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The LIM-Homeodomain Protein ISL1 Activates Insulin Gene Promoter Directly through Synergy with BETA2

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The LIM-homeodomain transcription factor ISL1 (islet factor 1) is essential for pancreatic islet cell and dorsal mesenchyme development. Mutations in ISL1 are associated with maturity-onset diabetes of the young and type 2 diabetes. Whether ISL1 plays a role in the insulin gene expression has not been fully elucidated. In the present study, we show that ISL1 can synergistically activate insulin gene transcription with BETA2 in pancreatic β cells. The protein–protein interactions of ISL1 and BETA2 are directly mediated by the LIM domains of ISL1 and the basic helix–loop–helix domain of BETA2. Deletion of the two LIM domains of ISL1 enhances the transcriptional activation of the insulin gene, indicating a key role for the homeodomain in activating the insulin promoter. Furthermore, ISL1 can bind with the A3/4 box in the rat insulin gene I promoter through its homeodomain. ISL1 expression is up-regulated at the mRNA level in type 2 diabetes (*db/db* mouse model) but down-regulated by dexamethasone in rat insulinoma cells. These results suggest that ISL1 is a transcriptional activator for insulin gene expression, and the interactions of ISL1 with BETA2 are required for the transcriptional activity of the insulin gene. Reduction in Isl1 gene expression appears to be involved in the impairment of insulin expression mediated by dexamethasone.

Keywords: ISL1; BETA2; insulin gene expression; protein interactions; transcriptional factors

Introduction

Insulin gene expression is tightly restricted to the β cells in the pancreatic islets of Langerhans and is precisely regulated by blood glucose levels.[1] This tissue specificity of insulin gene expression is conferred mainly by cis-acting regulatory sequences located within 300–400 bp upstream from the transcription start site of the insulin gene promoter,[2] which bind β-cell-restricted and ubiquitous transcription factors.[3] The mini-enhancer is one of the most important elements among the cis-acting regulatory sequences. It lies between −247 and −197 bp upstream from the transcription start site in the rat insulin I promoter and contains E2 and A3/4 boxes. ISL1 (insulin gene enhancer binding protein, islet factor 1), a LIM-homeodomain protein, binds the A3/4 box that is highly conserved and has been well documented to bind several homeodomain protein complexes in cell nuclei. There are three alternative copies of the TAAT or ATTA motif (TTAATAATCTAATTA), which are involved in ISL1 binding. BETA2, a basic helix–loop–helix (bHLH) transcription factor, binds the E2 box by forming a heterodimer with the ubiquitous bHLH protein E47.[4,5]

As a LIM-homeodomain protein, ISL1 contains two tandemly arrayed LIM domains near its N-terminus that form zinc-binding structures and a central homeodomain (HD) that contains 60 conserved amino acid residues responsible for specific DNA

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Abbreviations used: bHLH, basic helix–loop–helix; GST, glutathione *S*-transferase; HIT, hamster insulinoma tumor; DEX, dexamethasone; RIN, rat insulinoma; ISL1, islet factor 1; IgG, immunoglobulin G; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; RT, reverse transcription; ER, estrogen receptor; EDTA, ethylenediaminetetraacetic acid; STZ, streptozotocin.

binding. ISL1 is expressed in all four principal cell types of the endocrine pancreas, neurons in the central and peripheral nervous system, and cardiac precursors.[6–8] As a key transcription factor, the functions of ISL1 involve cell fate specification. Complete loss of dorsal pancreatic mesenchyme and endocrine islet cells was found in ISL1 knock-out mice embryos.[9] It has also been shown that ISL1 regulates the expressions of several genes in islet cells, such as proglucagon/glucagon (α cells), somatostatin (γ cells), amylin (δ cells), and kir6 (β cells).[10–13] However, it is still not clear whether the insulin gene, the most important gene in islet β cells, is regulated by ISL1.

Previous studies have indicated that ISL1 may also be involved in the regulation of insulin gene expression. It is defined as an insulin gene enhancer binding protein. MODY (maturity-onset diabetes of the young) 7 results from the insulin-releasing disability caused by a mutation of the ISL1 gene.[14,15] Nonsense mutations of the *ISL1* gene (Q310X) were found in a Japanese type 2 diabetic patient family.[16] Insulin-producing brain tumor cells can express ISL1 and BETA2 without expression of PDX1.[17] Combined expression of PDX1 and ISL1 can induce immature enterocytes to produce insulin.[18] These reports suggest that ISL1 may play a role in regulating insulin gene transcription. Our previous study has revealed that ISL1 could activate insulin gene expression by physical interaction with BETA2 in non-β cells.[19] Whether a similar activation can occur in β cells and the precise mechanism of synergistic activation of insulin gene expression by ISL1 and BETA2 remain to be elucidated.

