znf domain of ZHX1, when localized in the whole cell, it fused to the HDs of ZHX1 when it was localized in the nuclei (Figs. 1C and D). When the pGFP-ZHX1 (272–564) and pGFP-ZHX1 (565–873) were transfected, only the latter fusion protein became localized in the nuclei (Figs. 1E and F). These results indicate that HD3 through C-terminal region of ZHX1 includes the NLS. In addition, we made various constructs, including the pEGFP-ZHX1 (565–639), pEGFP-ZHX1 (640–733), pEGFP-ZHX1 (734–830), pEGFP-ZHX1 (831–873), pEGFP-ZHX1 (734–768), and pEGFP-ZHX1 (769–830). These plasmids were transfected into HEK293 cells and the subcellular localization of the GFP-ZHX1 fusion proteins was determined (Figs. 1G–L). Cells transfected with both the pEGFP-ZHX1 (734–830) and pEGFP-ZHX1 (734–768) expressed the GFP-ZHX1 fusion proteins in the nuclei (Figs. 1I and K).

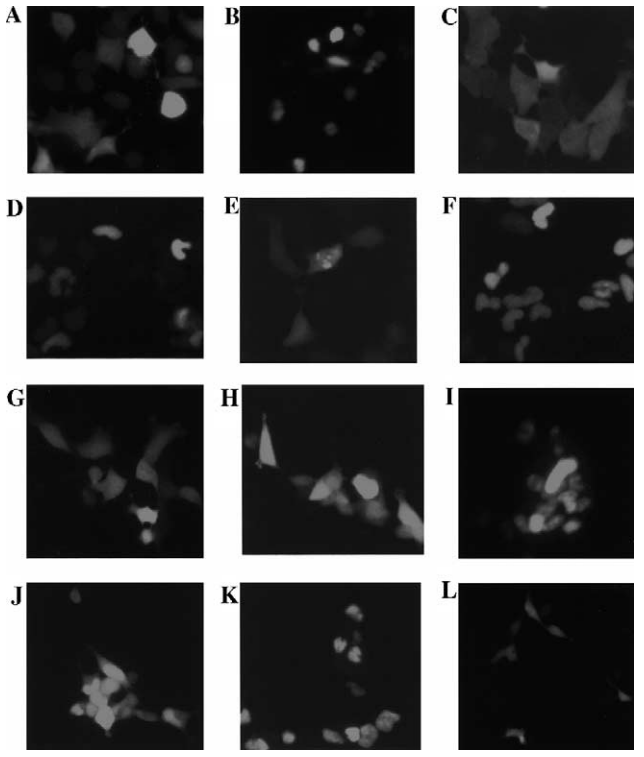

Fig. 1. Determination of nuclear localization signal of the human ZHX1. Expression plasmids (300 ng) which encode GFP alone or truncated ZHX1 proteins fused to the C-terminal of GFP were transfected into HEK293 cells. Forty-eight hours after transfection, the subcellular localization of GFP fusion protein was observed. (A) pEGFP-C1; (B) pEGFP-ZHX1 (1–873); (C) pEGFP-ZHX1 (1–271); (D) pEGFP-ZHX1 (272–873); (E) pEGFP-ZHX1 (272–564); (F) pEGFP-ZHX1 (565–873); (G) pEGFP-ZHX1 (565–639); (H) pEGFP-ZHX1 (640–733); (I) pEGFP-ZHX1 (734–830); (J) pEGFP-ZHX1 (831–873); (K) pEGFP-ZHX1 (734–768); (L) pEGFP-ZHX1 (769–830).

These results indicate that the amino acid sequence between 734 and 768 is necessary and sufficient for nuclear localization.

Dimerization domain mapping of the ZHX1

To map the dimerization domain of ZHX1, various ZHX1 proteins fused to the GAL4 AD were expressed in

