Contribution of a PS1-like element to the tissue- and cell-specific expression of the human GLP-1 receptor gene

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Abstract The GLP-1 receptor (GLP-1R) mediates the insulinotropic effects of the incretion hormone glucagon-like peptide 1 (7–36) amide (GLP-1). Recently, we cloned the 5′-flanking region of the human GLP-1R gene. To characterize tissue- and cell-specific *cis*-regulatory elements, we constructed a series of 5′-deletions of the promoter. The activity of these constructs was tested in different cell lines. An element with high homology to PS1 was found to repress GLP-1R promoter activity in fibroblasts and pancreatic D-cells, but was not active in pancreatic A- and B-cells. PS1 was described to inhibit activation of a D-cell-specific enhancer. Cloning the PS1-like element upstream a heterologous promoter (SV40) revealed that it is functionally active independently from this enhancer. Our data suggest that basal activity of the GLP-1R promoter is silenced in a tissue- and cell-specific manner by negatively acting *cis*-regulatory elements, including a PS1-like element.

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Key words: GLP-1 receptor promoter; *cis*-regulatory element; PS1; Silencer

1. Introduction

Glucagon-like peptide 1 (7–36) amide (GLP-1) arises as a post-translational product of proglucagon processing in intestinal cells and plays an important physiological role in the regulation of insulin secretion and proinsulin gene expression (for review see [1,2]). Its action is mediated by a stimulatory G-protein coupled receptor (GLP-1R) at the pancreatic β-cell, connected to the adenylate cyclase pathway in a glucose-dependent manner [3–6]. The GLP-1R is expressed in a cell- and tissue-specific manner in rat islets [7], rat lung [8], mouse small and large intestine, liver and kidney [9], human insulinoma [10], human islets [11,12], a human gastric tumor cell line [13] and human brain and heart [14]. It belongs to the family of seven transmembrane-spanning receptors that include secretin [15], parathyroid hormone [16], calcitonin [17] and glucagon receptors [18,19].

It is of special interest to study the cell- and tissue-specific expression of the GLP-1R since it is the target for a new therapeutic approach in diabetes mellitus type 2 [20–22]. Moreover, it could be shown that GLP-1R levels significantly influence glucose-mediated insulin release [23] and, therefore, knowledge would be valuable about the regulation of the GLP-1R gene. For this purpose we cloned the 5′-flanking sequences of the human GLP-1R gene ([24], accession No. U66062). Sequence analysis of this region revealed no TATA- or CAAT-box motif, but several other putative *cis*-regulatory elements, including three Sp1 binding sites, two of them activating gene transcription [25]. Sp1 is a well-known ubiquitous transcription factor [26,27] and seems to facilitate constitutive basal expression. Therefore, activation of gene transcription by Sp1 cannot be mainly responsible for cell- and tissue-specificity. There is evidence for the existence of additional regulatory elements in the GLP-1R promoter involved in negative control of transcription [24]. A candidate for one of these silencers in the GLP-1R promoter is an element in the region from −961 to −978, which is homologous to a recently characterized *cis*-regulatory element in the somatostatin gene (PS1) [28]. PS1 is located between −220 and −237 and decreases somatostatin gene promoter activity stimulated by a pancreatic D-cell-specific enhancer (SMS-UE) in an islet D-cell line and in fibroblasts, but not in an islet B-cell line [28]. The silencing activity of PS1 is not dependent on the position relative to SMS-UE. It was proposed that PS1 is involved in preventing somatostatin gene expression in non-islet cells. No data are available, demonstrating that PS1 exerts repressing functions independently from SMS-UE. Sequence analysis of the GLP-1R 5′-flanking region revealed no comparable enhancer. Therefore, it was the aim of this study to elucidate the role of the PS1-like element independently from SMS-UE and in the regulation of GLP-1R expression.

2. Materials and methods

2.1. Plasmid construction

A DNA fragment containing the GLP-1 receptor promoter ranging from −1632 to +37 cloned into the *Nhe*I and *Bgl*II sites of the pGL basic luciferase vector (Promega, Madison, USA) was used as template (T). 5′-Deletions were constructed using the erase-a-base system (Promega, Madison, USA) according to the suppliers protocol. Clones were analyzed by dideoxy chain termination sequencing (Sequenase, USB, Amersham, Cleveland, USA). Promoter fragments ranging from −1010 to +37 (containing the PS1-like element) and from −916 to +37 (missing the PS1-like element) were used for transient transfections.

