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¹H, ¹³C and ¹⁵N chemical shift assignments for the human Pitx2 homeodomain and a R24H homeodomain mutant

Thomas Doerdelmann, Douglas J. Kojetin, Jamie M. Baird-Titus, and Mark Rance

Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, OH 45267, USA

Abstract

The homeodomain is one of the most important eukaryotic DNA-binding motifs and has been identified in over one thousand proteins. Homeodomain proteins play critical roles in diverse biological processes, including cell differentiation and cell pattern formation. The human Pitx2 homeodomain binds several different DNA sequences and is a pivotal component of both the TGF- β and Wnt/ β -catenin signaling pathways. As the recognition of specific DNA sequences represents an essential biochemical function of all DNA-binding proteins, we have chosen the Pitx2 homeodomain model to investigate the mechanisms that convey biological specificity in these protein-DNA interactions. Here, we report complete chemical shift assignments of the human Pitx2 homeodomain and the R24H mutation that induces ring dermoid of the cornea syndrome.

Keywords

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

Biological context

The homeodomain is an evolutionarily conserved protein fold commonly found in transcription factors. Its 60 amino acid helix-turn-helix motif allows for DNA and RNA binding in both prokaryotic and eukaryotic organisms (Banerjee-Basu et al. 2003). Despite the diverse biological functions attributed to homeodomain-containing proteins, the function of the homeodomain alone is generally similar across the family. Homeodomains bind to short DNA fragments consisting of six base-pairs with the predominant consensus sequence TAATXY (where X and Y can be A, G, C or T). The Pitx2 homeodomain recognizes TAATCC as its consensus DNA sequence and is classified as a Bicoid-type, or K-50 class, homeodomain—defined as those homeodomains with a lysine in position 50.

The homeodomain-containing transcription factor Pitx2 is present in many developing embryonic tissues, including the heart (Su et al. 2004). Several human diseases, such as Rieger syndrome, iris hypoplasia and iridogoniodysgenesis are linked to mutations in the *Pitx2* gene (Saadi et al. 2001; Semina et al. 1996). These autosomal dominant diseases present themselves with anterior segment abnormalities including the eye, as well as the

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Mark.Rance@uc.edu .

Present Address: D. J. Kojetin Department of Molecular Therapeutics, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA

Present Address: J. M. Baird-Titus Department of Chemistry, College of Mount St. Joseph, 5701 Delhi Road, Cincinnati, OH 45233, USA

teeth. In addition, *Pitx2* also plays a critical role in left-right asymmetry and heart development. *Pitx2* $-/-$ null mice exhibit many heart defects including malpositioning of the heart, lack of atrial septation, hypoplasia of the ventricles and atrioventricular valve deformation (Kitamura et al. 1999). The severity of disease associated with *Pitx2* mutations is consistent with its role as a pivotal component of both the TGF- β and Wnt/ β -catenin signaling pathways, which control the activity of many transcription factors and cell cycle regulators. Not surprisingly, the homeodomain of *Pitx2* has been identified as a hotspot for mutations. The majority of these homeodomain mutations fall into three broad categories (1) those that modify DNA-binding ability (2) those that enhance or diminish transactivation capability and (3) those that impact protein stability. One such mutation that modifies DNA binding involves an amino acid substitution of arginine 24 to histidine (R24H) and causes the autosomal dominantly inherited ring dermoid of the cornea syndrome (MIM180550) (Xia et al. 2004). Unlike many other *Pitx2* homeodomain mutations the only clinical manifestations in R24H affected patients are observed in the eyes (Xia et al. 2004) suggesting that from a clinical perspective this may be considered a mild mutation. To better understand the molecular mechanisms of sequence specific DNA binding, we report the NMR assignments of the *Pitx2* homeodomain in its wildtype (wt) and R24H mutant isoforms. This is an important first step toward elucidating the basis of sequence specific DNA binding and will aid in our understanding in the evolution of ring dermoid of the cornea syndrome via the *Pitx2* R24H mutation.

