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Structural insights into the impact of two holoprosencephaly-related mutations on human TGIF1 homeodomain

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ABSTRACT

Human protein TGIF1 is an essential regulator of cell fate with broad roles in different tissues, and has been implicated in holoprosencephaly (HPE) and many cancers. The function of TGIF1 in transcriptional regulation depends on its three-amino acid loop extension (TALE) type of homeodomain (HD). Two missense mutations that led to P192A and R219C substitutions in TGIF1-HD were previously found in HPE patients and suggested to be the causes for these cases. However, how these mutations affected TGIF1 function has not been investigated from a structural view. Here, we investigated the roles of P192 and R219 in TGIF1-HD structure packing through determining the NMR structure of TGIF1-HD. Surprisingly, P192 and R219 were found to play roles in packing α1 and α2 to α3 together with A190 and F215 through side-chain interactions. Circular dichroism (CD) showed that P192A and R219C mutants displayed structural change and less folding compared with wild-type TGIF1-HD, and ^{1}H-^{15}N HSQC spectrum of P192A mutant exhibited chemical shift perturbations in all three helices of TGIF1-HD. Thus, it is suggested that P192A and R219C mutations led to structure disturbances of TGIF1-HD, which subsequently reduced the DNA-binding affinity of TGIF1-HD by 23-fold and 10-fold respectively, as revealed by the isothermal titration calorimetry (ITC) experiments. Our study provides structural insights of the probable pathogenesis mechanism of two TGIF1-related HPE cases, and evidences for the roles of P192 and R219 in HD folding.

1. Introduction

Homeodomains (HDs) are classical DNA-binding domains engaged by numerous development and disease-related transcription factors for binding sequence-specific DNA. Various structural studies on HDs in different transcription factors revealed a conserved structural architecture consisting of three α-helices connected by short loop regions, with the third helix for specific DNA binding [1]. The three-amino acid loop extension (TALE) homeodomains are atypical HDs that contain three more amino acids in the loop connecting helix α1 and α2, and could be divided into six classes including TGIF, MEIS, PREP, PBX, IRX and MKX [2].

Human TGIF1 (5′-TG-3′ interacting factor-1 or transforming growth-interacting factor-1) was originally characterized to be a transcriptional repressor that contains 272 amino acids [3]. Following study further identified five kinds of TGIF1 proteins with different length of N-terminus. They are encoded by twelve transcript variants of *TGIF1* gene resulted from alternative splicing, and the longest one contains 401 amino acids [4]. Nevertheless, all five kinds of TGIF1 have a TALE homeodomain located in the middle for specific DNA binding, and the same C-terminal part for interacting with various proteins. Through specific DNA binding and alternative interacting with various proteins involved in multiple cell signal pathways, TGIF1 controls cell proliferation, differentiation and apoptosis [3,5–8]. Abnormal TGIF1 function has been

Abbreviations: HPE, holoprosencephaly; TALE, three-amino acid loop extension; HD, homeodomain; TGIF1, 5′-TG-3′ interacting factor-1 or transforming growth-interacting factor-1; TGF-β, transforming growth factor-β; CD, circular dichroism; ITC, isothermal titration calorimetry; HSQC, heteronuclear single quantum coherence; CSP, chemical shift perturbation.

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implicated in many human diseases including holoprosencephaly (HPE) and cancers [9–12].

The TALE HD of TGIF1 is essential for its transcriptional repression of retinoid and TGF-β signaling [3,6]. TGIF1-HD was initially characterized to bind a retinoid X receptor (RXR) responsive element in the promoter of cellular retinol-binding protein II (CRBPII) for transcriptional repression in retinoid signaling, and the consensus motif bound by TGIF1-HD was determined as 5′-TGTCA-3' [3]. TGIF1-HD is also required for the repression of TGF-β signaling by TGIF1 [6]. So far, more than 1600 genes transcriptionally regulated by TGIF1 have been identified [9].

TGIF1 is one of the earliest four genes implicated in HPE pathogenesis, which is a severe human genetic disease with abnormal craniofacial development [13]. Many mutations of *TGIF1* gene that led to missense or truncation have been found in various HPE patients [12,14–17]. Among them, two missense mutations that led to P192A and R219C substitutions in TGIF1-HD, were suggested to be the causes for corresponding HPE cases [12,14]. Both P192A and R219C mutants reduced the DNA binding ability and expression level of TGIF1, and thereby impaired the repressive role of TGIF1 in retinoid and TGF-β signaling [17]. However, whether these mutations lead to structural disturbance remains to be investigated.

In this study, based on the first reported structure of TGIF1-HD, the roles of P192 and R219, two HPE-related residues, in structure packing were revealed. Both P192A and R219C mutations showed impaired DNA binding affinity and altered structure compared with wild-type TGIF1-HD. Furthermore, 2D ^{1}H-^{15}N HSQC spectrum of ^{15}N labeled P192A protein displayed chemical shift perturbations (CSPs) in all three helices, confirming the role of P192 in TGIF1-HD folding.