Effects of PS1 and mutated PS1 (PS1M) in a heterologous promoter were studied by annealing complementary oligonucleotides (MWG Biotech, Ebersberg, Germany) with appropriate ends (coding strand: CTA GCT GGC ATC **AA**G G**TT** CTT TGG TGG GGA for PS1; CTA GCT GGC ATC **GC**G G**GC** CTT TGG TGG GGA for PS1M. Essential nucleotides for activity and mutated nucleotides are printed in bold). The resulting double strands were ligated into pGL promoter luciferase vector, digested with *Nhe*I and *Bgl*II. Insertion of oligonucleotides was verified by dideoxy chain termination sequencing.

2.2. Transient transfections

The different promoter reporter gene constructs were transiently cotransfected with pSV-β-Gal vector (Promega, Madison, USA) into four cell lines (hamster insulinoma tumor cells, pancreatic B-cell line HIT; hamster glucagonoma cells, pancreatic A-cell line INR1G9; rat insulinoma tumor cells, pancreatic D-cell line RIN-1027-B2 and Chinese hamster lung fibroblasts, CHL) using DEAE-dextran or the Per-

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Fect Lipid method (Pfx-3) according to the suppliers protocol (Invitrogen, San Diego, USA). After transfection cells were plated into 35 mm dishes and incubated for 24 to 48 h in appropriate media (HIT: RPMI 1640/L-glutamine, 10% FCS, 5% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin; INR1G9 and RIN-1027-B2: RPMI 1640/L-glutamine, 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin; CHL: RPMI 1640/L-glutamine, 10% FCS, 1 mM sodium pyruvate, 1×non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin). All media and supplements were from GIBCO, BRL, Eggenstein, Germany). Cells were lyzed and β-galactosidase and luciferase activity was determined by chemoluminescense in a luminometer (Lumat LB 9501, Berthold, Bad Wildbad, Germany) using the appropriate substrates (Tropix, Bedford, USA). Cell lysates for β-galactosidase measurements were first incubated for 50 min at 48°C to inactivate endogenous β-galactosidase. All measurements were taken in duplicate and from at least five independent transfections. Luciferase activity levels, normalized with β-galactosidase activities are expressed as percent of control.

2.3. Nuclear extracts and DNase I protection experiments

Nuclear extracts from CHL, HIT, RIN-1027-B2 and INR1G9 cells were prepared according to Dignam et al. [29]. A 196 bp DNA fragment ranging from −1039 (*Sal*I) to −843 (*Pst*I) was isolated from plasmid T, radioactively endlabeled using T4 polynucleotide kinase and digested with *Van*91I (−877) to create a 162 bp fragment with a single radioactive label. For DNase protection the probe was mixed with 40 and 80 μg of nuclear extracts or BSA and treated according to the suppliers protocol (Core Footprinting System, Promega). G and G+A sequencing reactions were performed as described by Maxam and Gilbert [30]. The DNA fragments were resolved on a 6% denaturing polyacrylamide gel.

2.4. Electrophoretic mobility shift assay (EMSA)

A double stranded oligonucleotide PS1 (which was also used for cloning) was radioactively endlabeled using T4 polynucleotide kinase. Binding reactions with nuclear extracts from HIT, CHL, RIN-1027-B2 and INR1G9 cells were performed according to the suppliers protocol at room temperature (Gel Shift Assay System, Promega) for 30 min in the presence of 2 μg poly(dIdC). Competitors were present in 50- or 500-fold excess. Complexes were separated on a non-denaturing 4% polyacrylamide gel. For in-situ DNA-protein UV-crosslinking gels were exposed to UV light (312 nm) for 5 to 10 min. After exposure to X-ray films, gel slices were cut out and transferred to denaturing SDS polyacrylamide gels [31]. After electrophoresis the gels were dried and exposed. Molecular weights of the proteins are determined by comparison to pre-stained protein standards (BioRad, Hercules, USA).

