Identification of proteins that interact with NF-YA

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Abstract We used the yeast two-hybrid system to show that the serum response factor (SRF) and zinc-fingers and homeobox 1 (ZHX1) proteins interact with the A subunit of nuclear factor-Y (NF-YA). GST pulldown assays revealed that both proteins interact specifically with NF-YA in vitro. Amino acids located between 272 and 564, a region that contains two homeodomains, are required for the interaction of ZHX1 with NF-YA. Two different domains of NF-YA, a glutamine-rich region and a serine/threonine-rich region, are necessary for the interactions with ZHX1 and SRF, respectively.

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Key words: Transcription factor; Nuclear factor-Y; Serum response factor; Zinc-finger; Homeobox 1; Protein-protein interaction

1. Introduction

Nuclear factor-Y (NF-Y), also referred to as CCAAT binding factor, stimulates gene transcription from many promoters by binding to an inverted CCAAT box [1]. NF-Y, a ubiquitous heteromeric protein, is composed of three subunits, NF-YA, NF-YB, and NF-YC. NF-YB and NF-YC tightly interact with each other and their association precedes the association with NF-YA and the subsequent binding of the complex to the cognate DNA sequences [2]. NF-YB and NF-YC interact with other transcription factors or co-activators [3–6], but little is known about whether NF-YA interacts with proteins other than NF-YB and NF-YC.

To analyze the molecular mechanism of transcription activation by NF-Y, we determined whether the activation domain (AD) of NF-YA interacts with either a known or a novel transcription factor. Here we report the detection of zinc-fingers and homeobox 1 (ZHX1) and serum response factor (SRF) as NF-YA-interacting proteins, determine the minimal interaction domain of ZHX1 required for the association with NF-YA, and show that two different domains of NF-YA interact with SRF or ZHX1, respectively.

ZHX1 cDNA was isolated from a mouse liver library [7]. ZHX1 consists of 873 amino acids and contains two C2-H2-type zinc-fingers (Znf) and five homeodomains (HD). The mouse ZHX1 cDNA was cloned as a nuclear antigen recognized by the B92 monoclonal antibody, which was produced by immunization with cell lysates of the 14F1.1 mouse endothelial-adipose stromal cell, but the physiological role of this protein has not been determined [8].

The function of SRF is well-characterized. This transcription factor binds to and transactivates from the serum response element of the c-fos gene promoter [9,10]. Transactivation by serum stimuli is dependent upon the phosphorylation of p62TCF/Elk-1, which forms a ternary complex with SRF [9,10].

2. Materials and methods

2.1. Materials

The yeast two-hybrid system and human liver MATCHMAKER cDNA library were purchased from Clontech. Glutathione-agarose was purchased from Sigma. pGEX-5X-1 and Thermo Sequenase II DNA synthesizer. The nucleotide sequences are as follows: GSTzhx1s, 5′-GGGATCCCTAATCAGGGTTTACCAATGCAGTT-3′; 272zhx1s, 5′-CCGGGGATCCCCTAATCAGGGTTTACCAATGCAGTT-3′; YA141s, 5′-CCGGGGATCCCCCGGTAACTCTGCTGTCCAGCTGC-3′; 564zhx1as, 5′-CCGGGGATCCCTTCACTGTCCAGGGGTACCAAC-3′; ACT2-zhx1s, 5′-CATGGAAGGGCCCCGAGGATCC-3′; ACT2B1as, 5′-GGGGATCCCATATGGTATGCCTGCATTGCTAGTGAATGCCTGACGC-3′; YA112as, 5′-AAATTCTGGCTTGCCAT-3′; YA205s, 5′-CCGGCATATGGCAGTGAGGAGG-3′; YA141s, 5′-CCGGCATATGGCGTGCTGGAGG-3′; YA141s, 5′-CCGGGATCCCTAACGGGAGGAGG3′; ACT2B1s, 5′-AAATTCTGGCTTGCCAT-3′.