2. Materials and methods

2.1. Preparation of protein and DNA samples

The DNA fragment that encodes human TGIF1 (L171-E248) containing the homeodomain was cloned into an NESG-modified pET15 expression vector including a short N-terminal His-tag (MGHHHHHHSH) [18]. *U*-^{13}C, ^{15}N (NC) and *U*-^{15}N, 5% biosynthetically-directed ^{13}C (NC5) samples of TGIF1-HD were expressed and purified following standard protocols of NESG [19]. Briefly, BL21 (DE3) *E. coli* cells containing above plasmid construct were grown in MJ minimal medium at 37 °C until OD_{600} reached 0.6–0.8. Then, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was supplemented to induce protein expression at 17 °C overnight. The cells were harvested by centrifugation and lysed by sonication. The supernatant containing TGIF1-HD protein was further clarified by high speed centrifugation and filtration, and then purified using an ÄKTAxpress™ (GE Healthcare) equipped with a Ni-affinity column (HisTrap IMAC HP™ column) followed by a gel filtration column (HiLoad 26/60 Superdex 75). The purified protein was concentrated to a final concentration of 0.4 mM for study in the NMR buffer containing 10% v/v D_2O: 20 mM NH_4OAc, 100 mM NaCl, 5 mM $CaCl_2$, 10 mM DTT, and 0.02% NaN_3 at pH 4.5. The samples of P192A and R219C mutants were prepared using similar method as described above.

Single-stranded DNA (ssDNA) 5′-AGCTGTCAAAAC-3′ and its complementary DNA 5′- GTTTTGACAGCT-3′, purchased from Sangon Biotech Co., was dissolved with Milli-Q water and mixed at equimolar concentrations. The double-stranded DNA (dsDNA) was obtained through annealing above two ssDNA in a buffer containing 10 mM Tris, 50 mM NaCl, 1 mM EDTA, and pH 7.5. Annealing was conducted by heating the mixed DNA to 95 °C in a water bath for 5 min and then cooling down to room temperature overnight. After desalting, the DNA was lyophilized and re-suspended with the same buffer as that used in the NMR experiments to a final concentration of 5 mM.

2.2. Isothermal titration calorimetry (ITC) measurement

ITC was performed on a VP-ITC instrument (GE Healthcare). All samples were prepared in the same buffer as that used in the NMR experiments to avoid any heat change resulted from mixing buffers. The protein solutions of wild-type, P192A and R219C versions of TGIF1-HD in 100 μM were titrated at 298 K, into the reservoir containing 10 μM DNA respectively. Each titration experiment consisted of a preliminary injection of 0.5 μl followed by 24 injections of 1.2 μl. After converting the raw data into heat per injection, the curves were fitted using MicroCal Origin 7.0 (Microcal Software Inc.) to obtain the binding constant, stoichiometry and binding enthalpy. The first data point was not included when the titration curves were fitted. Dilution heat was subtracted by titrating protein solutions into buffer for each experiment. All ITC experiments were repeated in triplicate.

2.3. Chemical shift assignments

All NMR experiments on both NC and NC5 samples were carried out at 298 K. NMR data for backbone and side chain assignments were collected on a Varian Inova 600 MHz spectrometer with a 5-mm HCN cryogenic probe, and NOESY data for structure determination were collected on a Bruker Avance III 850 MHz spectrometer equipped with a conventional room temperature 5-mm HCN probe. Backbone assignments were determined from a series of spectra including 2D ^{1}H-^{15}N HSQC and ^{1}H-^{13}C HSQC, 3D HNCO, HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH and HBHA(CO)NH, while side chain assignments from spectra including 2D ^{1}H-^{13}C HSQC (aliphatic and aromatic), 3D HCCH-COSY, HCCH-TOCSY, (H)CCH-TOCSY and 4D^{13}C-^{13}C-HMQC-NOESY-HMQC. Stereo-specific isopropyl methyl assignments for all Val and Leu amino acids were achieved using 2D constant-time ^{1}H-^{13}C HSQC spectrum of NC5 sample [20]. All NMR data were processed using NMRPipe and analyzed with the Sparky program. The backbone and side chain resonances were automatically assigned using PINE Server from NMRFAM [21], followed by manual validation and correction. All assignments were further confirmed from NOESY spectra including 3D^{15}N-edited NOESY-HSQC and ^{13}C-edited NOESY-HSQC (optimized for aliphatic or aromatic carbons) with mixing time of 70 ms on the NC sample. Final assigned ^{1}H, ^{13}C and ^{15}N chemical shifts were deposited in the BioMagResBank (BMRB) with accession number 17971.

2.4. Structure calculation

NOE-based inter-proton distance restraints were determined automatically for TGIF1-HD using CYANA 3.0. Input for CYANA consisted of chemical shift assignments, NOESY peak lists from four NOESY spectra with peak intensities, the restraints for backbone phi (φ) and psi (ψ) torsion angle derived from chemical shifts of backbone atoms using the TALOS + software program [22]. Manual and iterative refinements of NOESY peak picking lists were guided using NMR RPF quality to assess "goodness of fit" between calculated structures and NOESY peak lists [23]. Towards the end of the iterative structure calculation process, hydrogen bond restraints for the NH–O and N–O distances were introduced based on identification of proximity of potential donors and receptors in early structure calculations. The 20 lowest energy structures calculated by CYANA 3.0 were further refined using restrained molecular dynamics in explicit water CNS 1.2 [24] and the PARAM19 force field, using the final NOE-derived distance constraints, TALOS-derived