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Functional analysis of transcriptional repressor Otx3/Dmbx1

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Abstract Otx3/Dmbx1 is a member of paired class homeodomain transcription factors. In this study, we found that Otx3/Dmbx1 represses the Otx2-mediated transactivation by forming heterodimer with Otx2 on the P3C (TAATCCGATTA) sequence in vitro. The 156 amino acid region (residues 1–156) of Otx3/Dmbx1 is required for its repressor activity, and interacts directly with Otx2. Co-localization of Otx3/Dmbx1 and Otx2 in brain sections was confirmed by in situ hybridization. These data suggest that Otx3/Dmbx1 represses Otx2-mediated transcription in the developing brain. We also identified the consensus binding sequence [TAATCCGATTA and TAATCC(N2-4)TAATCC] of Otx3/Dmbx1.

Keywords: Heterodimerization; Otx2; Otx3/Dmbx1

1. Introduction

Transcription factors containing a homeodomain (HD) are involved in genetic control of the development and maintenance of many cell types [1]. HD proteins of the Otx family (Otx1, Otx2, and Crx), the vertebrate homologues of Drosophila *orthodenticle* (*otd*), possess a K50 paired class HD, and play a critical role in the development of brain and eyes [2–4]. We previously identified Otx3/Dmbx1, a novel member of the Otx family [5]. Otx3/Dmbx1 expression is craniocaudally limited to the rostral region of the developing brain in mice from 7.5 to 11.5 days postcoitum (dpc). Otx3/Dmbx1 also is expressed in developing eyes, adult cerebellum, and pancreatic islets [5]. Recently, Ox3/Dmbx1 knockout mice were found to exhibit the neonatal lethality, dwarfism, and abnormal brain morphology [6], suggesting an important role of Otx3/Dmbx1 in brain development. During early organogenesis, the expression pattern of Otx3/Dmbx1 [5,7–11] overlaps those of Otx1 and Otx2 [2]. Using reporter gene assay, other members of Otx family (Otx1, Otx2, and Crx) were found to function as transcriptional activators [3,12–15], and we previously reported that Otx3/Dmbx1 represses Otx2-mediated transactivation [5]. In this study, we have investigated the biochemical and functional interactions between Otx3/Dmbx1 and Otx2 in vitro. We also screened its consensus binding sequences.

2. Materials and methods

2.1. Plasmids

cDNAs of mouse Otx3/Dmbx1 and Otx2 were obtained as described previously [5]. Series of Otx3/Dmbx1 mutants were subcloned into pGEX-4T (Amersham Bioscience, Uppsala, Sweden) and pFLAG-2 (Sigma–Aldrich, St. Louis, MO, USA) vectors. For generating fusion constructs of the Gal4 DNA binding domain (Gal4dbd) and Otx3/Dmbx1 or Otx2, series of Otx3/Dmbx1 deletion mutants or the C-terminus of Otx2 were subcloned into pFA-CMV vector (Stratagene, La Jolla, CA, USA).

2.2. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously [5]. A partial fragment of Otx3/Dmbx1 [amino acid (aa) 58–130] protein fused to glutathione *S*-transferase (GST) and nuclear extract from COS-1 cells transfected with FLAG-tagged Otx2 or Otx3/Dmbx1 (aa 1–156) were used. Nuclear extract was prepared as described previously, from about 2×10^6 transfected cells [16]. The oligonucleotide sequences used in EMSA were as follows: P3C(5′-GATCCTGAGTCTAATCCGATTAGTGTGCA-3′, 5′-GAGGACTCAGATTAGGCTAATCACACGTCTAG-3′), DR4(5′-GATCACGGGTCTTAATCCTTTAATCCATA-3′, 5′-GATCTATGGATTAAAGGATTAAGACCCGT-3′), DR5(5′-GATCTTAGCTTAATCCCCTTAATCCGTC-3′, 5′-GATCGACGGATTAAGGGGATTAAGCTAA-3′), DR6(5′-GATCTTATATAATCCCTTTTAATCCCTTT-3′, 5′-GATCAAAGGGATTAAAAGGGATTATATAA-3′).