reporter yeast strains. We used two SFY526 yeast strains harboring the pGBKT7 or pGBKT7-ZHX1 (1–873) as the reporter. While the yDBD strain harboring the pGBKT7 expresses GAL4 DBD alone, the yDBD-ZHX1 (1–873) strain expresses an entire coding region of ZHX1 fused to the GAL4 DBD. These yeast strains were transformed with the pACT2 and various GAL4 AD-ZHX1 fusion protein expression plasmids, after which, the β-galactosidase activities contained by them were determined. All the yDBD reporter strains that had been transformed with GAL4AD expression plasmids showed only marginal β-galactosidase activities. In contrast, when the pZHX1 (142–873) and pZHX1 (272–873) vectors, which express the HD1 through C-terminal regions of ZHX1 fused to the GAL4 AD, were transformed into the yDBD-ZHX1 (1–873), the resulting cells showed a high level of β-galactosidase activity (Fig. 2). Yeast transformed with the pZHX1 (565–873) vector, which expresses the HD3 through C-terminal region of ZHX1 fused to the GAL4 AD, contained no β-galactosidase activity. In contrast, yeast transformed with pZHX1 (272–564) and pZHX1 (272–432), which expresses the HD1 and HD2 region and the HD1 of ZHX1 fused to the GAL4 AD, respectively, exhibited a high level of β-galactosidase activity. No β-galactosidase activity was detected in yeast transformed with plasmids that encode either HD2 or the internal region between HD1 and HD2 of ZHX1 fused to the GAL4 AD (Fig. 2).

We next examined the in vitro dimerization of ZHX1 using GST pull-down assays (Fig. 3). We employed pGEX-5X-1, which expresses GST alone, and the pGST-ZHX1 (272–432) plasmid, which expresses the amino acid sequence between 272 and 432 of ZHX1 fused to the GST. In vitro-translated, ^{35}S-labeled ZHX1 was incubated with these purified proteins bound to glutathione–Sepharose. ZHX1 was found to bind the GST-ZHX1 (272–432) fusion protein but not to GST alone. In contrast, an unprogrammed reticulocyte lysate failed to bind to either protein (data not shown).

These results indicate that the amino acid sequence between 272 and 432, which encodes the HD1 region of

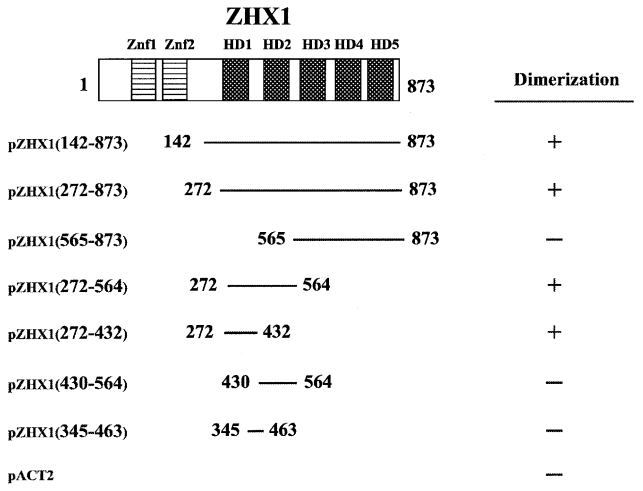

Fig. 2. Identification of the dimerization domain of ZHX1 using the yeast two-hybrid system. A schematic diagram of ZHX1 is illustrated at the *top*. The abbreviations show Znf and HD indicates the zinc-finger domain and homeodomain, respectively. The GAL4 AD-fusion constructs are depicted on the *left*. The + and − symbols indicate increased (+) and unchanged (−) levels of β-galactosidase activity, respectively, as compared to that of a yeast harboring a combination of pGBKT7 and pACT2.