The bHLH transcription factor BETA2 is expressed in pancreatic islet endocrine cells, intestinal cells, the pituitary gland, and a subset of neurons. BETA2 is a key regulator of development, differentiation, and survival of pancreatic cells.[20] The islets of mice homozygous for a targeted disruption of the *Beta2* gene have markedly diminished numbers of endocrine cells arranged in streaks, and they died from severe hyperglycemia within 3–5 days postpartum. BETA2 is also involved in insulin gene transcriptional regulation in β cells.[21] PDX1 and BETA2 interact with the coactivator p300 to activate the insulin promoter synergistically. The bHLH domain of BETA2 mediates the interaction with p300.[22]

In this study, we demonstrate that ISL1 is a novel transactivator of the insulin gene in pancreatic β cells. ISL1 stimulates transcriptional activation of the insulin promoter in coordination with BETA2. Both the LIM domain of ISL1 and the bHLH domain of

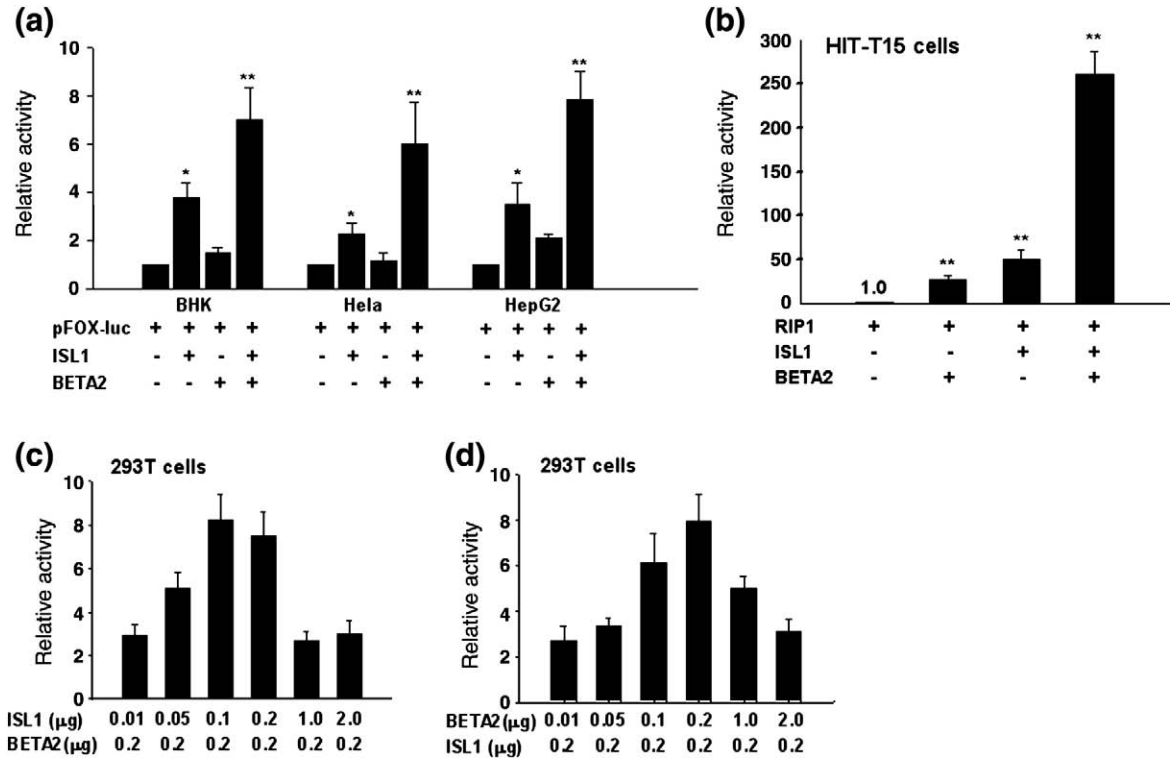

Fig. 1. ISL1 and BETA2 synergistically activate insulin promoters. (a) ISL1 and BETA2 synergistically activate mini-enhancer pFOX-luc in non-β cells. BHK, HeLa, and HepG2 cells were cotransfected with the mini-enhancer pFOX-luc reporter plasmid and the expression plasmids for ISL1 and/or BETA2. (b) HIT-T15 cells were cotransfected with an RIP1-luc reporter plasmid and the expression plasmids for ISL1 and/or BETA2. (c and d) ISL1 and BETA2 activate RIP1-luc in a dose-dependent manner. Numbers indicate amount of transfected plasmid (in micrograms). 293T cells were cotransfected with RIP1-luc reporter plasmid and the expression plasmids for (c) 0.2 μg BETA2 and various doses of ISL1 or (d) 0.2 μg ISL1 and various doses of BETA2. The total amount of DNA was kept constant using pcDNA3. At 48 h, the luciferase activities were determined. The activities of luciferase reporters (pFOX-luc or RIP-1-luc) were set as 1.0. pRL was used to normalize the transfection efficiency. Each bar represents mean±SD from triplicate independent experiments.