3. Results

3.1. Analysis of the tissue- and cell-specific activity of the human GLP-1R promoter by 5′-deletions

5′-Deletions of the human GLP-1R promoter and subsequent transient transfections of the constructs into HIT (pancreatic B-cell line, GLP-1R producing), InR1G9 and CHL cells (pancreatic A-cell line and fibroblasts, both GLP-1R non-producing cells) revealed that, by deletion to −1630, cell-specificity for pancreatic A- and B-cells is lost and that in the region from −1630 to −565 a tissue-specific silencer element is located [24]. Computer analysis of the sequence revealed an element from −978 to −961 with a high homology to PS1 (Fig. 1A). The essential nucleotides are completely conserved. To clarify the role of this PS1-like element, we constructed a series of 5′-deletions in the region from −1630 to −916 of the promoter upstream of a luciferase reporter gene. Activity of each construct was measured by transient transfections into different GLP-1R producing and non-producing cell lines. For comparison with published data, a pancreatic D-cell line (RIN-1027-B2) was included in the transfection experiments. 5′-Deletion from −1010 (contains the PS1-like element) to −916 (lacking the PS1-like element) (Fig. 1B) showed an increase in promoter activity in D-cells and fibroblasts, but no effects in B-cells (Fig. 2). These results are in good accordance to the published data for PS1 [28]. In the pancreatic A-cell line InR1G9 no effect can be observed by deletion of 94 bp containing the PS1-like element. These data suggest that the PS1-like element is involved in the tissue- and cell-specific expression of the human GLP-1R.

3.2. Activity of the PS1-like element independently from SMS-UE

In the somatostatin gene promoter PS1 represses the activity of a D-cell-specific enhancer (SMS-UE). The sequence of the GLP-1R promoter revealed no comparable enhancer, but the functionality of the PS1-like element seems likely. To evaluate, if it can function independently from SMS-UE, we cloned a 30 bp oligonucleotide containing the PS1-like element upstream a heterologous promoter (pGL promoter, luciferase gene under the control of an SV40 promoter, PS1). As a negative control another plasmid was constructed, containing a 30 bp oligonucleotide with mutated essential nucleotides of the PS1-like element (PS1M). Each construct was tested for promoter activity by transient transfections into different cell lines in comparison to the SV40 promoter (Prom) (Fig. 3). The PS1-like element repressed the activity of the SV40 promoter by about 40% in fibroblasts (CHL) and D-cells (RIN-1027-B2), but had no effects in A- (InR1G9) and B-cells (HIT). These data are in accordance with results obtained with 5′-deletions of the GLP-1R promoter. Cloning of the mutated PS1-like sequence did not alter the activity of the SV40 promoter in all four cell lines. Therefore, the PS1-like element can apparently act independently from SMS-UE and confers to cell- and tissue-specific expression of the human GLP-1R gene.

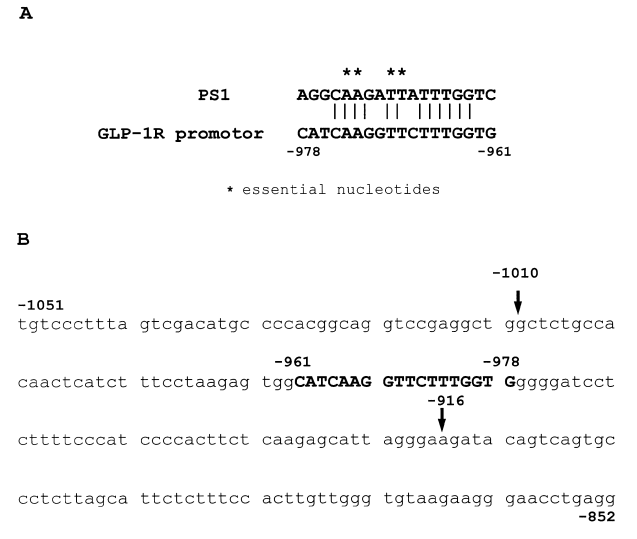

Fig. 1. A: Sequence homology between PS1 from the somatostatin gene and the PS1-like element found in the 5′-flanking region of the human GLP-1R gene. Asterics indicate essential nucleotides for activity, which are completely conserved in the GLP-1R gene. B: Partial sequence of the 5′-flanking region of the human GLP-1R gene. Numbers indicate the positions in relation to the main transcription start site, the arrows show the deletion constructs used for transient transfections. Bold capital letters represent the PS1-like element.

