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The homeodomain of PAX6 is essential for PAX6-dependent activation of the rat glucagon gene promoter: Evidence for a PH0-like binding that induces an active conformation

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ABSTRACT

The transcription factor PAX6 plays an important role in transcriptional regulation of the peptide hormone glucagon from pancreatic α-cells. PAX6 contains two DNA binding domains, the paired domain (PD) and the homeodomain (HD). While the interaction of the PD with the PAX6 responsive elements G1 and G3 in the rat glucagon gene promoter is well understood, the role of the PAX6 HD for PAX6 binding and function on G1 and G3 remains unclear. In EMSA studies the PAX6 HD was found to be mandatory for PAX6 binding to G1 but not to G3. Transient transfections with luciferase reporter gene constructs revealed the HD to be critical for proper function of PAX6 on both, G1 and G3. Transfection data with variant promoter constructs and limited proteolysis assays demonstrated that the DNA sequence located 5′ to the PD binding site plays an important role for PAX6 function and its conformation on the elements G1 and G3. Taken together, our data indicate a PH0-like binding of PAX6 to the glucagon promoter elements G1 and G3 where the HD binding site is abutted directly to the PD binding motif. The data suggest that the PH0-like binding induces a transcriptionally active conformation of PAX6.

1. Introduction

Type 2 diabetes mellitus is one of the most important diseases in the western world. In type 2 diabetic subjects, glucohomeostasis is disturbed by polygenetic reasons and is characterized by hyperglycemia and a proceeding resistance of tissues against the peptide hormone insulin. In normal individuals, blood glucose is regulated at a constant level by the peptide hormones insulin and glucagon. Both hormones are synthesized in the islets of Langerhans of the endocrine pancreas [1–3]. After its synthesis in pancreatic islet α-cells and its secretion into blood, glucagon acts on the liver to stimulate glycogenolysis and gluconeogenesis, increasing thereby hepatic glucose output. These actions of glucagon are opposite to those of insulin, the peptide hormone from pancreatic islet β-cells. A centerpiece in the coordination of the secretion of these two hormones is the direct inhibition of glucagon synthesis and secretion by insulin [1,2,4,5]. In insulin resistant or deficient states, glucagon synthesis and secretion becomes disinhibited leading to hyperglucagonemia contributing to fasting hyperglycemia in type 2 diabetes mellitus [1–3]. In addition to insulin, the β-cell secretory product zinc also inhibits glucagon exocytosis [6]. Therefore, the regulation of glucagon expression in pancreatic α-cells is an important aspect in the treatment of type 2 diabetes mellitus.

The transcription factor PAX6 belongs to the family of paired box proteins [4,5,7–9] and plays an important role in α-cell differentiation and α-cell-specific glucagon gene activation: in PAX6 knock out mice a total absence of α-cells was observed [9]. Internal mutations of the two PAX6 binding sequences in a luciferase reporter gene construct of the rat glucagon gene promoter resulted in a total loss of glucagon gene promoter activity [4,10–12], highlighting PAX6 as an essential transcription factor for glucagon synthesis. We recently showed that PAX6 is also a target for the antidiabetic action of thiazolidinediones [13], a new class of orally available drugs for treatment of diabetes mellitus [14]. Thiazolidinediones like rosiglitazone inhibit the transcriptional activity of PAX6 on glucagon gene transcription as ligands of PPARγ by a direct protein–protein interaction with PAX6 [13].

PAX6 is composed of an amino-terminal paired domain (PD) followed by a linker region (L), a homeodomain (HD) and a carboxyl-terminal transactivation domain (TAD). PAX6 binds to the pancreatic islet-cell specific enhancer sequences (PISCES) in the G1 and the G3 element of the rat glucagon gene promoter [4]. The TAD of PAX6 confers transcriptional activity by recruitment of coactivators like CBP

Abbreviations: PD, paired domain; HD, homeodomain; L, linker region; TAD, transactivation domain; PCR, polymerase chain reaction; PISCES, pancreatic islet cell-specific enhancer sequence; EMSA, electrophoretic mobility shift assay; PPARγ, peroxisome proliferator activated receptor γ; TK, thymidine kinase; DTT, dithiothreitol; RLU, relative luciferase units

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and p300 [15]. It has been shown that the PD of PAX6 binds the PISCES motif and that mutations in the PD binding sites of G1 and G3 result in a dramatically reduced transcriptional activity of the glucagon gene [16–19].

The role of the HD of PAX6 for the glucagon gene promoter activity is still unclear. The PAX6 HD belongs to the paired class of homeodomains that were shown to bind to a so called P3 site, consisting of two palindromic ATTA motifs separated by 3 nucleotides [20,21]. Homeodomains like the PAX6 HD were also shown to be involved in protein–protein interactions [22].