BETA2 are required for optimal interaction and synergy. Moreover, we show that the homeodomain of ISL1 mediates its binding with the mini-enhancer of the insulin promoter, and this binding can be enhanced by the presence of BETA2. We further demonstrate that the transcription level of the *Isl1* gene is down-regulated by glucocorticoids but hardly influenced by the circulating glucose concentrations, which is different from the regulation of insulin and *Pdx1* gene expressions. Our results ascertain a novel role of ISL1 in pancreatic β cells and describe the interaction and transcriptional synergy between ISL1 and BETA2. It extends the knowledge about ISL1 function in mature pancreatic islets after birth.

Results

ISL1 activates insulin promoter with BETA2 synergistically

We have previously shown that ISL1 could activate the rat insulin gene promoter synergistically with BETA2 in 293T cells. The effects in other cell lines, especially in β cells, have not been investigated. A luciferase reporter plasmid, pFOX, with the firefly luciferase gene inserted downstream of five copies of the rat insulin I mini-enhancer (−197 to 247 bp, containing E2 and A3/A4 boxes), pcDNA3-ISL1, and/or pCMV-BETA2 were cotransfected into non-β cell lines HeLa, BHK-21, or HepG2, which do not naturally express insulin or the islet-enriched transcription factors ISL1 and BETA2. A luciferase reporter plasmid RIP1 containing the rat insulin I promoter (0 to −410 bp) was transfected into a hamster pancreatic islet insulinoma cell line HIT (hamster insulinoma tumor)-T15 together with pcDNA3-ISL1 and/or pCMV-BETA2. Luciferase activity was measured. As shown in Fig. 1a and b, synergy between ISL1 and BETA2 was observed in all cell types tested. The activating efficiency of cotransfection of ISL1 and BETA2 expression plasmids was not only greater than either alone but also significantly greater than the sum of the individual activities of the two proteins expressed separately. Furthermore, the synergistic activation of insulin promoter exhibited a dose-dependent manner within 0.2 μg of ISL1 with a constant amount of BETA2 (Fig. 1c, left) or 0.2 μg of BETA2 with a constant amount of ISL1 (Fig. 1c, right).

These results indicate that ISL1 is able to activate the insulin promoter efficiently and there is clear evidence of synergistic activation between ISL1 and BETA2.

ISL1 interacts with BETA2 *in vivo* and *in vitro*

To investigate whether ISL1 and BETA2 might interact in activating insulin promoter, we cotransfected HIT-T15 cells with pCMV-FLAG-ISL1 and pCMV-BETA2, and BETA2 immunoprecipitates were immunoblotted with ISL1 antibody. ISL1 was detected in BETA2 immunoprecipitates and was absent in the control immunoglobulin G (IgG) immunoprecipitates (Fig. 2a). This suggests that ISL1 may form a complex with BETA2 *in vivo*. We further examined the binding of ISL1 to BETA2 *in vitro* by glutathione *S*-transferase (GST) pull-down assay. GST-ISL1 and GST proteins were immobilized on glutathione beads and incubated with ^{35}S-labeled BETA2. As shown in Fig. 2b, BETA2 was pulled down by GST-ISL1, but not by GST, demonstrating that ISL1 interacts with BETA2 physically *in vitro*.

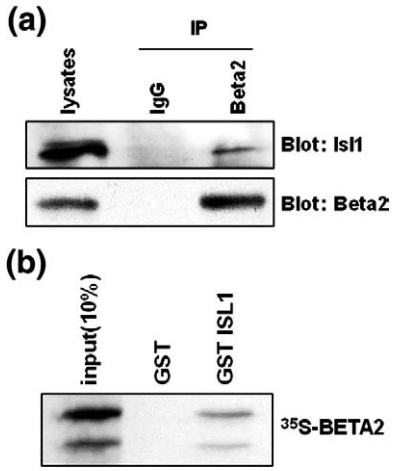

Fig. 2. ISL1 interacts with BETA2. (a) Co-immunoprecipitation of ISL1 and BETA2. Cell lysates from HIT-T15 cells transfected with the expression plasmids for ISL1 and BETA2 were immunoprecipitated with BETA2 antibody or normal IgG as a control. Immunoblot analysis of the lysates and immunoprecipitates was performed with ISL1 (upper panel) or BETA2 antibodies (lower panel). (b) GST pull-down experiment was performed as described in Materials and Methods. *In vitro* translated ^{35}S-labeled BETA2 was incubated with GST or GST-ISL1 proteins and analyzed by autoradiography.

The LIM domains of ISL1 are responsible for the interaction and transcriptional synergy with BETA2

The ISL1 protein comprises two LIM domains, one homeodomain (HD), and a carboxy-terminal transactivation domain (C domain) (Fig. 3a). To map the domains of ISL1 responsible for the interaction with BETA2, we performed GST pull-down experiments using ^{35}S-labeled BETA2 and various GST-ISL1 deletions or mutants. As shown in Fig. 3b, both the LIM1 and LIM2 domains of ISL1 can bind to BETA2, whereas the HD and C domains lacked the capability of interacting with BETA2. The disruption of the cysteine-composed double zinc-finger motif of the first LIM domain of ISL1 by the mutation C45G did not affect the binding to BETA2 (Fig. 3c). However, this mutation eliminated the synergy of ISL1 and BETA2 in the activation of the insulin promoter as demonstrated by the luciferase assay (Fig. 3d).