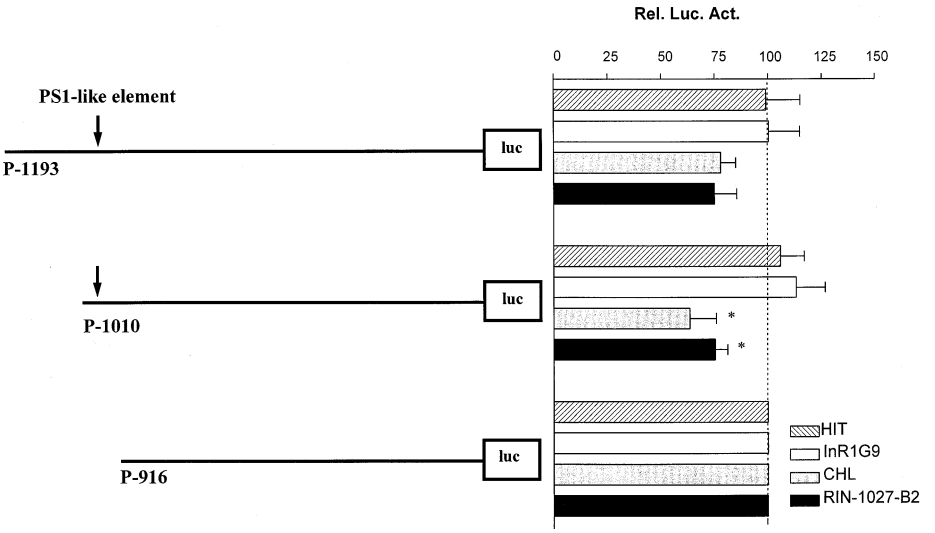

Fig. 2. Transient transfections of four cell lines with three 5′-deletion constructs of the GLP-1R promoter (schematic representation on the left side) as described in Section 2. The arrows indicate the position of the PS1-like element. The promoter/reporter gene construct missing the PS1-like element was set to 100%. Whereas no significant change in promoter activity occurred by deletion of the PS1-like element in HIT (B-cell line) and InR1G9 (A-cell line), a clear increase is observed in RIN-1027-B2 (D-cell line) and CHL (fibroblasts). The asterics indicate a P-value ≤ 0.015 in comparison to P-916. Luciferase activity was normalized by cotransfection with β-galactosidase. Results represent values from six independent transfections along with the standard deviation.

3.3. *DNase footprint and electrophoretic mobility shift analysis*

DNase footprint and electrophoretic mobility shift analysis was used to prove binding of nuclear proteins to the PS1-like sequence. With nuclear extracts of CHL cells, a protection in the region of the PS1-like element was seen (Fig. 4). Similar results were obtained with nuclear proteins of the other three cell lines (data not shown). To further characterize the proteins binding to the PS1-like sequence, nuclear extracts from all four cell lines were incubated with the endlabeled oligonucleotide containing the PS1-like sequence for electrophoretic mobility shift analysis. DNA-protein complexes were resolved on a 4% non-denaturing polyacrylamide gel. With all four nuclear extracts two retarded bands were found (S1 and S2), although in different intensities (Fig. 5). In CHL and RIN-1027-B2 cells the slower migrating signal S1 is more prominent, whereas the faster band S2 is more intense than S1 in

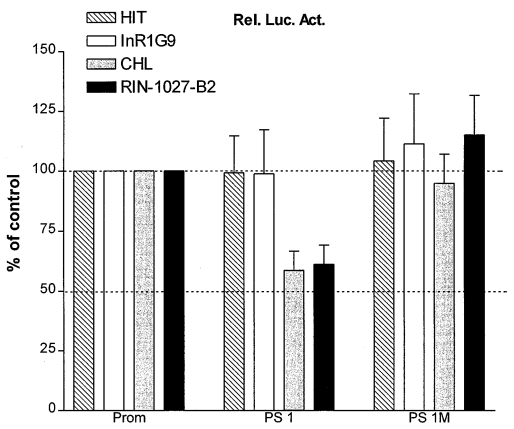

Fig. 3. Transient transfections of four different cell lines with PS1 (PS1) and PS1M (PS1M) cloned upstream an SV40 promoter, directing luciferase expression as described in Section 2. The control vector pGL2 promoter (Prom) was set to 100%. PS1 clearly decreases promoter activity in CHL (fibroblasts) and RIN-1027-B2 (D-cells) to about 60% of control. The mutated sequence PS1M has no influence on promoter activity in all four cell lines. Luciferase activity was normalized by cotransfection with β-galactosidase. Results represent values from eight independent transfections along with the standard deviation.

