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DNA-binding and transcriptional activities of human HSF4 containing mutations that associate with congenital and age-related cataracts

Yasuaki Enoki, Yuka Mukoda, Chie Furutani, Hiroshi Sakurai *

Division of Health Sciences, Kanazawa University Graduate School of Medical Science, 5-11-80 Kodatsuno, Kanazawa, Ishikawa 920-0942, Japan

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ABSTRACT

Heat shock transcription factor HSF4 is necessary for ocular lens development and fiber cell differentiation. Mutations of the human *HSF4* gene have been implicated in congenital and age-related cataracts. Here, we show that HSF4 activates transcription of genes encoding crystallins and beaded filament structural proteins in lens epithelial cells. Five missense mutations that have been associated with congenital cataract inhibited DNA-binding of HSF4, which demonstrates the relationship between *HSF4* mutations, loss of lens protein gene expression, and cataractogenesis. However, two missense mutations that have been associated with age-related cataract did not or only slightly alter HSF4 activity, implying that other genetic and environmental factors affect the functions of these mutant proteins.

1. Introduction

Cataracts are defined as lens opacities. Cataractogenesis has multiple causes and is often associated with a breakdown of the lens microarchitecture [1]. Lens proteins undergo a wide variety of alterations with age, and many of these are accelerated by oxidative, osmotic, and other stresses. In addition to environmental risk factors, genetic factors are also important in age-related cataract formation [2,3]. Congenital cataract is likely to cause childhood blindness. It is known that mutations in genes encoding two groups of proteins cause congenital cataract formation. The first group consists of structural proteins in the lens such as lens crystallins, beaded filaments unique to lens fiber cells, gap junction connexins important for intercellular communication in the avascular lens, and membrane water transporter aquaporin. The second group consists of transcription factors e.g. HSF4, MAF, FOXE3, and PITX3 that regulate lens development and fiber cell differentiation [4].

HSF4, despite being a member of the heat shock transcription factor (HSF) family, plays a role in cell growth and differentiation instead of stress-induced transcription of genes encoding heat shock proteins [5,6]. The N-terminal half of HSF4 contains a DNA-binding domain (DBD) with a 'winged' helix–turn–helix motif and hydrophobic repeat (HR-A and -B) regions with coiled-coil structures [7]. The HR-A/B regions mediate HSF4 trimerization. HSF4 trimers bind to the consensus heat shock element (HSE), made up of inverted repeats of the 5-bp sequence nGAAn, and to HSE-like sequences [7–9]. There are two HSF4 isoforms; HSF4b has the ability to activate transcription but not HSF4a [10]. Expression of HSF4 is restricted to the brain and lung [7,10]. *HSF4* knockout mice exhibit reduced expression of crystallins and beaded filaments in lens and develop cataracts [11–13].

Mutations of human *HSF4* have been associated with congenital cataract [14–18]. Furthermore, two missense mutations were identified by screening *HSF4* in 150 age-related cataract patients [19]. However, how these mutations affect HSF4 function remains unclear. In this study, we introduced missense mutations into *HSF4b* cDNA and characterized the molecular properties of the mutant proteins.

2. Materials and methods

2.1. In vitro polypeptide synthesis, chemical cross-linking, and electrophoretic mobility shift assay

Plasmid pcDNA-*HSF4b* was a derivative of pcDNA3.1(+) containing *HSF4b* cDNA [9]. Nucleotide changes were introduced into *HSF4b* by site-directed mutagenesis. HSF4b polypeptides were synthesized in vitro using the TNT T7 Coupled Reticulocyte Lysate System (Promega). The amounts of synthesized polypeptides were determined by immunoblot analysis with an anti-HSF4 antibody using purified recombinant HSF4b as a reference [9].