Abbreviations: NF-Y, nuclear factor-Y; NF-YA, A subunit of NF-Y; AD, activation domain; ZHX1, zinc-fingers and homeobox-1; SRF, serum response factor; Znf, zinc-fingers; HD, homeodomain; GST, glutathione S-transferase; PCR, polymerase chain reaction; DBD, DNA binding domain; SID, subunit interaction domain; SDHs, histidine-free synthetic dextrse; HNF4, hepatocyte nuclear factor 4

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cloned into the EcoRI/XhoI sites of the pGST-5X-1 to obtain pGST-ZHX1E/X. GST-zhx1s and GST-zhx1as oligonucleotides were annealed and phosphorylated. The double-stranded oligonucleotide was inserted into the EcoRI site of the pGST-ZHX1E/X to produce pGST-ZHX1(272/873), which expresses the amino acid sequence between 272 and 873 of ZHX1 ligated to glutathione S-transferase (GST). We employed the following method for construction of GAL4 AD-fusion protein expression vectors. The ACT2-zhx1s and ACT2-zhx1as oligonucleotides were annealed and phosphorylated. The pACT2-#111 was digested with EcoRI to remove a 390-bp insert that encodes amino acids 142–271 of ZHX1. The double-stranded oligonucleotide was ligated into the EcoRI site of this plasmid to obtain pZHX1(272–873). The pACT2 vector was digested with NcoI and EcoRI. The ACT2B1s and ACT2B1as oligonucleotides were annealed, phosphorylated, and ligated into the NcoI/EcoRI site of the pACT2 to produce the pACT2B1 plasmid. A polymerase chain reaction (PCR) was carried out using pBSII-hZHX1E/X as a template and the following combinations of primers: 565zhx1s and 873zhx1as, 272zhx1s and 564zhx1as, 272zhx1s and 432zhx1as, 430zhx1s and 564zhx1as, 272zhx1s and 390zhx1as, 564zhx1as and 390zhx1as, and YA140s and YA140as, YA61s and YA140as, YA31s and YA269as, YA61s and YA269as, YA141s and YA269as, YA172s and YA269as, and YA205s and YA269as to produce pYAI–204, pYAI–140, pYAI–112, pYAI–31, pYAI–61, pYAI–272, and pYAI–205, respectively. The nucleotide sequences of these constructs were determined. The resultant plasmids were digested with BamHI. After agarose gel electrophoresis, these fragments were ligated into the BamHI site of the pACT2B1 to produce pZHX1(565–873), pZHX1(272–564), pZHX1(430–564), and pZHX1(345–463), respectively. We also constructed GAL4 DNA binding domain (DBD) fusion protein expression vectors. PCR was carried out using the pGBT9(N/R/N)NF-YA1–269 as a template and the following combinations of primers: GAL4BD and YA140as, GAL4BD and YA112as, YA141s and YA140as, YA61s and YA140as, YA31s and YA269as, YA61s and YA269as, YA141s and YA269as, YA172s and YA269as, and YA205s and YA269as to produce pYAI–204, pYAI–140, pYAI–112, pYAI–31, pYAI–61, pYAI–272, and pYAI–205, respectively. pGEX-3H-SRF was a gift from Dr. Linda Sealy (Vanderbilt University). The EcoRI fragment of the pcDNA3/His-SRF encoding an open reading frame of SRF was subcloned into the EcoRI site of the pGEX-5X-1 to produce the pGST-SRF. PCR was carried out using pGEX-3H-SRF as a template with a combination of 412SRFas and 508SRFas primers. Amplified DNA was digested with BamHI and subcloned into the BamHI site of the pACT2B1 to produce pSRF(412–508). cDNA fragments that contain the open reading frame of NF-YA and NF-YB with Ndel and EcoRI ends were amplified by PCR for use in the in vitro transcription/translation system. These products were digested with Ndel and EcoRI and subcloned into the Ndel/EcoRI sites of pET-3a to obtain NF-YAET3a and NF-YBET3a, respectively. The nucleotide sequences of these constructs were determined by use of an automated DNA sequencer (PE Applied 373A DNA sequencer).