The deletion of the LIM1 domain affected neither the function of ISL1 alone nor the synergy of ISL1 with BETA2 in activating the insulin promoter (Fig. 3e). Surprisingly, the deletion of both the LIM1 and LIM2

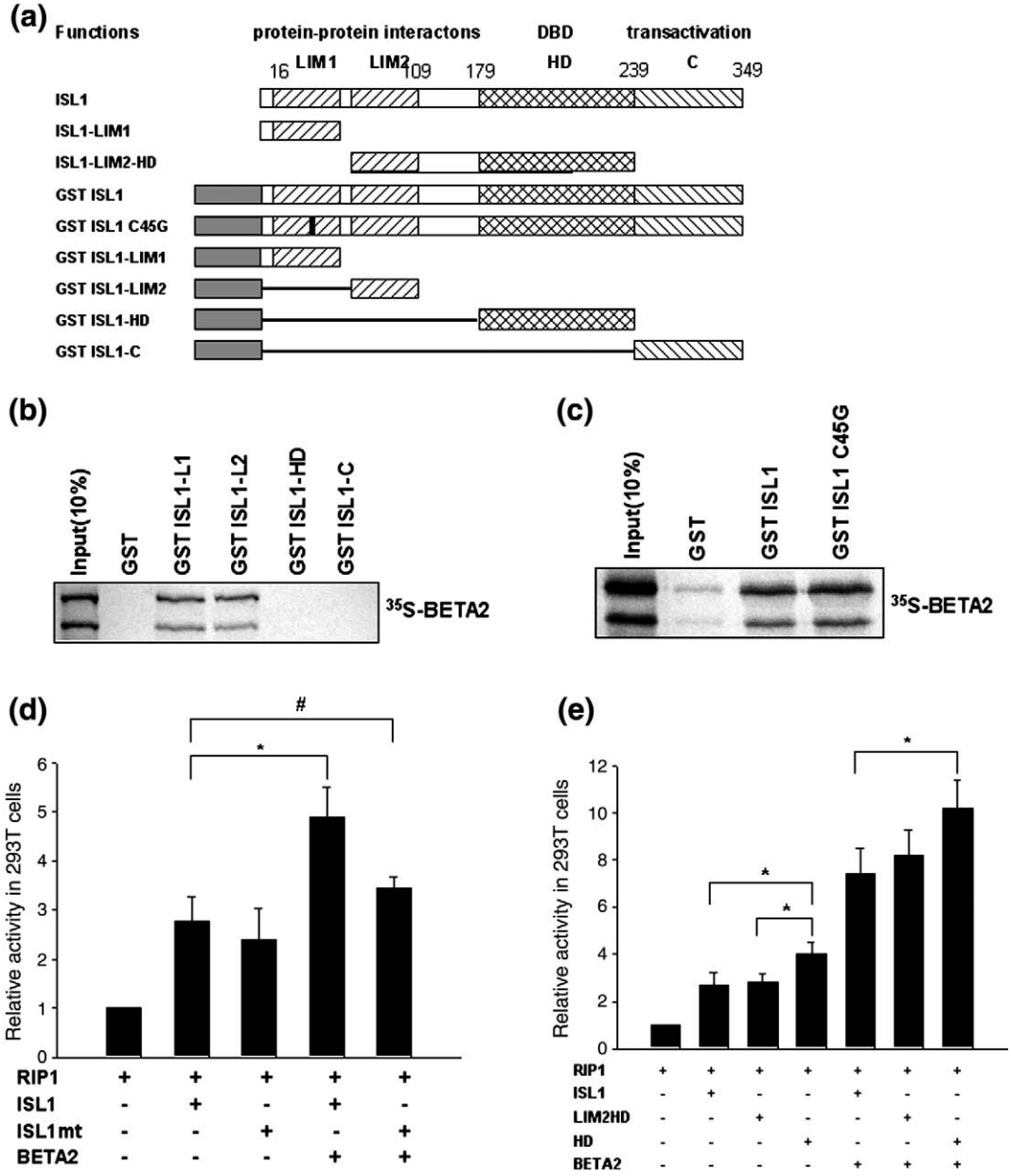

Fig. 3. LIM domains of ISL1 are responsible for the interaction with BETA2. (a) A schematic representation of ISL1 constructs used in this figure. The functions and names of the various domains are shown above the scheme of the full-length protein structure. In the ISL1 C45G construct, the mutation is depicted by the black rectangle. In the GST-ISL1 constructs, GST is depicted by a gray rectangle. (b) GST pull-down experiment analyzing the interaction between ^{35}S-labeled BETA2 and various fragments of ISL1 fused to GST. L1, LIM1; L2, LIM2. (c) GST pull-down experiments analyzing the interaction between ^{35}S-labeled BETA2 and GST-fusion proteins for wild-type or mutated ISL1. (d) Mapping of essential domains in ISL1 for the activation or synergistic activation of the insulin promoter with BETA2. 293T cells were transfected as indicated. (e) 293T cells were transfected as indicated. Luciferase activities were determined as described in Materials and Methods. Each bar represents mean±SD from three samples. *Statistical significance ($p<0.05$); #no significance ($p>0.05$).