For super-shift assay, anti-FLAG M2 antibody (Sigma–Aldrich) was incubated at 4 °C for 30 min with nuclear extract from COS-1 cells before mixing with radiolabeled probe.

2.3. Reporter assay

P3C-*tk*-luc and pFR-luc (Stratagene) were used as reporter plasmids. P3C-*tk*-luc was generated to express luciferase under control of the thymidine kinase (*tk*) minimal promoter linked to triple repeats of the consensus P3C binding sequence. GH3 cells or HeLa cells were used as the host strain. The reporter assay was performed as described previously [5].

2.4. Western blotting

COS-1, GH3, and HeLa cells transfected with each plasmid were sonicated in 2 × SDS sample buffer, separated by SDS–PAGE, and subjected to immunoblot analysis with anti-FLAG M2 antibody (Sigma–Aldrich) or anti-GAL4(DBD) (RK5C1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

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Abbreviations: HD, homeodomain; Gal4dbd, Gal4 DNA binding domain; aa, amino acid; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; *tk*, thymidine kinase; dpc, days postcoitum

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2.5. GST pull down assay

GST-fused partial fragments of Otx3/Dmbx1 (aa 1–156) and the C-terminus of Otx2 (aa 98–289) were purified as described previously [5]. Lysates of COS-1 cells transfected with FLAG-tagged or Gal4dbd-fused Otx2 or Otx3/Dmbx1 were incubated with 1 μg of GST-fusion protein pre-bound to glutathione-agarose beads in binding buffer [50 mM Tris–HCl, pH 7.5, 165 mM KCl, 1 mM $MgCl_2$, 10% (v/v) glycerol, 1% Triton X-100, 0.5% deoxycholic acid, 1 mM PMSF (phenylmethylsulfonyl fluoride)]. Bound proteins were eluted by adding 2 × SDS sample buffer and subjected to Western blotting using anti-FLAG M2 antibody (Sigma–Aldrich) or anti-GAL4 (DBD) (RK5C1) (Santa Cruz).

2.6. In situ hybridization

In situ hybridization of Otx3/Dmbx1 and Otx2 was performed as described previously [5]. Antisense probes for Otx2 and Otx3/Dmbx1 were designed to be complementary nucleotides 810–854 of mouse Otx2 (BC029667) and 984–1028 of mouse Otx3/Dmbx1 (NM_130865).

2.7. Random binding site selection

The Otx3/Dmbx1 binding sequence was selected from the random oligonucleotides, as described previously [17]. The sequences of the oligonucleotides used for binding site selection were 5′-CTCGG TACCTCGAGTGAAGCTAGC (N25) GGTAAGTCGGATCC GCGGTAAC-3′. Two hundred nanograms of the GST-fused partial fragment Otx3/Dmbx1 (aa 58–130) protein was bound to 0.3 μg of double-stranded random oligonucleotides. Enrichment of binding sites was performed by filter-binding method. After five rounds of selection, amplified products were subcloned and sequenced.

3. Results

3.1. Otx3/Dmbx1 forms homodimer and heterodimer with Otx2 on the P3C sequence

EMSA showed that both full length Otx2 and Otx3/Dmbx1 form monomer and homodimer on the P3C probe (Fig. 1A, lanes 1 and 3). When both Otx2 and Otx3/Dmbx1 were mixed with the probe, a distinct band corresponding to the size of the Otx3/Dmbx1–Otx2 heterodimer appeared between the Otx2 homodimer and the Otx3/Dmbx1 homodimer (Fig. 1, lane 5). Western blot analysis confirmed that nuclear extract from COS-1 cells contained Otx2 and Otx3/Dmbx1 equally (Fig. 1B).