For the chemical cross-linking analysis, HSF4b polypeptides (0.4 ng) in 4 μl of 25 mM Hepes-KOH, pH 7.6, 70 mM NaCl, 2 mM EDTA, and 5% glycerol were incubated at 37 °C for 20 min, treated with EGS (ethylene glycol bis-(succinimidylsuccinate)) at room temperature for 20 min, electrophoresed on a SDS-polyacrylamide gel, and subjected to immunoblot analysis [9]. The experiments were performed at least three times with similar results.

* Corresponding author. Tel.: +81 76 265 2588; fax: +81 76 234 4369.
E-mail address: sakurai@kenroku.kanazawa-u.ac.jp (H. Sakurai).

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For the electrophoretic mobility assay, the binding reaction was carried out in an 8-μl mixture containing 0.5 ng of HSF4b polypeptides, 25 mM Hepes-KOH, pH 7.6, 90 mM NaCl, 2 mM EDTA, 5% glycerol, 100 ng of poly(dI–dC), and 0.01 ng of ^{32}P-labeled HSE oligonucleotide at 37 °C for 20 min. The samples were electrophoresed and subjected to phosphorimaging [9,20]. The experiments were performed at least four times with similar results.

2.2. Cell culture and transfection

HeLa cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and 2.5 μg/ml Fungizone (Invitrogen) at 37 °C in a 5% CO_2 atmosphere [9,20]. SRA01/04 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 10 μg/ml gentamycin, and 2.5 μg/ml Fungizone at 37 °C in a 5% CO_2 atmosphere [21]. To express the HSF4b–VP16 fusion protein in HeLa cells, the activation domain of herpes simplex virus VP16 (amino acid residues from 413 to 490) was fused in-frame to the C-terminus of HSF4b in the pcDNA3.1(+) vector [9]. Transient transfection was performed using HilyMax reagent (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Expression of HSF4b–VP and HSF4b was verified by immunoblot analysis using anti-VP16 and anti-HSF4 antibodies, respectively [9].

2.3. Luciferase assay

The HSE-SV40p-*LUC* reporter genes contained 3P-, gap-, and step-type HSE oligonucleotides inserted upstream of the SV40 promoter fused to the firefly luciferase gene [9,20]. The luciferase reporter genes *BFSP1-LUC*, *BFSP2-LUC*, *CRYAB-LUC*, and *CRYGC-LUC* contained the following promoter regions respectively: −1044 to −43 of *BFSP1*, −998 to −48 of *BFSP2*, −586 to +3 of *CRYAB*, and −734 to +3 of *CRYGC* (relative to the translation initiation site) [9]. Luciferase assays were performed as previously described [9,20]. Experiments were carried out at least eight times, and statistical significance was determined using the Student's *t*-test.

2.4. Reverse transcription (RT)-PCR analysis

SRA01/04 cells grown in 60-mm dishes were transfected with a DNA mixture containing 1.1 μg of pcDNA-*HSF4b* and 7.7 μg of carrier pcDNA3.1(+) DNA. After 15 h, total RNA was isolated from the cells using the FastPure RNA Kit (TAKARA BIO). RNA (1.6 μ) was reverse transcribed with oligo-dT primer (1 μM) using AMV reverse transcriptase (Promega) in a 20-μl reaction mixture for 1 h at 42 °C. Two microliters of sample was used for PCR (25-μl mixture) with gene-specific primers. The PCR products were separated by polyacrylamide gel electrophoresis and visualized by staining with ethidium bromide. The amounts of PCR products were compared after normalizing each sample to the amount of *GAPDH* PCR product [9,20]. The experiments were performed at least three times with similar results.