domains together enhanced the transcriptional activation of insulin reporter greatly, indicating a key role of HD domain in activating insulin promoter. Furthermore, the synergistic effect of ISL1 and BETA2 was enhanced by approximately 40% in the absence of the LIM1 and LIM2 domains, indicating the involvement of HD in the transcriptional synergy. A previous report indicated that the LIM domain mediated the recruitment of some co-repressors. We propose that the deletion of LIM domains might change the interaction of ISL1 with other proteins simultaneously or impair some inhibitory roles to the homeodomain. The surprising synergy between ISL1 without LIM interaction domains and BETA2 also suggests that there could be some other unidentified proteins in the complex of ISL1 and BETA2, which

probably mediate the synergic activity between ISL1 and BETA2, as it has been reported that the homeodomain of ISL1 could also interact with other proteins.[23] However, the detailed mechanism remained to be investigated.

Taken together, our data show that the two LIM domains of ISL1 associate with BETA2 in the course of protein–protein interaction. The HD domain of ISL1 appears to be involved in the transcriptional synergy.

The bHLH domain of BETA2 is required for its interaction with ISL1

To define which part of BETA2 is required for interaction with ISL1, we used a series of BETA2 deletions in GST pull-down experiments. The deletions of BETA2 were translated and labeled in a reticulocyte lysate system *in vitro* and tested for their ability to bind to the GST-ISL1 affinity resin. As shown in Fig. 4a, the interaction only happened if the fragment contained at least residues 1–158, indicating that the bHLH domain of BETA2 protein is crucial in mediating the interaction with ISL1. Luciferase assay showed that the deletion of any domain of BETA2 eliminated its synergistic effect with ISL1 to the insulin promoter (Fig. 4b), indicating that every domain was essential for synergy.

The homeodomain of ISL1 contributes to DNA binding

To evaluate the direct binding of ISL1 to the mini-enhancer of rat insulin I, we performed electrophoretic mobility shift assays (EMSAs). Nuclear extracts from 293T cells transfected with expression plasmids for ISL1, LIM2-HD, and HD, respectively, were probed with a radiolabeled oligonucleotide containing the A3/4 box of the insulin promoter. As shown in Fig. 5a, ISL1 could bind to the A3/4 box. The specificity of binding was confirmed by their attenuated band after the addition of ISL1 antibody. The deletion of the LIM1 domain partly decreased the DNA binding ability of ISL1, whereas the HD alone could still bind to the A3/4 probe, demonstrating that the HD of ISL1 is crucial for DNA binding as previously reported.[24] Furthermore, to determine whether the transcriptional activity of ISL1 is regulated by its DNA binding ability, we employed ISL1 expression plasmid (pcDNA3-ISL1) in combination with wild-type insulin mini-enhancer, mutant A or mutant E (mutations in A3/4 box or E box, Fig. 5b) reporter vectors to transfect 293T cells for luciferase assays. Consistent with the EMSA results, mutant A3/4 box markedly impaired both the insulin reporter activation mediated by ISL1 alone (Fig. 5c) and the synergistic