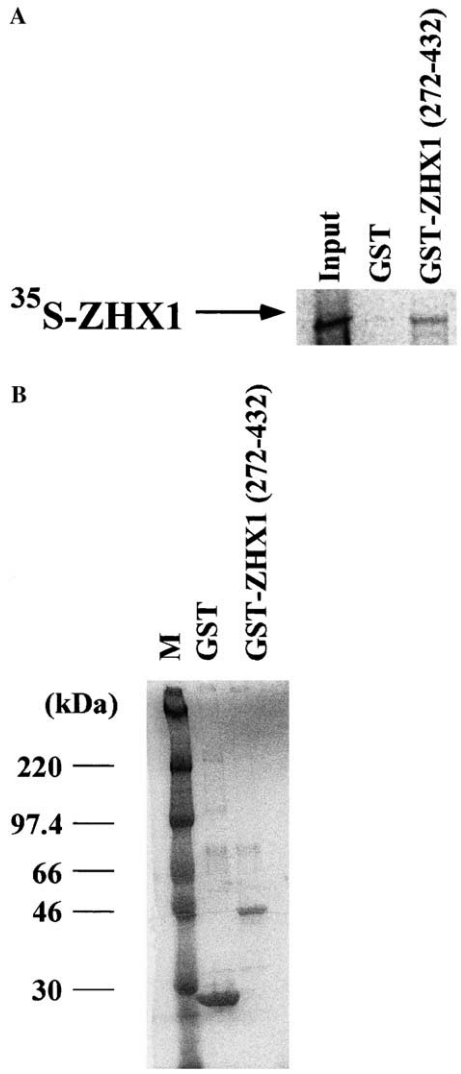

Fig. 3. Determination of dimerization domain of ZHX1 using the GST pull-down assays. (A) In vitro-translated, ^{35}S-labeled, ZHX1 was incubated with Sepharose beads containing bound GST or GST-ZHX1 (272–432) fusion protein. The beads were washed thoroughly and the bound protein was eluted and analyzed by SDS–PAGE. The interaction signal was detected with the FUJIX BAS-2000 image analyzing system. The signal in the lane marked "Input" represents 10% of the protein added to the reactions shown in the other lanes. (B) The relative purity and amounts of GST (28 kDa) and GST-ZHX1 (272–432) (45 kDa) proteins were determined by gel-staining with Coomassie brilliant blue R-250, respectively.

ZHX1 is essential for the formation of a dimer, both in vivo and in vitro.

Determination of the transcriptional activity of human ZHX1 and mapping of the minimal repressor domain

Since the issue of whether or not ZHX1 is a DNA-binding protein is unclear, the binding nucleotide sequence remains unknown. Thus, to evaluate the transcriptional role of ZHX1, we employed a mammalian one-hybrid system. The 5× GAL4-GL3 Control reporter plasmid, in which five copies of the GAL4-binding site were inserted upstream of the SV40 promoter in the pGL3-Control [14]. We also prepared effector plasmids, pSG424 and pGAL4-ZHX1 (1–873), which express GAL4 DBD alone or an entire coding region of the human ZHX1 fused to the C-terminal of the GAL4 DBD, respectively. When the 5× GAL4-GL3 Control and various amounts of the pGAL4-ZHX1 (1–873) were co-transfected into HEK293 cells, the luciferase activity was decreased in a concentration-dependent manner (Fig. 4). Maximal inhibition was obtained with 100 ng pGAL4-ZHX1 (1–873). In contrast, when the pGAL4-ZHX1 (1–873) was co-transfected with a pGL3-Control in which five copies of GAL4-binding sites were missing, the luciferase activity remained unchanged (Fig. 4).

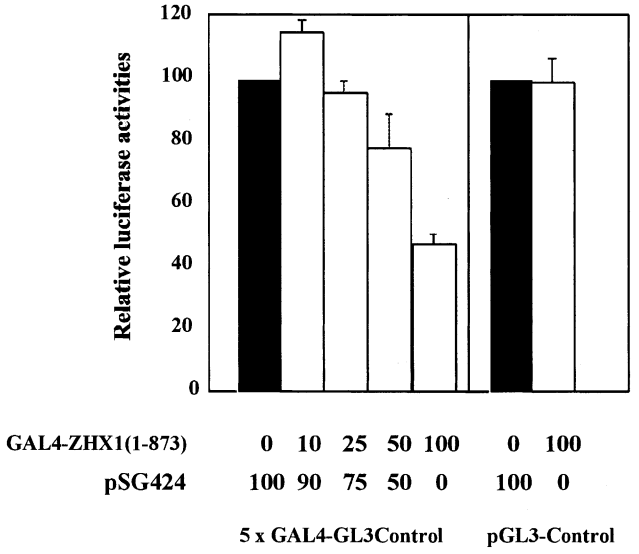

Fig. 4. ZHX1 is a transcriptional repressor. HEK293 cells were co-transfected with 2 ng pRL-CMV, 100 ng SV40 promoter-directed expression vector (shown on the *column*), and 100 ng of the 5× GAL4-GL3 Control or pGL3-Control reporter plasmid. The pSG424 and pGAL4-ZHX1 (1–873) express GAL4 DBD alone and the entire coding sequence of ZHX1 fused to the C-terminal of the GAL4 DBD, respectively. Forty-eight hours after transfection, the cells were harvested and both firefly and sea pansy luciferase activities were determined. A value of 100% was assigned to the promoter activity of the reporter plasmid in the presence of 100 ng pSG424, respectively. Each column and bar represents the mean and standard error of at least three transfection experiments.