2.4. Yeast two-hybrid assays

HF7c or SFY526 cells were transformed with the indicated bait plasmid by a TE/LiAc-based high efficiency transformation method [12]. Human liver cDNA library screening, using the pGTB9(N/R/N)NF-YA1–269 bait plasmid, was performed as described [11]. HF7c cells harboring a bait plasmid were transformed with the indicated GAL4 AD expression plasmids for use in HIS3 reporter gene assays. SFY526 cells harboring the indicated bait plasmid were transformed with various GAL4 AD expression plasmids for use in β-galactosidase assays. Quantitative β-galactosidase assays, using o-nitrophenyl-β-D-galactoside as the substrate, were carried out on permeabilized cells [13].

2.5. GST binding assays

TOPP3 cells were transformed with pGEX-5X-1, pGST-ZHX1(272–873), or pGST-SRF. The preparation of the GST fusion protein, and the GST binding assays, were performed as described [11].

3. Results

3.1. Isolation of NF-YA-interacting proteins in a yeast two-hybrid library screen

The amino acid sequence 1–269, which encodes the AD but not the DBD or subunit interaction domain (SID) of NF-YA [14], was fused to the GAL4 DBD and this chimeric protein was employed as the bait to screen a human liver cDNA library using the yeast two-hybrid system. Approximately 1×10^6 independent clones were screened. Three clones, #111, #112, and #120, showed His^+ and β-gal positive properties [11]. We isolated plasmids that encode GAL4 AD fusion protein from these clones and designated them pACT2-#111, pACT2-#112, and pACT2-#120, respectively. pACT2-#111 encodes the amino acid sequence between 142 and 873 of human ZHX1 [11]. The nucleotide sequences of pACT2-#112 and pACT2-#120 are identical to human SRF. pACT2-#112 and pACT2-#120 contain the nucleotide sequence 1078–2640 of SRF, which corresponds to the amino acid sequence 241–508. This region includes the C-terminal AD of SRF and the domain that interacts with RAP74 [15]. It does not contain the DBD, dimerization domain, or the domain required for interaction with p62/Elk-1. Hereafter pACT2-#111, and pACT2-#112 and pACT2-#120 were named pZHX1(142–873) and pSRF(241–508), respectively.

We next examined the specificity of the interaction between NF-YA and ZHX1 or SRF using the yeast two-hybrid assay (Table 1). We employed two bait plasmids, pGTB9(N/R/N) which expresses the GAL4 DBD, and pGTB9(N/R/N)YA1–269 which expresses the GAL4 DBD ligated to the AD of NF-YA, and three prey plasmids, pACT2 which expresses the GAL4 AD, and pZHX1(142–873) or pSRF(241–508), which express the GAL4 AD fused to the cloned ZHX1 or SRF cDNAs, respectively. When the yeast HF7c strain containing pGTB9(N/R/N) was transformed with pACT2, pZHX1(142–873), or pSRF(241–508), the transformants did not grow on histidine-free synthetic dextrose (SD-His) plates (Table 1). Yeast harboring a combination of pGTB9(N/R/N)YA1–269 and pACT2 also did not grow on SD-His plates. In contrast, yeast containing a combination of pGTB9(N/R/N)YA1–269 and pZHX1(142–873) or pSRF(241–508) grew on SD-His plates. We then determined β-galactosidase activity in yeast SFY526 strains that harbor the same combina-

<table>
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<tr>
<td>pSRF(241–508)</td>
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Table 1

HIS3 reporter gene expression and β-galactosidase activities of yeast strains harboring various combinations of GAL4 DBD and AD fusion plasmids.
tions of plasmids. As shown in Table 1, yeast containing both pGBT9(N/R/N)YA1-269 and pZHX1(142-873) or pSRF(241-508) plasmids expressed higher β-galactosidase activity than the other yeasts tested. These results indicate that the AD of NF-YA specifically interacts with ZHX1 and SRF in vivo.