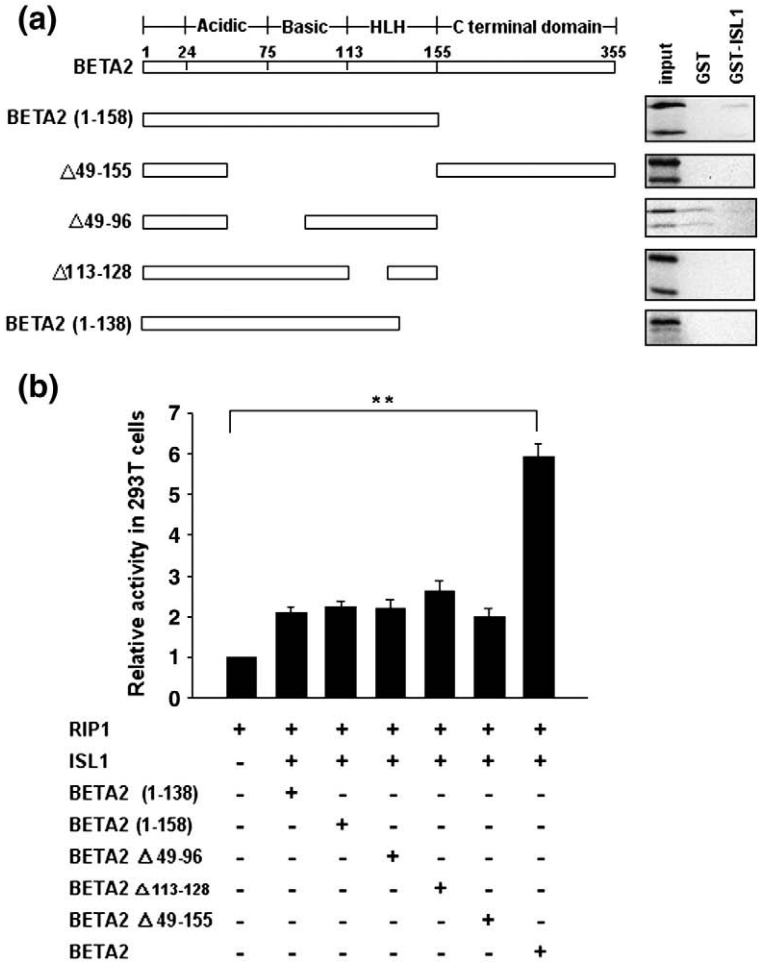

Fig. 4. bHLH domain of BETA2 is responsible for the interaction with ISL1. (a) GST pull-down assay showed that bHLH domain of BETA2 is crucial for interaction with ISL1. The structure of BETA2 and its truncations and/or deletions is shown schematically. (b) HeLa cells were cotransfected with an insulin-luciferase reporter plasmid and the expression plasmids for ISL1, BETA2, and its truncations and/or deletions as indicated for 48 h. Luciferase activities were determined. Each bar represents mean± SD from triplicate experiments.

