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Drosophila distal-less negatively regulates dDREF by inhibiting its DNA binding activity

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Abstract

The *Drosophila* DNA replication-related element binding factor (dDREF) is required for expression of many proliferation-related genes carrying the DRE sequence, 5′-TATCGATA. Over-expression of dDREF in the eye imaginal disc induces ectopic DNA synthesis, apoptosis and inhibition of photoreceptor cell specification, and results in rough eye phenotype in adults. In the present study, half dose reduction of the *Distal-less* (*Dll*) gene enhanced the dDREF-induced rough eye phenotype, suggesting that *Dll* negatively regulates dDREF activity in eye imaginal disc cells. Biochemical analyses revealed the N-terminal (30aa to 124aa) and C-terminal (190aa to 327aa) regions of Dll to interact with the DNA binding domain (16aa to 125aa) of dDREF, although it is not clear yet whether the interaction is direct or indirect. Electrophoretic mobility shift assays showed that Dll thereby inhibits DNA binding. The repression of this dDREF-function by a homeodomain protein like Dll may contribute to the differentiation-coupled repression of cell proliferation during development.

Keywords: DNA replication-related element binding factor (DREF); Distal-less; DNA binding; Proliferation; Transcription factor

1. Introduction

Coordinated expression of many cell proliferation-related genes is required for the cellular shift from a resting to a proliferating state [1–5]. A candidate regulatory factor thought to be involved in this process is the transcription regulatory factor named DREF (DNA replication-related element-binding factor) found in *Drosophila* [6] and human [7]. *Drosophila* DREF (dDREF) consists of a homo-dimer of a polypeptide of 709 amino acid residues [6], which binds specifically to the DRE sequence (5′-TATCGATA) in the promoters of many DNA replication- and cell proliferation-related genes to activate their transcription [6,8,9]. The N-terminal 115 amino acid residues of dDREF containing the domain for specific DRE-binding and dimer formation act together as a dominant-negative effecter against endogenous dDREF [10]. We have established transgenic fly lines carrying cDNAs coding for either the N-terminal region of dDREF [10] or the full-length dDREF [11]. Expression of full-length dDREF in eye imaginal discs caused ectopic DNA synthesis in cells behind the morphogenetic furrow, that are normally postmitotic, and abolished photoreceptor specifications of R1, R6 and R7 [11]. Furthermore, dDREF expression resulted in apoptosis of imaginal disc cells in the region where commitment to R1/R6 cells takes place, suggesting that failure of differentiation of R1/R6 photoreceptor cells might cause apoptosis [11]. Consequently, the adult eyes exhibited an abnormal morphology, the rough eye phenotype [11]. Using transgenic flies, we have screened for mutations that enhance or suppress the dDREF-induced rough eye phenotype. Candidates for dDREF-interaction include cell cycle regulators such as dE2F [9], and

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regulators of chromatin-structure such as dMi-2, brahma, osa and moira [11]. Genetic interactions between dDREF and Distal-less (Dll) have also been observed [11]. *Dll* carrying the homeobox is the first genetic signal for limb formation from imaginal discs in *Drosophila* development [12].

In the present report, we provide several lines of evidence indicating that Dll specifically binds to the DNA-binding domain of dDREF and thereby inhibits its activity as a transcriptional activator. Repression of dDREF, a key regulatory factor for proliferation-related genes, by Dll may explain differentiation-coupled reduction of cell proliferation during development.

2. Materials and methods

2.1. Fly strains and genetic crossing

Fly stocks were maintained at 25 °C on standard food. The Canton S fly was employed as the wild-type strain. Dll^5 and Dll^9 alleles were obtained from the Bloomington, Indiana stock center. The UAS-dDREF fly was described earlier [11] along with the transgenic fly line (line number 16) carrying pGMR-GAL4 on the X chromosome [13]. Female flies expressing DREF (GMR-GAL4/GMR-GAL4; UAS-DREF/UAS-DREF; +/+) were crossed with individual males carrying mutant alleles for the *Dll* gene, and F1 progeny were allowed to develop at 25 °C.

2.2. Oligonucleotides

Dll-N: 5′-GGCGCTAGCATGGACGCCCCCGACGCCCCG
Dll-C: 5′-CTGCTCGAGTTAGACCGCCGGCCAAACAGT
These primers were used for the polymerase chain reaction (PCR) to amplify Dll cDNA.
UAST-SEQ: 5′-AGTAACCAGCAACCAAG

2.3. Plasmids

The series of plasmids expressing GST-dDREF (GST-dDREF$_{1–708}$, GST-dDREF$_{16–603}$, GST-dDREF$_{16–251}$, GST-dDREF$_{16–205}$, GST-dDREF$_{16–185}$, GST-dDREF$_{16–165}$, GST-dDREF$_{16–145}$, GST-dDREF$_{16–125}$, GST-dDREF$_{16–105}$, GST-dDREF$_{16–85}$, GST-dDREF$_{250–579}$ and GST-dDREF$_{250–612}$) were as described earlier [6,14].

The template plasmids for *in vitro* transcription/ translation reactions were pBS-DREF carrying a full length DREF cDNA in pBluescript II (Strategene) [14] and pBS-Dll carrying a full length Dll cDNA in pBluescript II.