We next used in vitro GST binding assays to test for an interaction of NF-YA and ZHX1 or SRF. In vitro translated, 35S-labeled NF-YA or NF-YB was incubated with purified GST, GST-ZHX1(272/873) or GST-SRF proteins bound to glutathione-agarose (Fig. 1). NF-YA bound to GST-ZHX1(272/873) and GST-SRF, but not to GST alone. In contrast, NF-YB failed to bind to either protein. These results indicate that NF-YA specifically interacts with ZHX1 or SRF in vitro and in vivo.

3.2. Determination of the domain of ZHX1 or SRF that interacts with NF-YA

Various truncated ZHX1 proteins fused to the GAL4 AD were expressed in a reporter yeast strain in order to map the domain of ZHX1 that interacts with the AD of NF-YA. We used an SFY526 yeast strain harboring the pGBT9(N/R/N)YA1-269 as the reporter. When the pZHX1(272-873) vector, which expresses the HD1 through HD5 regions of ZHX1 fused to the GAL4 AD, was transformed into the reporter yeast, the resultant cells showed a high level of β-galactosidase activity (Fig. 2A). Yeast transformed with the pZHX1(565-873) vector, which expresses the HD3 through HD5 regions of ZHX1 fused to the GAL4 AD, showed no β-galactosidase activity. In contrast, yeast transformed with pZHX1(272-564), which expresses the HD1 and HD2 regions of ZHX1, exhibited high β-galactosidase activity. No β-galactosidase activity was detected in yeast transformed with plasmids that encode either HD1 or HD2, or the internal region between HD1 and HD2 of ZHX1 fused to the GAL4 AD. These results indicate that the amino acid sequence located between 272 and 564, and encoding the HD1 and HD2 regions of...
ZHX1, is essential for the physical interaction with the AD of NF-YA.

The amino acid sequence between 412 and 508 of SRF is known to interact with the RAP74 subunit of TFIIF [15]. We tested whether the same domain of SRF interacts with NF-YA. pSRF(412^508) expresses the amino acid sequence located between 412 and 508 of SRF fused to the GAL4 AD. Yeast harboring pGBT9(N/R/N)YA1^269 transformed with the pSRF(412^508) showed no β-galactosidase activity (Fig. 2B). This indicates that the domain of SRF necessary for interacting with NF-YA is not identical to that required for the interaction with RAP74.

3.3. Mapping the domains of NF-YA that interact with ZHX1 or SRF

As shown in Fig. 3, the AD of NF-YA consists of a glutamine-rich domain and a serine/threonine-rich domain [14]. Various truncated forms of NF-YA ligated to the GAL4 DBD were prepared to determine which domain is required for the interaction with ZHX1 or SRF. Yeast strain SFY526 containing pZHX1(142^873) or pSRF(241^508) was used as the reporter. These cells were transformed with pYA1^140, which expresses the amino acid sequence located between 1 and 140 of NF-YA ligated to the GAL4 DBD. Interestingly, the yeast harboring pZHX1(142^873) showed high β-galactosidase activity, but cells with pSRF(241^508) were inactive. This indicates that the domain of NF-YA required for the interaction with ZHX1 is different from that required for SRF.

