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¹H, ¹³C and ¹⁵N chemical shift assignments for the human Pitx2 homeodomain in complex with a 22-base hairpin DNA

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Abstract

As one of the most important eukaryotic DNA-binding motifs, the homeodomain has been identified in over one thousand proteins. Transcription factors containing homeodomains play critical roles in diverse biological processes and are often implicated in human genetic disorders. The human Pitx2 protein is a central downstream transcription factor in both the TGF- β and Wnt/ β -catenin signaling pathways, and involved in the regulation of cell differentiation, cell pattern formation and tissue maintenance. To better understand how Pitx2 regulates its target genes, we are investigating the mechanisms that confer DNA-binding specificity on its homeodomain. Here, we report complete ¹H, ¹³C and ¹⁵N protein chemical shift assignments of the human Pitx2 homeodomain in complex with a non-consensus DNA sequence.

Keywords

Pitx2; Homeodomain; Axenfeld-Rieger; DNA-binding; NMR

Biological Context

As one of the four major DNA-binding motifs, the homeodomain is an evolutionarily conserved protein fold that is an integral component of both prokaryotic and eukaryotic transcription factors (Banerjee-Basu et al. 2003). It plays important roles in diverse biological processes ranging from embryonic pattern formation to cell-type specification, and differentiation. The conserved 60 amino acid domain achieves its regulatory role by binding to short 6 base pair DNA sequences with a common TAAT core followed by 2 variable base pairs that confer specificity.

Given the number of different homeodomain-containing regulatory proteins, the plethora of cellular processes they regulate, and the limited number of 6 base pair combinations, it has been suggested that regulation occurs on multiple levels (Biggin and McGinnis 1997; Zhao et al. 2000). These levels include the target DNA sequence, homeodomain composition, and DNA binding cooperativity with other DNA-binding cofactors. As each homeodomain protein utilizes its own unique combination of strategies, understanding these strategies will be a major step towards understanding the principles that govern homeodomain-mediated cellular processes. In the case of the Pitx2 homeodomain, it recognizes TAATCC as its consensus DNA sequence, yet it is capable of binding to various other sequences including

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TAAGCC, AAATCC or TAGTCC (Chaney 2005). As part of our investigation of the mechanisms that confer DNA-binding specificity on the homeodomain motif, we report the NMR assignments of the Pitx2 homeodomain bound to a non-consensus TAAGCT DNA sequence. This sequence has not been identified as a native target of the Pitx2 homeodomain, yet *in vitro* experiments indicate a nanomolar affinity (unpublished). NMR chemical shift assignment is an important next step toward elucidating the structural and biophysical properties that govern DNA-binding for the Pitx2 homeodomain, and comparison with our free homeodomain and consensus DNA-bound homeodomain solution structures will improve our understanding of sequence specific DNA-binding.

Methods and experiments

Protein Expression and Purification

Proteins were expressed in *Escherichia coli* BL21DE3star cells (Invitrogen) from a pET-28 expression vector as a fusion protein containing a N-terminal Tobacco Etch Virus (TEV) protease-cleavable 6xHisTag with a C-terminal tail (EFIVTD). Expression conditions were as described previously (Chaney et al. 2005). Cells were harvested by centrifugation at 2500g for 15 minutes. Harvested cells were resuspended in 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic at pH 7.4 (PBS) + 10mM imidazole. Resuspended cells were lysed via sonication. The lysate was cleared by centrifugation at 25,000g for 30 minutes at 4°C. Cleared lysate was applied to a pre-equilibrated 5 ml HisTrap HP column (GE Healthcare). The column was washed with 10 column volumes (c.v.) of PBS+10mM imidazole. An additional wash step of 10 c.v. with PBS+100 mM imidazole was performed. The target fusion protein was eluted with 10 c.v. of PBS+500 mM imidazole. Protein concentrations were estimated via A_{278} ($\epsilon_{278} = 18350 \text{ cm}^{-1} \text{ M}^{-1}$) and the eluate was adjusted to contain 10% (v/v) glycerol, 5 mM β -mercaptoethanol and 5 mM EDTA. In-house produced TEV protease was added for fusion tag removal at a 1:25 ratio and cleavage was performed at 4°C over 4 hours. Cleaved protein was loaded on a 1 ml HiTrap SP FF (GE Healthcare) cation exchange column, washed with buffer (10 mM NaH_2PO_4 , 400 mM NaCl, pH 7.0), and eluted with the same buffer containing a higher salt concentration (10 mM NaH_2PO_4 , 1 M NaCl, pH 7.0). Purity was determined to be >98% by SDS-PAGE. The purified protein was dialyzed overnight at 4°C into 10 mM NaH_2PO_4 , 1 mM EDTA, pH 7.0.