The HA tag was cut out from pACT2 (Clontech) by digestion with *Bgl*II and *Xho*I, then cloned into *Bgl*II and *Xho*I sites of pUAST [15] to create the plasmid pUAST-HA. The full-length Dll cDNA was amplified by RT-PCR using total cellular RNA isolated from 9 to 12 h *Drosophila* embryos using TRIZOL (Invitrogen) and primers Dll-N and Dll-C. Amplified fragments were digested with *Nhe*I and *Xho*I, and the digested DNA fragment was cloned into *Nhe*I and *Xho*I sites of the pUAST-HA to create the plasmid pUAST-HADll. This was cut with *Nhe*I and blunt-ended by digestion with Mung bean nuclease, then the DNA fragment was cut out by digestion with *Xho*I. The isolated fragment was inserted between the *Eco*RI site which was blunt-ended by treatment with T4 DNA polymerse and the *Xho*I site of pGEX4T-1 (Amersham) to create the plasmid pGST-Dll$_{1–317}$. In this procedure, the original *Eco*RI site in pGEX4T-1 was regenerated in pGST-Dll$_{1–317}$.

The plasmid pGST-Dll$_{1–317}$ was cut with *Eco*RI and *Fsp*I, then the isolated DNA fragment was inserted into *Eco*RI and *Sma*I sites of the pGEX4T-1 to create the plasmid pGST-Dll$_{1–124}$. The plasmid pGST-Dll$_{1–317}$ was also cut with *Sma*I and *Xho*I, blunt-ended, and then self-ligated to create pGST-Dll$_{1–29}$, cut with *Sma*I and *Fsp*I and inserted into *Sma*I site of pGEX4T-1 to create the plasmid pGST-Dll$_{30–124}$, with *Xho*I and *Fsp*I, and inserted into *Sma*I and *Xho*I sites of pGEX5X-3 (Amersham) to create pGST-Dll$_{125–327}$, with *Sma*I and *Fsp*I, and inserted into *Sma*I site of pGEX5X-3 to create pGST-Dll$_{125–189}$ and with *Sma*I and then self-ligated to create pGST-Dll$_{\Delta 30–189}$.

All plasmids were propagated in *Escherichia coli* (*E. coli*) XL-1 Blue, isolated by standard procedures [16] and further purified with a QIAGEN Plasmid Midi Kit (QIAGEN). DNA sequencing was carried out with a BigDye-^{TM} Terminator v3.0 Cycle Sequencing Standard kit (Applied Biosystems) using an ABI PRISM^{TM} 310 NT Genetic Analyzer (Applied Biosystems). When necessary, chemically synthesized oligonucleotides (17 mer) were used as sequencing primers.

2.4. GST pull down assay

In vitro transcription and translation reactions were carried out with the TNT-coupled reticulocyte lysate system (Promega) in the presence of [^{35}S] methionine and the sizes and amounts of the *in vitro* translation products were analyzed by SDS-PAGE.

GST-dDREF or GST-Dll fusion proteins were produced in *E. coli* XL1-blue as described previously [17] and the amounts of each fusion protein were estimated by SDS-PAGE. Glutathione beads (15 μl) equilibrated in binding buffer (50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 10% glycerol, 150 mM NaCl, 0.1% NP-40, 5 mM β-mercaptoethanol, 0.5 mg/ml BSA, 1 mM phenylmethylsulfonyl fluoride) were incubated for 16 h at 4 °C with *E. coli* extracts containing approximately 1 μg of GST, GST-dDREF or GST-Dll recombinant protein. After washing two times with the same buffer, a 6 μl aliquot of *in vitro* translated Dll or dDREF polypeptide was added and the mixture was incubated at 25 °C for 1 h. After washing five times with 100 μl of the same buffer, bound proteins were eluted by boiling in a sample solution consisting of 0.0625 M Tris–HCl (pH 6.8), 2% SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.001% bromophenol blue, then separated by SDS-PAGE. After Coomassie Brilliant Blue-staining to verify that equal amounts of the fusion proteins were loaded, the gels were dried and ^{35}S-labeled proteins were detected by autoradiography.

2.5. Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were performed as described earlier [18] with minor modifications. A reaction mixture containing 20 mM HEPES pH 7.6, 100 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 10% glycerol, 1 μg of salmon sperm DNA was used with Kc cell nuclear extracts. ^{32}P-labeled probes (10,000 cpm) were incubated in 15 μl of reaction mixture. To test for effects of Dll protein on DNA binding activity of dDREF, Kc cell nuclear extracts were incubated with the purified GST or GST-Dll proteins for 15 min at 4 °C, before adding the probe. DNA–protein complexes were electrophoretically resolved on 4% polyacrylamide gels in 100 mM Tris/borate pH 8.3, 2 mM EDTA containing 2.5% glycerol at 25 °C. The gels were dried and then autoradiographed.

2.6. Scanning electron microscopy

Adult flies were anesthetized, mounted on stages and observed in a Hitachi S-100 scanning electron microscope in the low vacuum mode.

3. Results

3.1. Genetic interaction between dDREF and Distal-less

Transgenic fly lines expressing dDREF in eye imaginal discs exhibit a rough eye phenotype but normal viability and fertility (Fig. 1B) [11]. This dDREF expression system, therefore, can be used as a genetic tool to identify mutations that modify the rough eye phenotype. By modifier screening, we previously demonstrated that half-dose reduction of certain members of *trithorax* group genes, such as *brahma,*