Methods and experiments

Protein expression and purification

Proteins were expressed from a pet28 expression vector as a His₆-TEV-GS-*Pitx2*homeodomain-EFIVTD fusion protein in *Escherichia coli* BL21DE3star cells (Invitrogen). Expression conditions were as described previously (Chaney et al. 2005). Cells were harvested by centrifugation at 2,500g for 15 min. 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) + 10 mM imidazole. Resuspended cells were lysed via sonication. The lysate was cleared by centrifugation at 25,000g for 30 min, 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 + 10 mM imidazole. Next, an additional wash step of 10 c.v. with PBS + 100 mM imidazole was performed. Target fusion protein was eluted with 10 c.v. of PBS + 500 mM imidazole. Protein concentrations were estimated via A₂₇₈ ($\epsilon_{278} = 18,350 \text{ cm}^{-1} \text{ M}^{-1}$) 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 h. Cleaved protein was then loaded on a 1 ml HiTrap SP FF (GE Healthcare) cation exchange column, washed with washing buffer (10 mM NaH₂PO₄, 400 mM NaCl, pH 7.0), and eluted with buffer containing a higher salt concentration (10 mM NaH₂PO₄, 1 M NaCl, pH 7.0). Purity was determined to be >98% by SDS-PAGE. The eluted homeodomain was dialyzed overnight at 4°C into 10 mM NaH₂PO₄, 150 mM Na₂SO₄, 1 mM EDTA, pH 7.0 for NMR studies. Proteins were then concentrated utilizing Amicon Ultra15 spin filters (Millipore) with a 3 kDa molecular cut-off.

Nuclear magnetic resonance spectroscopy

Final sample concentrations were approximately 1–1.5 mM, in 90% H₂O/10% D₂O solution containing 10 mM NaH₂PO₄, 150 mM Na₂SO₄ and 1 mM EDTA, at pH 7.0. All NMR experiments were carried out on Varian Inova 500, 600 and 800 MHz spectrometers. The sample temperature was set to 295 K. The ¹H chemical shifts were referenced to internal DSS and the ¹³C and ¹⁵N shifts were referenced indirectly (Wishart et al. 1995). Assignment

of backbone and side-chain resonances were obtained utilizing the following experiments: HNCACB, HNCA, CBCA(CO)NH, HNCO, HBHA(CO)NH, (H)CC(CO)NH-TOCSY, H(CC)(CO)NH-TOCSY, HCCH-TOCSY. A pH titration of the histidine side chains was performed using a long-range ^1H - ^{15}N HSQC experiment. Raw data was processed utilizing NMRPipe (Delaglio et al. 1995) and spectra were analyzed via NMR-ViewJ (Johnson 2004).

Assignment and data deposition

The ^1H - ^{15}N HSQC spectra for the human wt and R24H Pitx2 homeodomain are 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 (96%) were obtained for side-chain nuclei. The majority of residues exhibited strong, well-dispersed backbone amide resonances, indicative of a protein with a defined three-dimensional structure. For the mutant protein, most residues exhibit only small chemical shift changes suggesting that this mutation does not impact the core fold. Distinct differences in chemical shifts are observed around the point of mutation as indicated in Fig. 1. Mapping the backbone amide chemical shift changes onto the projected secondary structure profile suggests local changes in the region towards the end of helix 1 and between helices 1 and 2 (Fig. 2). Additional changes towards the end of helix 3 cannot directly be explained through physical proximity. The pK_a of H24 was determined to be 6.76 ± 0.08 , suggesting that the effects of the R24H mutant may be of electrostatic nature. The pK_a of H7 was 6.57 ± 0.04 .

The ^1H , ^{13}C and ^{15}N chemical shifts have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu/>) under accession number 17147 (wt) and 17145 (R24H).

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Fig. 1. Overlaid 2D ^1H - ^{15}N HSQC spectra of uniformly ^{15}N -labeled Pitx2 homeodomain wildtype (*red*) and R24H mutant (*black*) in 10 mM NaH_2PO_4 , 150 mM Na_2SO_4 , pH 7.0. The spectra were recorded at 600 MHz ^1H frequency at a temperature of 295 K. Backbone resonance assignments are indicated in *one-letter* amino acid code for the wt protein. *Straight lines* indicate resonances that have shifted significantly in the mutant isoform. Locations of peaks not visible at the contour level chosen are indicated with a *box*

Fig. 2.

Weighted chemical shift changes of the Pitx2 homeodomain R24H mutant. Backbone $^1\text{H}_\text{N}$

and ^{15}N chemical shift changes $\Delta_{\text{av}} \left(\Delta_{\text{av}} = \left[\left(\Delta\delta_{\text{HN}}^2 + \Delta\delta_{\text{N}}^2 / 25 \right) / 2 \right]^{1/2} \right)$ relative to the backbone chemical shifts of the free wildtype protein