Table 1
Determination of the repressor domain of ZHX1

GAL4DBD-ZHX1 fusion protein expression plasmid	Relative luciferase activities (%)
pSG424	100
pGAL4-ZHX1 (1–873)	52.7 ± 3.1
pGAL4-ZHX1 (1–271)	90.5 ± 6.8
pGAL4-ZHX1 (272–873)	67.5 ± 7.5
pGAL4-ZHX1 (272–564)	90.0 ± 8.6
pGAL4-ZHX1 (565–873)	78.8 ± 3.5
pGAL4-ZHX1 (1–873)ΔNLS	59.5 ± 4.8
pGAL4-ZHX1 (1–873)ΔDD	74.3 ± 3.7
pGAL4-ZHX1 (565–639)	92.7 ± 7.7
pGAL4-ZHX1 (640–733)	94.5 ± 11.2
pGAL4-ZHX1 (734–768)	125.0 ± 11.7
pGAL4-ZHX1 (769–830)	99.3 ± 10.5
pGAL4-ZHX1 (831–873)	79.3 ± 3.5

These results show that the GAL4-ZHX1 fusion protein represses luciferase expression by specifically binding to the GAL4-binding sites and indicates that ZHX1 functions as a transcriptional repressor.

We then mapped the repression domain of ZHX1 by co-transfection of the 5× GAL4-GL3 Control with the effector plasmids, which express the GAL4 DBD fusion protein with various ZHX1 deletion mutants (Table 1). The relative activities with the pSG424 plasmid are shown as 100%. The presence of pGAL4-ZHX1 (1–873) caused a decrease in luciferase activity by about 52%. We then analyzed the N-terminal deletion constructs. The pGAL4-ZHX1 (272–873) repressed the luciferase gene expression by an extent similar to pGAL4-ZHX1 (1–873). In contrast, although the pGAL4-ZHX1 (565–873) showed weak repressor activity, the level was approximately half of that of the pGAL4-ZHX1 (1–873). We also analyzed the successive C-terminal deletion constructs of ZHX1. Neither pGAL4-ZHX1 (272–564) nor pGAL4-ZHX1 (1–271) showed repressor activities. We then constructed the pGAL4-ZHX1 (1–873)ΔDD, in which amino acid sequence between 272 and 432 corresponding to the dimerization domain of ZHX1 was truncated from the pGAL4-ZHX1 (1–873). Although the pGAL4-ZHX1 (1–873)ΔDD showed weak repressor activity, the level was same as that of the pGAL4-ZHX1 (565–873) (Table 1). These results suggest that the dimerization domain of the ZHX1 per se does not have any repressor activity but dimerization is prerequisited for the full activity. Furthermore, to map a minimal repressor domain in the amino acid sequence between 565 and 873, we made various constructs, including the pGAL4-ZHX1 (565–639), pGA L4-ZHX1 (640–733), pGAL4-ZHX1 (734–768), pGAL4-ZHX1 (769–830), and pGAL4-ZHX1 (831–873). Only the pGAL4-ZHX1 (831–873) showed repressor activity and the level was same as that of the pGAL4-ZHX1 (565–873). Finally, we examined the issue of whether the NLS of ZHX1 is required for its transcriptional repressor activity. We prepared the pGAL4-ZHX1 (1–873)ΔNLS, in which

amino acid sequence between 734 and 768 corresponding to the NLS of ZHX1 was truncated from the pGAL4-ZH X1 (1–873). The pGAL4-ZHX1 (1–873)ΔNLS repressed luciferase expression by an extent similar to pGAL4-ZHX1 (1–873).

These results indicate that the amino acid sequence between 831 and 873 encoding the acidic region of the human ZHX1 is responsible for repressor activity and that dimerization but not the NLS is prerequisited for the full activity in this assay system.

Discussion

The amino acid sequence of ZHX1 contains some putative structural motifs, which serve as transcription factors, including the Znf motif, HD, and basic and acidic regions. It has been reported that the Znf motif is required for binding to the cognate DNA sequence via a Zn^{2+} molecule, that the HD consists of a conserved 60 amino acid motif and functions as the DBD, that the basic region is also the DBD or NLS, and that the acidic region is involved in the transcriptional activity [4,15–17]. In this study, we determined some of the features of ZHX1 involved in nuclear localization, dimerization, and transcriptional activity, and mapped their minimal domains. These domains, defined by molecular dissection, are summarized in Fig. 5.