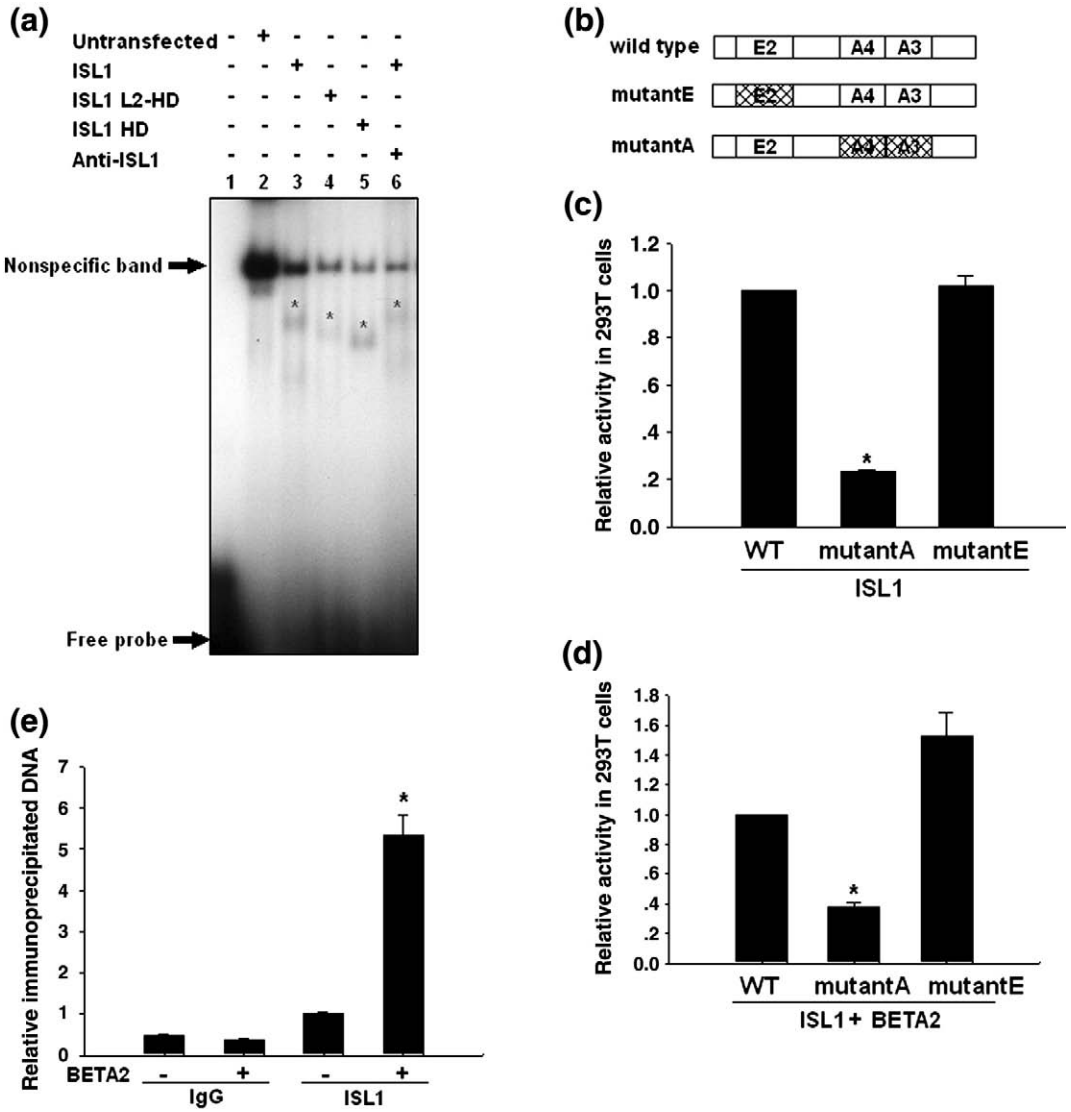

Fig. 5. BETA2 elevates ISL1 DNA binding activity. (a) ISL1 and its fragment proteins were tested by EMSA for the ability to bind the ^{32}P-labeled rat insulin A3/4 probe. Asterisks represent specific bands. (b) Schematic representation of mini-enhancer pFOX constructs used in (c) and (d). (c) Effect of the insulin I promoter mutations on the activity of ISL1. 293T cells were cotransfected with expression plasmids for ISL1 and mini-enhancer-luciferase reporter plasmid for wild type or mutant A or mutant E. (d) Effect of insulin I promoter mutations on the synergy between ISL1 and BETA2. 293T cells were cotransfected with the expression plasmids for ISL1 and BETA2 and mini-enhancer-luciferase reporter plasmids for wild type or mutant A or mutant E. (e) BETA2 and ISL1 recruitment to the insulin I promoter analyzed by ChIP assay. Soluble chromatin was prepared from 293T cells that were transfected with 4 μg of pRIP1-luc reporter plasmid, 2 μg of pCMV-FLAG-ISL1, and/or 2 μg of pCMV-BETA2 plasmid, followed by immunoprecipitation with antibodies against FLAG. The final DNA extractions were amplified using pairs of primers that cover the ISL1 binding sites of the insulin promoter by real-time PCR with normal IgG as a control. The data obtained were normalized to the corresponding DNA input.

effect of ISL1 with BETA2 (Fig. 5d). In contrast, no obvious effects were observed on mutant E box (Fig. 5c and d). These results indicate that the A3/4 box of the insulin promoter is a key region for ISL1 activation effect and ISL1 synergism with BETA2.

BETA2 elevates ISL1 DNA binding activity

To gain insight into the exact mechanism underlying the synergistic activation of insulin promoter by ISL1 with BETA2, we employed promoter chromatin immunoprecipitation (ChIP) assays. The FLAG antibody specifically immunoprecipitated the insulin I promoter when FLAG-ISL1 alone or both FLAG-ISL1 and BETA2 were expressed. The amplification region covered one ISL1 binding site (TTAATAA) located at −228 to −239 bp in rat insulin I promoter. As shown in Fig. 5e, the presence of BETA2 significantly accentuated the loading of ISL1 on the insulin promoter compared to ISL1 alone, suggesting that BETA2 facilitated ISL1 binding affinity with the insulin promoter.

Isl1 and Beta2 are expressed at different levels in pancreatic islets from STZ mice and *db/db* mice

To investigate the possible effects of ISL1 on the regulation of insulin gene expression in pathological conditions, we prepared total RNA from streptozotocin (STZ) mice (representing type 1 diabetes) and *db/db* mice (inherited obesity, representing type 2 diabetes) and examined the mRNA expressions of insulin, Isl1, and Beta2 by real-time reverse transcription (RT)-PCR analysis. As shown in Fig. 6a, the mRNA expression levels of insulin and Beta2 were reduced in STZ mice, while the Isl1 mRNA level was slightly increased without significance. As shown in Fig. 6b, the mRNA expression level of Isl1 was increased in hyperinsulinemic *db/db* mice, while Beta2 expression was essentially unchanged. Although these results show the expression changes of Isl-1 and Beta2 in animal models of diabetes, it is