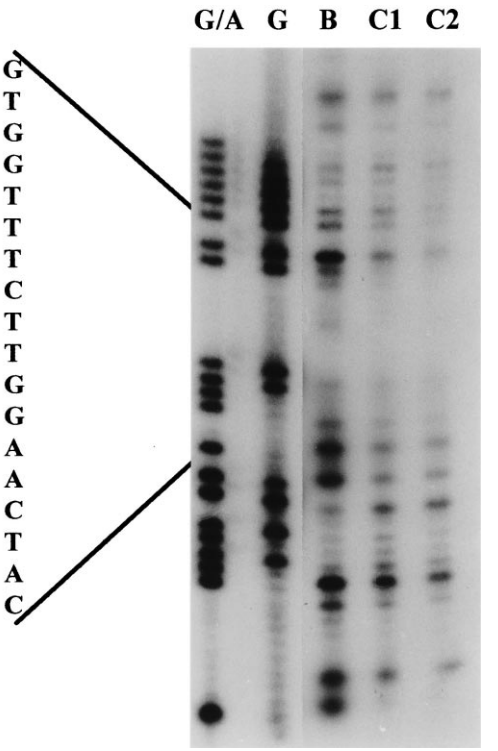

Fig. 4. DNase footprint analysis with nuclear extracts from CHL cells. The DNA fragment, containing the PS1-like element, was incubated with 60 μg BSA (lane B) or with 40 μg (lane C1) and 80 μg (lane C2) nuclear proteins from CHL cells, partially digested with DNase I and resolved on a denaturing 6% gel as described in Section 2. Sequencing reactions of the fragment were run in parallel on the gel (lanes G/A and G). The protected region is indicated with the sequence of the PS1-like element.

InR1G9 and HIT cells. Both signals can be competed by addition of an excess of PS1-like oligonucleotide and to a lesser extent by an excess of PS1M oligonucleotide. These data point to a functional DNA-protein complex in fibroblasts and D-cells present in band S1.

3.4. In-situ DNA-protein UV-crosslinking

To characterize the proteins binding to the PS1-like oligonucleotide, DNA-protein complexes S1 and S2 were covalently linked with UV light in native gels. After exposure the gel slices were cut out and transferred to a denaturing SDS gel. Analysis of complex S1 revealed that with nuclear extracts from all four cell lines two proteins with a molecular weight of 120 and 95 kDa bind to the oligonucleotide (Fig. 6). Although the amounts of nuclear proteins and radioactively labeled oligonucleotide are the same during all binding reactions, the intensity of the 95 kDa proteins is clearly higher in CHL and RIN-1027-B2 cells than in cells which show no effect of the PS1-like sequence. Therefore, the 95 kDa protein probably mediates the repression of gene transcription. With band S2 similar results are obtained: two nuclear proteins from all four cell lines bind to the DNA with a molecular weight of 110 and 65 kDa, but there is no difference in the intensity.