Of the three putative NLSs of human ZHX1, an arginine-rich region that is encoded by the region between 734 and 768 was the *bona fide* NLS (Fig. 1). Although the amino acid sequence around both ends in the NLS is replaced with different amino acid residues among the human, rat, and mouse, the core region is conserved among these species [1–3]. In many other genes, it has been reported that the NLS was mapped in the basic region, which is lysine-rich [18]. The region has been reported to be associated with nuclear importing proteins such as importin α and then to be translocated from the cytoplasm to the nucleus [17]. The issue of whether ZHX1 interacts with importin α or a closely related protein via the arginine-rich region in the process of nuclear localization is of considerable interest.

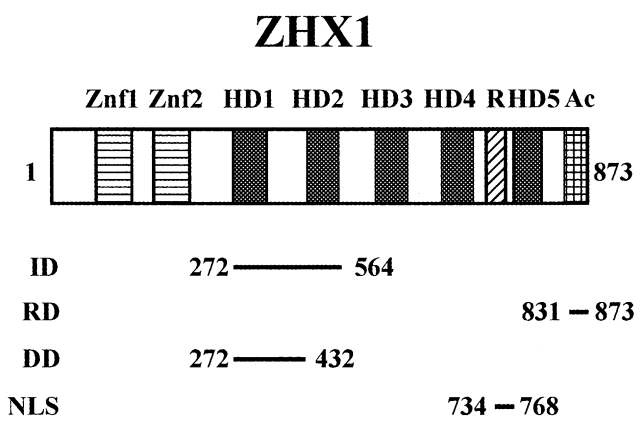

Fig. 5. Schematic representation of the structure and functional domains of ZHX1. A schematic diagram of ZHX1 is depicted. Znf, zinc-finger motif; HD, homeodomain; R, arginine-rich region; Ac, acidic region; ID, interaction domain with NF-YA; RD, repressor domain; DD, dimerization domain; NLS, nuclear localization signal.

The minimal dimerization domain of ZHX1 was mapped to the HD1 region. Interestingly, the region is part of the interaction domain with NF-YA [5]. A region containing HD1 through HD2 of ZHX1 interacted with the glutamine-rich region of NF-YA [5]. The amino acid sequences of HD1 and HD2 are completely identical among the human, rat, and mouse [1–3]. While the HD was originally reported as the DBD, it is known to be a domain that interacts with other transcription factors or molecules, which are involved in the signal transduction pathway [4,19–21]. Therefore, it is likely that the HDs of ZHX1 function as a dimerization domain as well as an interacting domain. The issue of whether the dimerization of ZHX1 competitively interferes with or cooperatively enhances an association of ZHX1 with NF-YA remains to be determined.

The Znf motif is required for binding to the cognate DNA sequence via a Zn^{2+} molecule [15]. Although ZHX1 has two Znf motifs, it is not clear that ZHX1 is, in fact, a DNA-binding protein. Since some of the Znf proteins, including the cyclic AMP-response element-binding protein-binding protein, do not bind to the DNA [22], the possibility that ZHX1 is not be a DNA-binding protein, cannot be excluded.

Using a mammalian one-hybrid system, it is clear that ZHX1 functions as a transcriptional repressor. This is the first demonstration of the biological function of ZHX1. Generally, some specific motifs such as the glutamine-rich, serine/threonine-rich, and proline-rich regions, as well as acidic regions mediate transcriptional activity [16,23–25]. An acidic region of the human ZHX1 was responsible for transcriptional repressor activity. Glutamic acid and aspartic acid residues in the region were conserved among the human, rat, and mouse [1–3]. Dimerization but not the NLS of ZHX1 was prerequisited for full activity (Table 1).

What is the physiological role of ZHX1? Thus far, we have no evidence to answer this question. However, it has been reported that the level of ZHX1 mRNA is increased in growing T cells by treatment with IL-2 [6]. We speculate that the induced ZHX1 directly interacts with endogenous transcription factors and extinguishes their activities, and that it leads the cell to transit the differentiated status to an undifferentiated status. In addition, it has been reported that transcription regulatory regions interact with co-factors to function as transcriptional repressors [26,27]. These cofactors include mSin3A/B, the nuclear co-repressor/silencing mediator of receptor transcription, and histone deacetylases. When ZHX1 acts as a transcriptional repressor, it would interact with these

corepressors and repress gene transcription. The identification of target genes of ZHX1 and ZHX1-interacting proteins will be required to address this question.

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