Pax6 and Pdx1 form a functional complex on the rat somatostatin gene upstream enhancer

Frank Gellert Andersen\(^a\), Jan Jensen\(^a\), R. Scott Heller\(^a\), Helle Vestergård Petersen\(^a\), Lars-Inge Larsson\(^b\), Ole Dragsbæk Madsen\(^a\), Palle Serup\(^a\),* 

\(^a\)Department of Developmental Biology, Hagedorn Research Institute, Niels Stensenvej 6, DK-2820 Gentofte, Denmark
\(^b\)Department of Molecular Cell Biology, State Serum Institute, Copenhagen, Denmark

Received 29 December 1998

Abstract The somatostatin upstream enhancer (SMS-UE) is a highly complex enhancer element. The distal A-element contains overlapping Pdx1 and Pbx binding sites. However, a point mutation in the A-element that abolishes both Pdx1 and Pbx binding does not impair promoter activity. In contrast, a point mutation that selectively eliminates Pdx1 binding to a proximal B-element reduces the promoter activity. The B-element completely overlaps with a Pax6 binding site, the C-element. A point mutation in the C-element demonstrates that Pax6 binding is essential for promoter activity. Interestingly, a block mutation in the A-element reduces both Pdx1 binding and promoter activity. In heterologous cells, Pdx1 potentiated Pax6 mediated activation of a somatostatin reporter. We conclude that the β3-cell-specific activity of the SMS-UE is achieved through simultaneous binding of Pdx1 and Pax6 to the B- and C-elements, respectively. Furthermore, the A-element appears to stabilise Pax6 binding.

© 1999 Federation of European Biochemical Societies.

Key words: Pax6; Pdx1; Somatostatin; Pancreatic islet; Transcription

1. Introduction

Cell-specific expression of the glucagon, insulin, somatostatin, and pancreatic polypeptide genes by the four classical cell types of the islets of Langerhans is regulated at the transcriptional level [1–5]. The promoters of the glucagon, insulin, and somatostatin genes are composed of a combination of positively and negatively acting cis-elements [6–12]. Isolated positively acting cis-elements confer tissue- (islet-specific cell type- (α-, β-, δ-) specific expression [13–17].

Thus, activity of the somatostatin promoter in islet β- and δ-cell lines relies on a composite enhancer element termed the somatostatin-upstream enhancer (SMS-UE) or TSE-1 (tissue-specific element I) spanning nucleotides −115 to −75 (see Fig. 1) [11,18–20]. Additional positively acting A/T-rich cis-elements (TAAT1/TSE-II and TAAT2) further upstream are also contributing to promoter activity [18,21,22]. The activity of these elements is dependent on the integrity of a cyclic AMP response element (CRE) located at position −48 to −41 [11,18]. Additionally, negatively acting factors suppress activity in non-islet cells [12]. The SMS-UE is a tripartite element consisting of the subdomains SMS-UE-A, -B, and -C [11,19]. A so called PISCES motif pancreatic islet cell enhancer sequence) is found in the C-element of the SMS-UE [13]. Interestingly, PISCES motifs are also found in promoter elements within the glucagon and insulin genes [13]. These elements, however, display different cell-type-specific activities [20]. Thus, while the glucagon gene G1-element shows preferential activity in α-cells, the glucagon gene G3-element has a broad islet cell-specific activity. In contrast, the insulin gene C2-element and the SMS-UE show β3-cell-specific activity [20]. To begin to unravel the mechanism by which the SMS-UE confers cell-type specificity, we analysed the factors binding to this element in a somatostatin-producing cell line. We found by antibody supershift experiments that the major complexes formed on the A-element were produced by Pdx1 and Pbx proteins. On the B- and C-elements we observed a complex composed of Pdx1 and Pax6 binding simultaneously. This complex was also formed by recombinant Pdx1 and Pax6. By mutational analysis of the SMS-UE, we found a correlation between formation of the Pdx1-Pax6 complex and promoter activity. Interestingly, small mutations that abolished Pdx1 and Pbx binding to the A-element did not reduce promoter activity. However, a block mutation that also reduced Pax6 binding to the SMS-UE did inhibit promoter activity. Furthermore, Pdx1 enhanced Pax6 mediated activity of a somatostatin reporter in heterologous cells. Together, these data suggest that the Pdx1-Pax6 complex is functional and required for cell-specific activity of the SMS-UE.