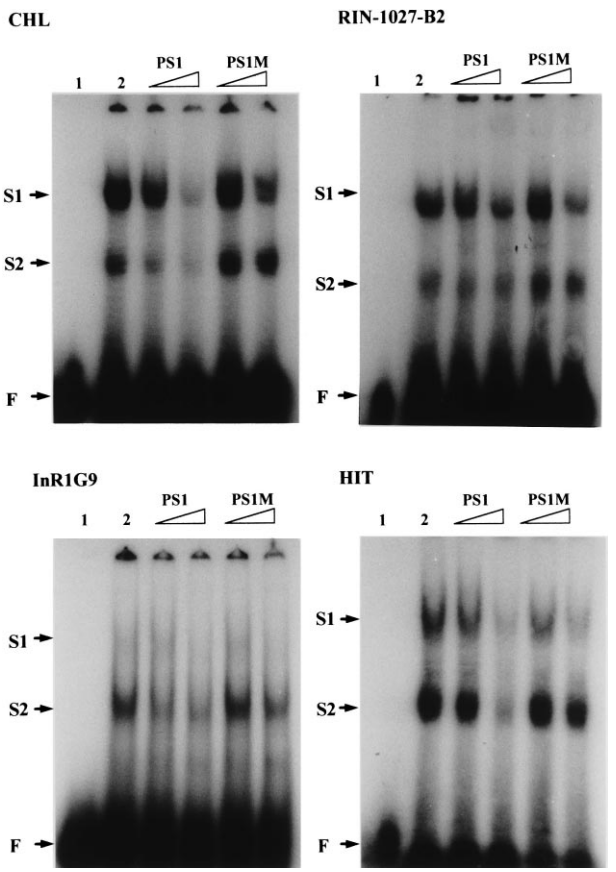

Fig. 5. Electrophoretic mobility shift analysis of an oligonucleotide containing the PS1-like sequence as described in Section 2. Lanes 1 in each panel show the free oligonucleotide, in lanes 2 nuclear extracts of the above indicated cell line were added, the following lanes show the competition with increasing amounts of unlabeled PS1 or PS1M oligonucleotide (50- and 500-fold excess). Retarded DNA-protein complexes are indicated by arrows S1 and S2, the free oligonucleotide by F. Band S1 is clearly more prominent in CHL and RIN-1027-B2 cells in comparison to S2 than in HIT and InR1G9 cells.

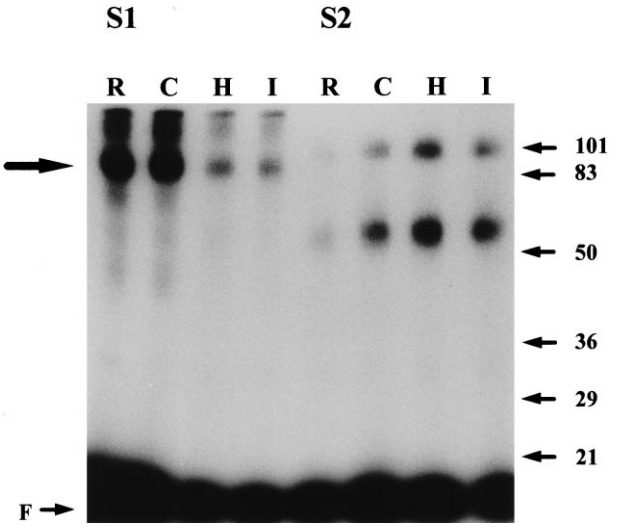

Fig. 6. In-situ DNA-protein UV-crosslinking of band S1 and S2 with nuclear proteins from four different cell lines as described in Section 2. Lane R: RIN-1027-B2 cells, lane C: CHL cells, lane H: HIT cells, lane I: InR1G9 cells. Lane F indicates the free oligonucleotide, arrows at the right side the protein markers with the molecular weight in kDa. With all four nuclear extracts proteins of the same molecular weight were linked to the radioactively labeled oligonucleotide for S1 (120 and 95 kDa) and S2 (110 and 65 kDa). The 95 kDa protein is more prominent in CHL and RIN-1027-B2 cells (large arrow on the left side).

4. Discussion

In the region from −1630 to −565 of the 5′-flanking sequence of the human GLP-1R gene, a tissue-specific, negatively acting *cis*-regulatory element is apparently located, as shown by transient transfections of fibroblasts with different 5′-deletion constructs [24]. With computer analysis a sequence was found, exhibiting high homology to the earlier characterized silencer PS1 [28]. Since PS1 represses the activity of a D-cell-specific enhancer (SMS-UE) of the somatostatin gene in fibroblasts and shows no effect in B-cells, it seems a possible candidate for the tissue-specific regulation of the GLP-1R gene expression. No sequence was found in the 5′-flanking region of the human GLP-1R gene comparable to SMS-UE, therefore, PS1 should be functionally active independently from SMS-UE. The first hints for functionality of the PS1-like element were promoter activities in transient transfections of different 5′-deletion constructs of the human GLP-1R promoter. Removing the PS1-like sequence by deletion from −1010 to −916 lead to a clear increase of promoter activity in fibroblasts and islet D-cells and no effect in islet B-cells. These results are in accordance with earlier published data from Vallejo et al. [28]. In islet A-cells no repression by the PS1-like sequence could be observed. A basal transcription of the human GLP-1R gene is repressed in a cell- and tissue-specific manner by the PS1-like element.