3. Results

3.1. Oligomerization and HSE-binding of HSF4b in vitro

HSF4 mutations associated with congenital cataract formation are located in the DBD (A20D, R74H, and I87V), linker region (L115P and R120C), and HR-A (R176P) (Fig. 1A) [14,16,17]. Two mutations identified in age-related cataract patients, Q62R and R117H, are located in the DBD and linker region, respectively [19]. The effects of these mutations on HSF4b oligomerization were analyzed using in vitro-synthesized polypeptides (Fig. 1B). Wild-type HSF4b polypeptides treated with EGS, a chemical cross-linker, were detected as two bands on SDS-polyacrylamide gels (Fig. 1C). The faster migrating band with a molecular size of 60 kDa was a monomeric form of HSF4b. The slower migrating band had a molecular size of 180 kDa, which corresponds to the size of an HSF4b trimer. The Q62R, R74H, I87V, and R117H mutations did not affect the formation of HSF4b trimers. However, oligomerization was markedly inhibited in the A20D, L115P, and R120C mutant proteins and completely inhibited in the R176P mutant protein under the assay conditions. Therefore, HSF4b oligomerization is modulated by the DBD, linker region, and HR region.

Electrophoretic mobility shift assays were conducted to determine the HSE-binding ability of HSF4b polypeptides. Probe oligonucleotides were synthetic model HSEs consisting of three inverted repeats of nGAAn that were either continuous (3P), containing one (gap), or two (step) skips between three repeats (Fig. 1D). As shown previously [9], wild-type HSF4b polypeptides were capable of binding to continuous 3P-type HSE and discontinuous gap- and step-type HSEs (Fig. 1E). HSF4b containing the A20D mutation bound to continuous HSE, but not to discontinuous HSEs. In contrast, HSF4b-Q62R exhibited slightly higher binding affinity for discontinuous HSEs than wild-type HSF4b. The I87V and R117H mutations did not significantly affect binding of HSF4b to the three HSE types; however, the R74H, L115P, R120C, and R176P mutations completely inhibited binding. These results show that the eight mutant HSF4b polypeptides exhibit different abilities to oligomerize or bind to HSEs in vitro.

3.2. Expression of model reporter genes by HSF4b–VP

It has been shown that the DBD of *Saccharomyces* HSF regulates not only the DNA-binding ability but also the transactivating ability of the activation domain [22]. To further analyze the effects of HSF4 mutations on protein–DNA interaction, HSF4b fused to the constitutively active VP16 activation domain was cotransfected with reporter gene into HeLa cells. HSF4b–VP fusions induce expression of HSE-SV40p-*LUC* reporter genes, which contain model HSEs upstream of the SV40 promoter-luciferase gene fusion, depending on the DNA-binding ability, even though the mutations may affect the function of the HSF4b activation domain [9]. Expression of HSF4b–VP was shown by immunoblot analysis (Fig. 3A). Cotransfection of wild-type HSF4b–VP caused a 9.4-, 4.7-, and 4.1-fold increase in luciferase activity of reporter genes that contained HSE3P, HSEgap, and HSEstep, respectively (Fig. 3B). Consistent with the results of the electrophoretic mobility shift assays, HSF4b–VP containing the A20D mutation was capable of inducing expression of reporter gene containing continuous 3P-type HSE, but not discontinuous gap- and step-type HSEs. Three mutations slightly affected reporter gene activity: the Q62R mutation appeared to enhance expression of reporter genes containing discontinuous HSEs, while the I87V and R117H mutations appeared to inhibit expression of all three reporters. Other mutations almost completely inhibited the transcription activating ability of HSF4b–VP. Note that, despite the same amount of plasmid DNA being used for transfection, protein levels of the transcriptionally active HSF4b–VP derivatives (WT, Q62R, I87V, and R117H) were reduced compared with those of the inactive derivatives (A20D, R74H, L115P, R120C, and R176P) (see Fig. 2A), implying that the expression of HSF4b–VP is negatively regulated by an unknown feedback mechanism. Whatsoever the reason, the induction of expression by mutant HSF4b–VP fusions acting on model HSEs varied according to their DNA-binding abilities.

3.3. Transcription of lens protein genes by HSF4b

To examine the transcriptional activity of HSF4b, wild-type and mutant proteins were expressed in the human lens epithelial cell line SRA01/04 (Fig. 3A). The mRNA levels of *CRYGC* encoding γC-crystallin were significantly increased by wild-type HSF4b, as determined by RT-PCR (Fig. 3B). HSF4b containing the A20D, R74H, L115P, R120C,