2. Materials and methods

2.1. Plasmids

pCMX-STF1 [23] containing the rat Pdx1 cDNA under control of the CMV promoter was a gift from J. Leonard, pSG5-Pax6 containing the quail Pax6 cDNA under control of the SV 40 promoter was a gift from S. Saule. The somatostatin reporters were constructed by a multistep procedure. Initially a BgII restriction site was inserted between the Sa/I and PstI sites in pCAT-Basic (Promega) creating pCAT-Basic-B2. A 124 bp BgII-Xhol fragment containing the somatostatin gene sequences from −71 to +53 was isolated from pl1.4-CAT [18] and inserted into BgII and Xhol digested pCAT-Basic-B2, creating p71-CAT-Basic-B2. Double stranded oligonucleotides representing wild-type or mutant somatostatin gene sequences from −72 to −122 (see Fig. 1) were inserted into BgII digested p71-CAT-Basic-B2. The 4X(GAL4-SMS-UE)-CAT reporter was designed using mutimerised olignonucleotides (see Fig. 6A) inserted in the correct orientation into the BgII site of the somatostatin minimal reporter construct, p32.5-CAT [18]. GAL4-Pdx1, GAL4-Cdx2 fusion constructs were generated by PCR and inserted in frame into pG424 [24] using the BamHI and Xhol cloning sites. The Isl-1 cDNA [25] was inserted into the EcoRI site of pSG424. GAL4-VP16 has been described previously [26]. All constructs were verified by dyeoxy cycle sequencing.

2.2. Transfections

Transient transfection assays were done using Lipofectamine (Gibco-BRL) according to the manufacturer’s instructions. Briefly, ap-
The recombinant proteins were produced by transiently transfecting NIH 3T3 cells with 3–5 in sodium orthovanadate) using 175 μl per well. After shaking for 10 min at room temperature, 33 μl 2.1 M KCl, 46% glycerol buffer was added and shaking was allowed to proceed for 10 min at room temperature. The contents of the wells were adjusted to 15% glycerol using a 50% stock, collected and centrifuged at 20000 × g for 10 min at 4°C. The supernatant was collected and stored at −80°C. Protein content was measured using the Bradford assay (Bio-Rad).

2.4. Antiserum and electrophoretic mobility shift assays

The anti-Pxd1 antiserum was a gift from S. Saule and has been described previously [32]. The bg11 anti-Pax6 antiserum was used in standard EMSA. The reaction buffer contained 12.5% glycerol, 30 mM Tris-HCl, 75–100 mM KCl, 20 mM NaCl, 1 mM MgCl2, 20–50 μg/ml poly-dIdC and 20–50 μg/ml poly-dGdC. 2–4 μg/ml hering sperm DNA, 0.1% Nonidet NP-40, 3 mM DTT. For recombinant proteins up to 10 μg of whole cell extract was used, and all reactions were adjusted to the same protein concentration using extracts from untransformed cells. Antisera were used in a 1/10 dilution in the presence of 15 mM NaN3 using 2–3 μl per binding reaction as described previously [28]. Binding reactions were separated on a 5% polyacrylamide gel as previously described [28]. The gels were dried and analyzed by autoradiography. Gel images were scanned using Adobe Photoshop 3.0 and figures prepared in Canvas 5.0.2.