A hairpin-forming DNA sequence 5'-GCTAAGCTGCTTGCAGCTTAGC-3' was purchased from IDTDNA and heated to 95°C for 15 minutes followed by rapid cooling on ice. An equimolar amount of DNA was added to the dialyzed protein and the complex was then concentrated utilizing Amicon Ultra15 spin filters (Millipore) with a 3kDa molecular cut-off.

Nuclear Magnetic Resonance Spectroscopy

Final sample concentrations were approximately 1–1.5 mM, in 90% H_2O /10% D_2O solution containing 10mM NaH_2PO_4 (pH 7.0) and 1 mM EDTA. All NMR experiments were carried out on Varian Inova 500, 600 and 800 MHz spectrometers. The sample temperature was set to 295K. ^1H chemical shifts were referenced to internal DSS, and the ^{13}C and ^{15}N shifts were referenced indirectly (Wishart et al. 1995). Assignment of backbone and side-chain resonances were obtained utilizing the following 3D NMR experiments: HNCACB, HNCA, CBCA(CO)NH, HNCO, HBHA(CO)NH, (H)CC(CO)NH – TOCSY, H(CC)(CO)NH – TOCSY, HCCH – TOCSY. Data were processed with NMRPipe (Delaglio et al. 1995) and spectra analyzed with NMRViewJ (Johnson 2004).

Assignment and data deposition

The ^1H - ^{15}N HSQC spectrum for the human wild-type Pitx2 homeodomain bound to the TAAGCT DNA-sequence is shown in Fig. 1. The native homeodomain sequence corresponds to resonances 1 – 60. Complete backbone assignments were obtained for residues 2 – 66, and near complete assignments (>95%) were obtained for side-chain nuclei. Indicative of a properly folded protein, the majority of residues displayed strong, well-dispersed amide backbone resonances. The amide side chain of asparagine at position 51 is shifted noticeably out of the range typically observed for asparagine and glutamine side chains. This behavior has previously been observed for other homeodomains bound to DNA, including both the Pitx2 and the Bicoid homeodomains bound to their TAATCC consensus DNA (Chaney et al. 2005; Baird-Titus et al. 2006), but was not observed for the DNA-free protein (Doerdelmann et al. 2010). Mapping the backbone amide chemical shift changes between DNA-bound and free Pitx2 homeodomain onto the projected secondary structure profile (Fig. 2) suggests local changes in the N-terminal arm and throughout helix 3. These changes are in agreement with our previous findings and the common observation that the homeodomain utilizes its N-terminal arm and helix three to make specific DNA contacts.

The ^1H , ^{13}C and ^{15}N chemical shifts have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu/>) under accession number 17452.

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Fig. 1.
 2D ^1H - ^{15}N HSQC spectrum of uniformly ^{15}N -labeled Pitx2 homeodomain bound to the TAAGCT DNA duplex. The spectrum was recorded at 600 MHz ^1H frequency at a temperature of 295K in 10 mM NaH_2PO_4 , pH 7.0 buffer. Backbone resonance assignments are indicated in one-letter amino acid code. Straight lines indicate glutamine and asparagine amide side chain resonances. A significantly shifted asparagine 51 side chain is indicated in red letters.

Fig. 2. Weighted chemical shift changes of the TAAGCT-bound Pitx2 homeodomain. Backbone ^1H and ^{15}N chemical shift changes Δ_{av} ($\Delta_{av} = [(\Delta\delta_{\text{HN}}^2 + \Delta\delta_{\text{N}}^2/25)/2]^{1/2}$) relative to the backbone chemical shifts of the free wild-type protein