3.2. The residues 1–156 of Otx3/Dmbx1 is required to repress the Otx2-mediated transactivation

To determine the region of Otx3/Dmbx1 required for repression of Otx2-mediated transactivation, we performed reporter gene assay (Fig. 2). Western blot analysis confirmed that each mutant was clearly expressed in GH3 cells (Fig. 2A-b). Mutational analysis revealed that wild type, ΔC27, ΔC90, ΔC150, ΔC200, and Δ220 repress Otx2-mediated transactivation (Fig. 2A-c, lane 2 as 100%) by 74%, 71%, 52%, 77%, 88%, and 88%, respectively (Fig. 2A-c, lanes 3–8), while Δ251 and ΔN repress transactivation to a lesser degree of 20.0% and 30.8%, respectively (Fig. 2A-c, lanes 9 and 10). This indicates that the residues 1–156 (which contains the N-terminus, HD, and the following 31 amino acids) of Otx3/Dmbx1 is required for expression of repressor activity on the P3C sequence. To further determine

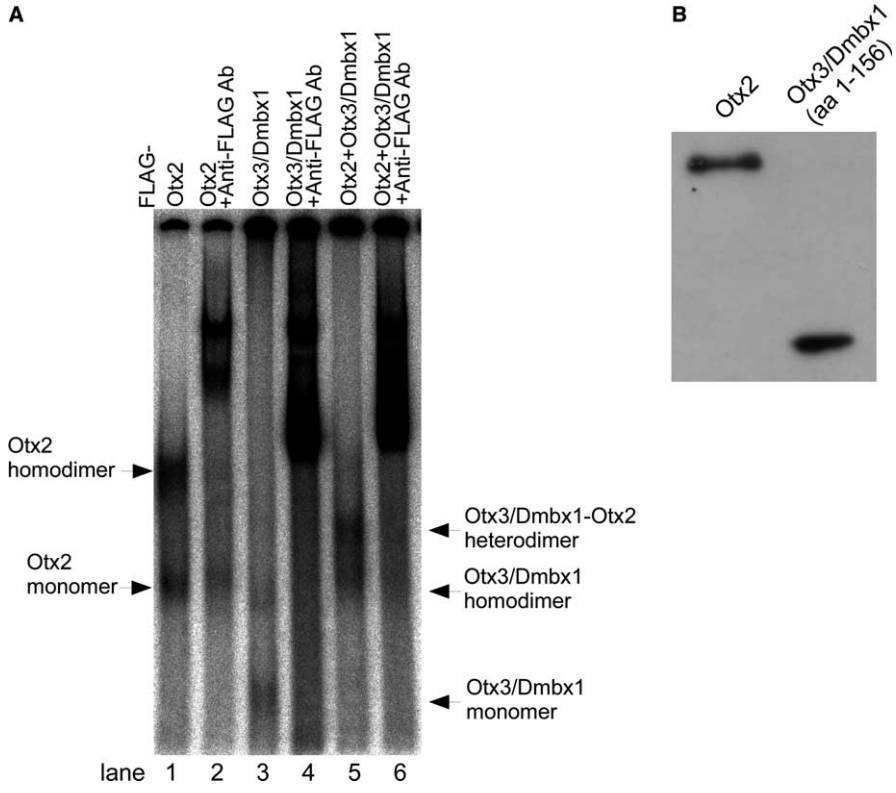

Fig. 1. DNA binding properties of Otx3/Dmbx1. (A) EMSA using nuclear extracts obtained from COS-1 cells transfected with FLAG-tagged Otx3/Dmbx1 (aa 1–156) and/or FLAG-tagged full-length Otx2 and ^{32}P-labeled P3C probe. The specificity of the band shifts was confirmed by super-shift assay using anti-FLAG M2 antibody (lanes 2, 4, and 6). (B) Expression of Otx2 and Otx3/Dmbx1 in COS-1 cells. Nuclear extract from COS-1 cells was analyzed by Western blotting with anti-FLAG M2 antibody.