3. Results

3.1. Pdx1 and Pbx proteins can bind the isolated SMS-UE-A element

The SMS-UE of the somatostatin gene contains a Pax6 binding site [20,34] and a Pdx1 binding site [21,23] that have been shown to confer preferential transcriptional activity in β- and δ-cell lines [20]. As the binding site of the Pax6 paired domain and Pdx1 binding site only comprise the SMS-UE-B and -C elements it seemed possible that an SMS-UE-A element binding factor contributed to promoter activity along with Pax6 and Pdx1. We therefore initially identified the factors capable of binding to the A-element. The sequence of the A-element (TGTATTAGTTT) resembles the TAAT2 element (TGTATTAGTTA, differences underlined) which is known to bind Pdx1, either as a monomer or as a complex with one of the Pbx proteins [22]. By performing EMSA using the A-element probe (see Fig. 1) with nuclear extracts isolated from βT3-csom, an insulin- and somatostatin-producing subculture of the islet cell line βT3C, we found that one major complex was formed with βT3C-som extract that was absent from extracts prepared from the glucagon-producing cell line αTC1.9. In addition, two minor complexes were present in both αTC1.9 and βT3C-som extracts (Fig. 2). Addition of antisera specific for either Pdx1 or Pbx showed that the β-cell-specific complex was due to Pdx1 binding while the two minor complexes were Pbx immunoreactive (Fig. 2). In contrast to the TAAT2 element, Pbx/Pdx1 dimer formation was not observed on the A-element and this was found to be due mainly to the nucleotide differences at position seven and to a minor degree also at position 11 between the two sites (F.G. Andersen, manuscript in preparation). A point mutation at position three of the Pbx site (M-A1) as well as a block mutation at positions 4–7 (M-A3) abolished both Pdx1 and Pbx binding to the A-element (Fig. 2, lanes 10 and 11).

3.2. Pax6 and Pdx1 forms a ternary complex with the SMS-UE

To test whether a Pax6 and Pdx1 containing complex could form on the SMS-UE, we performed EMSA using the ABC-
probe spanning the entire SMS-UE (Fig. 1) in the presence of βTC3-som nuclear extract. Two major complexes S1 and S2 were observed, however, on shorter exposures (not shown) the S1 complex was resolved into two complexes of slightly different mobilities, S1a and S1b (Fig. 3, lane 1). Inclusion of Pax6 or Pdx1 antisera in the binding reaction identified the S1a complex as Pdx1 and the S1b complex as Pax6 (Fig. 3, lanes 3–5). The S2 complex contained both Pdx1 and Pax6 (Fig. 3, lanes 3–5). The Pax6 antiserum failed to completely shift the S2 complex (Fig. 3, lane 4). This is due to the presence of a Pdx1 dimer generated by co-occupancy of the A- and B-elements (see Fig. 2, lane 2 and Fig. 4, lanes 12–14). However, full length recombinant Pdx1 often failed to generate this complex, most likely due to insufficient amounts of Pdx1 produced in the transfected fibroblasts. The identification of complex S2 as a Pax6-Pdx1-DNA ternary complex was corroborated by the finding that recombinant Pdx1 and Pax6 formed a complex with a mobility identical to the S2 complex (Fig. 3, lane 9). In agreement with a previous study [28] we observed that rat Pdx1 migrates slightly faster than mouse Pdx1 (Fig. 3, compare lanes 1 and 7).

3.3. Sequence requirements for Pax6 and Pdx1 binding to the SMS-UE: intact A-, B-, and C-elements are required for optimal Pax6 binding

In order to examine the functional relevance of Pax6 and Pdx1 binding to the SMS-UE, we introduced a set of mutations in either the A-, B-, or C-elements (see Fig. 1) and tested the effect of these on binding of recombinant proteins as well as promoter activity. Plasmids encoding wild type Pax6 and a truncated version of rat Pdx1 (Pdx1(ΔN)) deleting most of the activation domain of Pdx1 [35] were used to generate recombinant proteins in NIH-3T3 cells. Pdx1(AN) was used to ensure different migration of Pax6 and Pdx1 monomers. The M-A1 mutation which abolished Pdx1 binding to the A-element (see Fig. 2) had no effect on Pax6 binding or Pax6-Pdx1-DNA ternary complex formation (Fig. 4, lanes 7 and 8). In contrast, the M-A3 mutation, which also abolished Pdx1 binding to the A-element (see Fig. 2), reduced Pax6 binding as well as Pax6-DNA complex formation (Fig. 4, lanes 13–16). This was also clearly seen using βTC3-som nuclear extract (data not shown). The M-A4 mutation led to a similar reduction in complex formation as the M-A3 mutation (Fig. 4, lanes 17–20). Conversely, the M-A2 mutation resulted in increased Pax6 and Pdx1 monomer, and Pax6-Pdx1-DNA complex formation (Fig. 4, lanes 9–12). The importance of a functional Pdx1 binding B-element for ternary complex formation was demonstrated using the M-B1 mutation (Fig. 1). Specific targeting of the Pdx1 consensus site, leaving the Pax6 consensus unaffected, resulted in reduced Pdx1 binding and abolishment of the Pax6-Pdx1-DNA complex, without influencing Pax6 monomer formation (Fig. 4, lanes 21–24). The M-A1B1 mutation targeting both the A- and B-element Pdx1 sites resulted in severe reduction of Pdx1 complex formation, but left Pax6 monomer binding unchanged (Fig. 4, lanes 25–28). The M-B2 mutation abolished the Pax6 complex as well as Pdx1 binding to the B-element and consequently Pax6-Pdx1-DNA ternary complex formation (Fig. 4, lanes 29–32). Similarly, when using βTC3-som nuclear extract no Pax6 immunoreactive complex were observed (data not shown). Finally, the M-C mutation abolished formation of the Pax6 complexes without affecting Pdx1 binding (Fig. 4, lanes 33–36). The same effect was seen using βTC3-som nuclear extract (data not shown).