To prove functionality of the PS1-like element independently from SMS-UE, a double stranded oligonucleotide containing the PS1-like sequence was cloned upstream an SV40 promoter of a luciferase expression vector (PS1). As a negative control a plasmid served containing a mutated and there-

fore inactivated form of PS1 (PS1M). Transient transfections of different cell lines with these constructs clearly revealed a 40% reduction of gene expression in pancreatic D-cells and fibroblasts and no effect in pancreatic B- and A-cells. The mutated PS1-like sequence did not influence promoter activity in all cell lines. Therefore, it was shown that the PS1-like sequence resembles a *cis*-regulatory element, which can repress transcription independently from a D-cell-specific enhancer and in a cell- and tissue-specific manner.

To characterize the proteins, which can interact with the PS1-like element, we performed DNase footprint analysis and found a protection centered around the PS1-like element. Electrophoretic mobility shift analysis with nuclear extracts from all four cell lines revealed two retarded DNA-protein complexes. However, the slower migrating complex S1 was more intense in pancreatic D-cells and fibroblasts. UV-crosslinking experiments revealed that there was no difference in proteins binding to the PS1-like sequence with all four nuclear extracts for complex S2, but in complex S1 a 95 kDa protein was more prominent in D-cells and fibroblasts, putatively resembling the functional transcription factor. The same proteins from complex S1 and S2 can also bind to the mutated oligonucleotide PS1M including the 95 kDa protein (data not shown). Possibly, a conformational change of the protein structure is necessary for functional activity. Still, it cannot be excluded that all proteins found are in vitro artefacts and that the functional transcription factor can only bind in vivo or is masked by unspecific proteins.

PS1 was first characterized as a repressor of somatostatin gene expression in fibroblasts and islet D-cells [28]. The published in vivo data are in accordance with our results concerning repression of gene transcription in different cell lines, but the in vitro data of proteins binding to PS1 are different. Vallejo et al. [28] identified two proteins of 120 and 130 kDa by UV-crosslinking in fibroblasts, an additional protein of 100 kDa in islet B-cells and several more small proteins with a molecular weight between 40 and 45 kDa in D-cells. We found proteins of 120 and 95 kDa in all four cell lines linked to the oligonucleotide, although the protein of 95 kDa was more prominent in fibroblasts and D-cells. These differences may be due to the different cell lines used or to the substitution of dTTP by Br-dUTP. Br-dUTP leads to a more efficient UV-crosslinking of proteins to DNA, but we worked without this substitution to be as close as possible to the in vivo situation. Another difference maybe due to the sequence of the used oligonucleotides, which have a high homology, but are not identical and, therefore, can give rise to another binding specificity.

Silencer elements are important regulators of cell-specific expression of a number of genes. For example, neuron-specific expression of SCG10 [32,33] and type II Na^+ channel [34] appears to be due to selective repression and de-repression and activation by non-cell-specific enhancers. Both PS1 and the PS1-like element bind to ubiquitously distributed nuclear proteins, and the availability of the proteins seems to determine the repressing action of the PS1-like element. They do not act as absolute repressors, which are functionally active independently from other factors [35], but rather interact with other regulatory elements. The effect of PS1 on the somatostatin gene expression is compensated in part by a D-cell-specific enhancer and the PS1-like element is not able to repress the activity of the GLP-1R promoter completely. Similar mechanisms are found for the regulation of the genes coding for lysozyme [36] and prolactin [37]. The interaction of positively and negatively acting factors defines the pattern of gene expression in different cell types [38–42]. PS1 might be developmentally regulated and be involved in determining transcription of different genes during pancreatic islet cell maturation, since it does not repress gene expression in A- and B-cells. The sequential appearance of hormones in pre-B-cells and later in mature cells points to a differentiation of first A- and B-cells and then later of D-cells [43].

Repression of gene transcription seems to be an important regulatory mechanism in the islet cell-specific gene expression during pancreatic development. Insulin [44,39] and gastrin [38] transcription, for example, is directed by silencers in interaction with positive regulatory elements. A similar situation is found for the expression of the human GLP-1R. Transcription of the gene is under the control of different, cell-specific silencers like PS1 and ubiquitous enhancers like Sp1, which interact to enable the tissue- and cell-type-specific transcription of the gene.

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