PAX proteins use different combinations of their DNA-binding domains to stimulate gene transcription on different promoters. The PAX6 homologous protein *eyeless* in *Drosophila melanogaster* induced also eye development when its homeodomain was absent [23]. In contrast, an isoform of PAX6 in *C. elegans* lacking the PD seems to be essential for the development of the peripheral nervous system [24].

In site selection assays, the PD and HD of PAX6 were shown to bind cooperatively to certain DNA motifs. In this class of PAX6 binding motifs the HD binding site was abutted with 0 bases spacing to the PD binding motif in reverse orientation and therefore named PH0-site [25]. Further evidence for a PH0-like binding of PAX6 was found in footprint assays of the proximal PAX6 binding site L1–170 in the L1 promoter [26].

In this study, we addressed the question how the PAX6 HD is involved in transcriptional activation of the glucagon gene promoter by PAX6. We particularly investigated the evidence for a PH0-like binding conformation of PAX6 on the elements G1 and G3. We show that the PAX6 HD plays an important role for PAX6 activity on glucagon gene transcription and that there is strong evidence for a PH0-like binding scenario.

2. Materials and methods

2.1. Plasmid constructs

The plasmids −350GluLuc [27], −169GluLuc [28], −350/−150GluLuc [28] have been described previously. The constructs −350/−150G3PH0mut1 and −350/−150G3PH0mut2, carrying a mutation in the G3 element of the glucagon gene promoter were prepared by PCR from −350/−150GluLuc. Two PCR fragments were generated with the primer pairs −350/−150_for/−350G3mut1_rev and −350G3mut1_for/−350/−150_rev (−350/−150G3PH0mut1) or −350/−150_for/−350G3mut2_rev and −350G3mut2_for/−350/−150_rev (−350/−150G3PH0mut2) (Table 1). The 3′-overhang of the first fragment and the 5′-overhang of the second fragment, which carried the desired mutation, were annealed in a primerless PCR [29]. The final fragments were obtained in a subsequent PCR with 2 µl of the primerless PCR reaction batch as template and the primer pair −350/−150_for/−350/−150_rev. The fragments were digested with BamHI and HindIII and cloned into the BamHI/HindIII sites of pT81Luc [30]. For −169G1PH0mut1 and −169G1PH0mut2 carrying a mutation in the G1 element of the glucagon gene promoter, PCR fragments were generated using the primer pairs −169_for/−169G1mut1_rev and −169G1mut1_for/−169_rev (−169G1PH0mut1) or −169_for/−169G1mut2_rev and −169G1mut2_for/−169_rev (−169G1PH0mut2). After a primerless PCR the final fragments were amplified in a PCR using the primer pair −169_for/−169_rev. The fragments were digested with BamHI and XhoI and cloned into the BamHI/XhoI sites of pXP2 [30]. The expression vector pBat14.mPax6 containing the murine Pax6 cDNA was kindly provided by Dr. M. German (University of California, San Francisco, CA). To construct the plasmid pBAT-dHD the PAX6 HD from amino acid 214 to 271 was replaced by a XhoI restriction site. Two fragments were obtained by using pBAT14.mPax6 as PCR template in combination with the following primer pairs: primer pair 1 Pax6fl-5/Pax6dHD-3 as upstream primer and downstream primer, respectively, and primer pair 2 Pax6dHD-5/Pax6fl-3. PCR products were gel-purified via an agarose gel, digested with XhoI and ligated with T4-Ligase (Fermantas, Munich, Germany). After another round of gel purification, the ligation product was digested with BamHI and HindIII and cloned into the BglII/HindIII sites of pBAT14. The same procedure was used to construct the plasmid pBAT-dH3 where the third helix of the PAX6 HD from amino acid 251 to 271 was replaced by a XhoI restriction site. The primer pairs used were: primer pair 1 Pax6fl-5/Pax6dH3-3 and primer pair 2 Pax6dHD-5/Pax6fl-3. For pBAT-dPD lacking the PD (amino acids 23 to 136), the primer pairs Pax6fl-5/Pax6dPD-3 and Pax6dPD-5/Pax6fl-3 were used. The plasmid pBAT-3Ala was constructed by PCR-mediated in vitro mutagenesis according to the manufacturer's instructions for the Quick-Change Site-Directed Mutagenesis kit (Stratagene, Heidelberg, Germany) using pBAT14.mPax6 as template and the specific primers HD-mut-CS and HD-mut-NCS. A schematic representation of the constructs is shown in Fig. 1A. For in vitro transcription/translation the plasmids pcDNA3-Pax6-WT, pcDNA3-dHD, pcDNA3-3Ala and pcDNA3-dH3 were constructed by PCR using the primer pair 5′-pGEX and 3′-pGEX as upstream and downstream primers, respectively, and the corresponding pBAT-constructs (see above) as templates. The PCR products were digested with BamHI and cloned into the BamHI site of pcDNA3 (Promega, Mannheim, Germany). The plasmid pcDNA3-PD encoding the PAX6 PD (amino acids 1–136) was prepared by PCR using the primer pair Pax6PDfor/Pax6PDrev2 (Table 1) and the plasmid pcDNA3-Pax6-WT as template. The PCR product was digested with HindIII and EcoRV and cloned into the HindIII/EcoRV sites of pcDNA3. The following plasmids were prepared in analogy: pcDNA3-HD encoding the PAX6 HD (amino acids 210–268) was generated by using the primer pair