We then determined the minimal domain of NF-YA required for the interaction with ZHX1. We constructed C-terminal deletion mutants of NF-YA fused to the GAL4 DBD. pYA1–140 and pYA1–112 express the amino acid sequences located between 1 and 140 and 1 and 112 of NF-YA, respectively. The reporter yeast transformed with pYA1–140 was the only one that showed high β-galactosidase activity. This indicates that amino acid sequence 1–140 of NF-YA is sufficient for an interaction with ZHX1. Additional definition was accomplished by using pYA31–140 and pYA61–140, which express the amino acid sequences 31–140, and 61–140 of NF-YA, respectively. Yeast harboring pYA31–140 exhibited high β-galactosidase activity; pYA61–140 was inactive. These results indicate that the NF-YA 31–140 amino acid sequence, which contains a glutamine-rich domain, is the minimal domain required for the interaction with ZHX1.

The domain of NF-YA required for an interaction with SRF was also studied using successive N-terminal deletion mutants of NF-YA ligated to the GAL4 DBD. pYA31–269, pYA61–269, pYA141–269, pYA172–269, and pYA205–269 express amino acid segments 31–269, 61–269, 141–269, 172–269, and 205–269 of NF-YA, respectively. The reporter yeast harboring pSRF(241–508) was transformed with each of these plasmids. High β-galactosidase expression was noted in cells transformed with pYA31–269, pYA61–269, and pYA141–269. These results indicate that the amino acid sequence located at positions 141–269 of NF-YA, which contains a serine/threonine-rich domain, is the minimal domain required for the interaction with SRF.

4. Discussion

ZHX1 and SRF interact with NF-YA. ZHX1 contains two Znf and five HDs, and belongs to a divergent homeobox protein family [7,11]. The ZHX1 amino acid segment 272–564, which encodes HD1 and HD2, contains the NF-YA-interacting domain. Generally, the HD contains a conserved 60 amino acid motif that functions as a DBD [16]. However, the HD can also interact with other transcription factors [17–19], and with proteins not generally considered to be transcription factors [20,21]. For example, some protein kinases function as co-repressors by interacting with HD-bound transcription factors [20]. Also, calreticulin, a chaperone protein, binds to the HD of thyroid transcription factor-1 and enhances its transcriptional activity [21]. The NF-YA-interacting domain of ZHX1 might be part of a transcription factor network that could lead to the discovery of other ZHX1-interacting protein(s).
A synergistic interaction may occur between the Y-box and the SRE element in the human β-actin and thrombospondin 1 gene promoters [22,23]. Our results show that the proteins that bind to these elements interact. Although the region of SRF we cloned contains a RAP74-interacting domain, the minimal domain of SRF required for the interaction with NF-YA is not identical with the domain required for SRF-RAP74 interaction. In contrast, the AD of SRF interacts with ATF 6 through a domain that is larger than that required for the SRF-RAP74 interaction [24]. It is possible that the SRF 241–508 domain may interact with both NF-YA and ATF 6.

Two different domains of NF-YA, the glutamine-rich region and serine/threonine-rich region, interact with ZHX1 and SRF, respectively. The C-terminal region of NF-YA contains domains that interact with NF-YB and NF-YC, and it has a DBD. Thus NF-YA has several domains required for protein-protein interactions and it too may participate in a complicated transcription factor-network system that involves several other proteins. Recent reports show that Sp1 and hepatocyte nuclear factor 4 (HNF4) are NF-YA-interacting proteins [25,26]. Although the minimal domain required for the interaction of HNF4 with NF-YA was not determined, the glutamine-rich region of NF-YA is necessary for the interaction with Sp1 [25]. ZHX1 and SRF can now be added to the list of NF-YA-interacting proteins.

We postulate that NF-Y is one of the factors involved in cAMP-mediated transcriptional stimulation of the rat type II hexokinase gene promoter [27]. SRF activity is induced by serum stimuli and by cAMP [9,10]. It is possible that interactions between NF-Y and SRF or ZHX1 may be regulated by cAMP. It remains to be determined whether interactions among these transcription factors affect the promoter activity in target genes, if ZHX1 and SRF simultaneously interact with NF-YA, and if Sp1, in addition to these proteins, can competitively interact with NF-YA. We are performing experiments to address these questions.

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References