3.4. Mutations that disrupt the Pax6-Pdx1 complex formation impair promoter activity

To analyse the functional importance of ternary complex formation we introduced the mutations used in Fig. 2C into a somatostatin reportor construct (−122/+53) linked to CAT. We then assayed promoter activity by transiently transfecting βTC3-som cells with wild type and mutant constructs. The M-A3, M-B2, and M-C mutations that all affected Pax6 binding and reduced or abolished ternary complex formation impaired SMS-UE mediated activity to a level seen with a −71 reporter construct (Fig. 5A). The M-B1 mutation which compromises
Pdx1 binding to the B-element and the M-A1B1 mutation which affects Pdx1 binding to the A- as well as the B-element, both without affecting Pax6 binding, resulted in an approximately 50% reduction in promoter activity (Fig. 5A). The M-A4 mutation also showed a 50% reduction in promoter activity, a slightly higher activity than observed for the M-A3 mutation although these two mutations appeared to affect Pax6 binding similarly. The M-A1 mutation did not affect promoter activity, suggesting that binding of Pdx1 and Pbx proteins to the A-element does not contribute to promoter activity in islet cells. A single mutation, M-A2, resulted in increased promoter activity (Fig. 5A) which correlated with increased formation of the Pax6-Pdx1-DNA ternary complex on this mutant, in addition to increased binding of Pax6 and Pdx1 monomers.

3.5. Pdx1 potentiates Pax6 mediated activation of a somatostatin reporter

To further test whether Pax6 and Pdx1 were capable of interacting functionally on the somatostatin promoter we transfected NIH-3T3 cells with a somatostatin reporter construct in the presence or absence of Pax6 and Pdx1 expression
vectors. Transient transfection analysis revealed that Pdx1 could enhance Pax6 mediated transcriptional activation of the 3122SOM-CAT reporter construct (Fig. 5B). Mutation of the Pax6 binding site (3122SOM(M-C)-CAT) reduced Pax6 mediated activity by 50% and abolished the potentiation by Pdx1 (Fig. 5B). A 371 somatostatin reporter was only activated 1.4-fold by Pax6 and Pdx1 did not potentiate this activity (not shown).