Table 1
Sequences of primers used in this study

Primer	Sequence
−350/−150_for	5′-CGTAGGATCCATGGCCAAATAGCACATCAA-3′
−350/−150_rev	5′-CGTAAAGCTTACCGGGGTGAGACCTCAG-3′
−350G3mut1_rev	5′-TGAAACCCTACTTCAGCTCT-3′
−350G3mut1_for	5′-TAGGGTTTCACGCCTGACTG-3′
−350G3mut2_rev	5′-TGAACCACTACTTCAGCTCT-3′
−350G3mut2_for	5′-TAGTGGTTCACGCCTGACTG-3′
−169_for	5′-CGTAGGATCCCTCTGAGGTCTCACCCCGG-3′
−169_rev	5′-CGTACTCGAGCAGGTGGAGCTCCTTTGG-3′
−169G1mut1_rev	5′-ATACCTTTCTCATCTGTAAA-3′
−169G1mut1_for	5′-AGAAAGGTATATTGTCAGCG-3′
−169G1mut2_rev	5′-ATAACCTTCTCATCTGTAAA-3′
−169G1mut2_for	5′-AGAAGGTTATATTGTCAGCG-3′
Pax6fl-5	5′-GAGTCGGATCCGGAGGCTGCCAACCAGCT-3′
Pax6fl-3	5′-CGCCGCAAGCTTTACTGTAATCGAGGCCA-3′
Pax6dHD-3	5′-CGCCGCTCGAGATTTCTTTGCAGCTTCCG-3′
Pax6dHD-5	5′-CGCGCCTCGAGAGGAACCAGAGAAGACAG-3′
Pax6dH3-3	5′-GCGGCCTCGAGAGGTAGATCTATTTTGGC-3′
Pax6dPD-3	5′-GATTCCTCGAGCGGCAGTGGCCGCCCGTT-3′
Pax6dPD-5	5′-GATTCCTCGAGATGGGCGCAGACGGCATG-3′
HD-mut-CS	5′-CCTGAAGCAAGAATACAGGCATGGTTTGCTGCTCGAAGGCAAATGG-3′
HD-mut-NCS	5′-CCATTTGGCCCTTCGAGCAGCAAACCATGCCTGTATTCTTGCTTCAG-3′
5′-pGEX	5′-GAGTCGGATCCATGCAGAACAGTCAC-3′
3′-pGEX	5′-GAGCGGGATCCTTACTGTAATCGAGG-3′
Pax6PDfor	5′-ATAAAGCTTATGCAGAACAGTCACAGCGGAG-3′
Pax6PDrev2	5′-ATAGATATCTTACTGTTGCTTTTCGCTAGCCAGG-3′
Pax6HDfor2	5′-CTGAAGCTTATGCTGCAAAGAAATAGAACATC-3′
Pax6HDrev	5′-ATAGATATCTTATCTTCTCCATTTGGCCCTTCG-3′
Pax6Linker_rev	5′-ATAGATATCTTACTTCCGCTTCAGCTGAAG-3′
PDBac_for	5′-ATAGGATCCATGCAGAACAGTCACAGCGGAG-3′
PDBac_rev	5′-ATACTCGAGTTACTGTTGCTTTTCGCTAGCCAGG-3′
Pax6wtBac_rev	5′-ATACTCGAGTTACTGTAATCGAGGCCAGTACTG-3′
X1-for	5′-ATCGAGGGCCGGAGTACCAGTGTCTAC-3′
X1-rev	5′-ACTCCGGCCCTCGATGATAGGAATGTGACTA-3′
X5-for	5′-ATCGAGGGCCGGATGAACAGTCAGCCCATG-3′
X5-rev	5′-CATCCGGCCCTCGATGTGCGGAGGGGTG-3′
X6-for	5′-ATCGAGGGCCGGTCGGGGACCACTTCAAC-3′
X6-rev	5′-CGACCGGCCCTCGATCTGACTGTTCATGTG-3′

Recognition sites of enzymes are underlined and mutations of the wild type sequences are highlighted in grey.