3.6. The activation domains of Pdx1 and Cdx2/3 can co-operate with Pax6 when fused to the GAL4 DNA binding domain

The activation domain of Pdx1 has been mapped to mainly three highly conserved sequences within the N-terminal 75 amino acids [35,36]. These sequences are also required by Pdx1 to synergistically activate insulin-enhancer mediated transcription together with products of the E2A gene. To determine whether the activation domain of Pdx1 was required for transcriptional co-operativity with Pax6, we used a fusion protein between the GAL4 DNA binding domain (GAL4-DBD) and the activation domain of Pdx1. As a reporter, we constructed a synthetic promoter containing four copies of the SMS-UE with the B-element replaced by a GAL4 binding site. The multimerised oligonucleotide was inserted in front of the somatostatin minimal promoter in p2.5-CAT (Fig. 6A). This reporter could be activated by Pax6 (Fig. 5B) while the parent vector could not (data not shown). Activation by GAL4-Pdx1 and transcriptional synergy between GAL4-Pdx1 and Pax6 was readily observed on this reporter (Fig. 6B). This activity of the Pdx1 activation domain was conserved between Xenopus laevis and rat Pdx1 (Fig. 6B). The cooperativity observed between Cdx2/3 and Pax6 on the glucagon promoter (see accompanying manuscript) prompted us to examine whether the activation domain of Cdx2/3 could also synergise with Pax6 on this synthetic reporter. Use of GAL4-Cdx2/3 fusion constructs revealed that the N-terminal activation domain of Cdx2/3 [37] was indeed capable of cooperating with Pax6 on the synthetic promoter when fused to the GAL4-DBD (Fig. 6B). A GAL4-VP16 fusion construct was highly active on this reporter but only capable of modest cooperativity with Pax6 (Fig. 6B).

4. Discussion

Pdx1 has previously been reported to stimulate somatostatin gene transcription [21-23,38]. Three Pdx1 binding sites are located within approximately 400 bp of the somatostatin 5'-flanking region [21,23]. Nevertheless, the first 120 bp of the promoter is sufficient to confer islet cell-specific expression in transfection assays [11]. In such a construct mutational inactivation of the B-element Pdx1 binding site reduces promoter activity substantially [11]. Similarly, in a construct including 1400 bp of 5'-flanking sequence, inactivation of the Pdx1 site in the SMS-UE B-element reduces promoter activity by 70% [18]. However, as several conserved bases in the Pax6-PD consensus site are also affected by this mutation it cannot be excluded that the Pax6 binding is also affected. We now show that the mutational sensitivity of the C-element is due to the disruption of the Pax6 binding. Moreover, we show that this Pax6 binding site is overlapping with a Pdx1 binding site and that these two sites can be co-occupied. Furthermore, formation of the Pax6-Pdx1-DNA ternary complex is reduced when nucleotides outside the Pdx6-PD consensus site are mutated (i.e. the M-A3 and M-A4 mutation in the A-element) or deleted (not shown). The reduced ternary complex formation correlates with reduced promoter activity. In contrast, a point
mutation (M-A1) which reduces Pdx1 and Pbx binding to the A-element (similar to the M-A3 mutation) has no effect on promoter activity, suggesting that binding of Pdx1 or Pbx to the A-element is not required for promoter activity. One possible explanation for the sensitivity of the A-element to the M-A3 and M-A4 mutations but not the point mutation (M-A1) could be that Pax6 contacts this element perhaps through its homeodomain. This interaction could be selectively affected by the M-A3 and M-A4 mutations. The increased binding of Pax6 to the M-A2 mutation and correlating increase in promoter activity supports that A-element sequences (which are located outside the paired domain recognition sequence) are involved in determining the affinity of Pax6 towards the SMS-UE. The mode of Pax6 binding to the SMS-UE is currently under investigation. Our data do not support a model involving binding of Pbx proteins to the A-element. It is, however, a possibility that binding of Pbx proteins to the A-element might modulate the activity of the SMS-UE in other cell types. We have shown that Pax6 mediated activation of a somatostatin reporter in heterologous cells can be potentiated by Pdx1. Together, our data suggest that the SMS-UE comprises a composite Pdx1/Pax6 binding site and that this contributes to the cell specificity of the somatostatin promoter. In contrast to the insulin and glucagon 5'-flanking regions which in transgenic mice direct expression to the appropriate cell type [2,3], the somatostatin 5'-flanking region (−4000/+53) drives expression of the non-transforming K1 mutant SV40 T-antigen to β-cells rather than δ-cells (M. Low, R. Ventimiglia, and R.H. Goodman, personal communication). As δ-cells are thought to arise from a Pdx1 positive, insulin-producing precursor [39,40], we propose that islet-specific activity of the somatostatin 5'-flanking region gene relies on co-operation between Pdx1 and Pax6. The expression of Pdx1 and Pax6 in newly formed δ-cells (data not shown, [41]) is consistent with such a model. However, additional mechanisms, possibly mediated by cis-elements upstream of −4000 bp or in the 3'-region, must be required to limit activity to δ-cells.

References