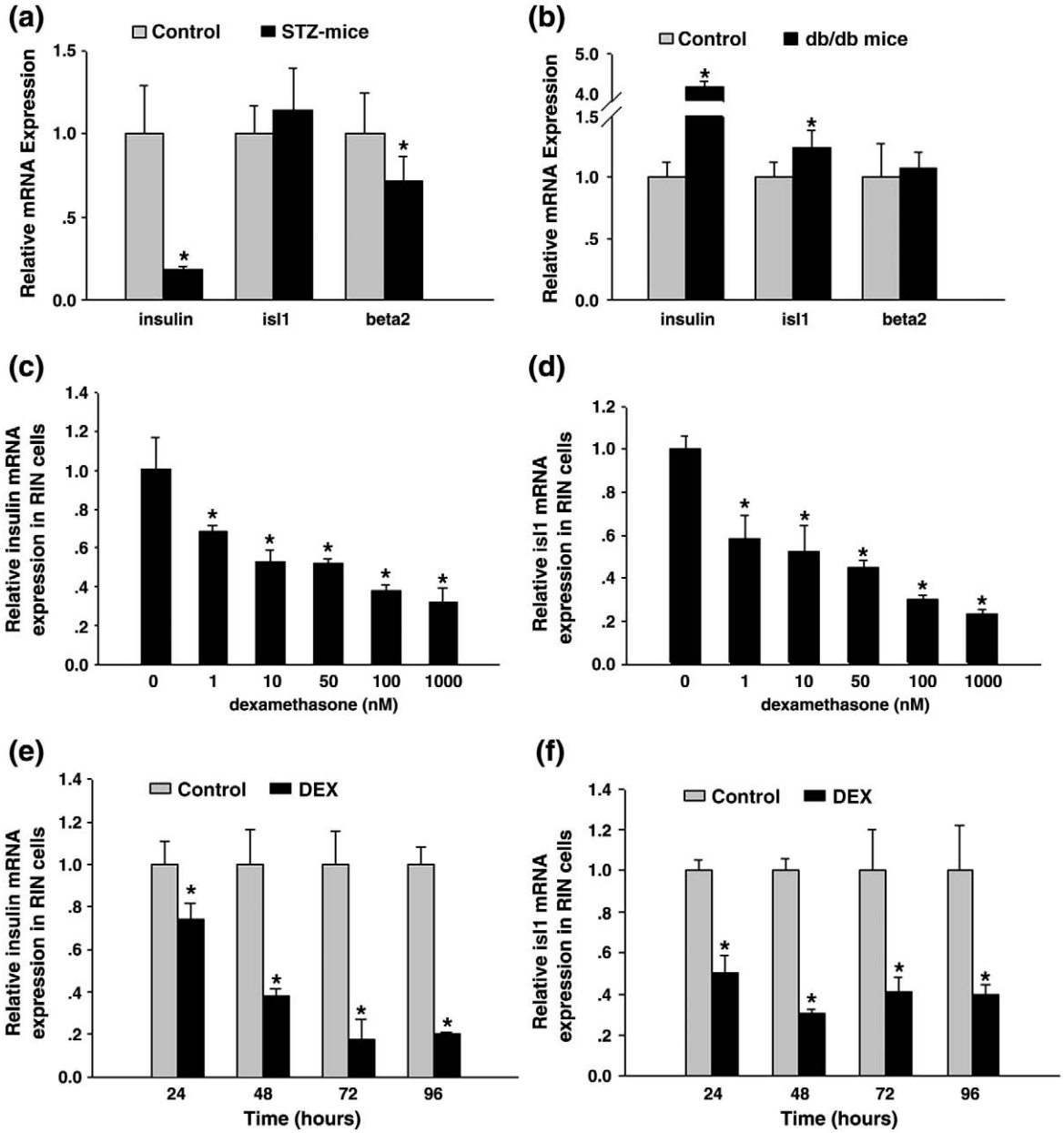

Fig. 6. Insulin, Isl1, and Beta2 gene expressions in pancreatic islets from STZ mice and *db/db* mice and effects of DEX on insulin and Isl1 mRNA expression in RIN cells. Relative amounts of insulin, Isl1, and Beta2 mRNA levels in pancreatic islets from (a) STZ mice and (b) genetically obese *db/db* mice were measured by real-time RT-PCR. mRNA levels were compared with those in the control group. (c) and (d) show the level of mRNA expressions measured by real-time RT-PCR in RIN cells treated with DEX at various concentrations (0–1000 nM) for 48 h. (e) and (f) show the level of mRNA expressions measured by real-time RT-PCR in RIN cells treated with 100 nM DEX for different periods of time (0–96 h). The level of gene expression was normalized to 18S RNA. Each bar represents mean±SD from three samples; *statistical significance ($p<0.05$).

premature to conclude that the expression of Isl1 might be associated with insulin or glucose level in type 2 diabetes at present. More investigation yet remains to be explored.

Glucocorticoid reduces the expression of Isl1 in rat insulinoma cells

It has been reported that glucocorticoids are physiological inhibitors of insulin synthesis at the transcription level.[25,26] The inhibitory effect of dexamethasone (DEX) on insulin gene expression was confirmed in the rat pancreatic islet insulinoma cell line (RIN) by real-time RT-PCR. As shown in Fig. 6c and e, DEX strongly inhibited insulin gene mRNA expression in a dose-dependent manner (0–1000 nM) at 48 h or in a time-dependent manner (0–96 h) at 100 nM. Similar to insulin, real-time RT-PCR analysis revealed that the Isl1 mRNA levels were also down-regulated by DEX treatment in a dose-dependent manner (0–1000 nM), and the lowest expression level was observed at 48 h with 100 nM DEX (Fig. 6d and f). These results show that DEX inhibits Isl1 gene expression, consistent with the effect of DEX on the inhibition of insulin gene transcription.

Discussion

ISL1 is well known as a LIM-homeodomain structure protein and is one of the key factors in the control of development of the four endocrine cell lineages of pancreas and dorsal mesenchyme.[9] Although it was originally defined as insulin gene enhancer binding protein, its roles in regulating insulin gene transcription and the exact regulation mechanism are still unknown.

ISL1 promotes synergistic activation with BETA2 on insulin promoter-driven reporter activity in non-β cells and β cells

Previous studies indicated that mutations in the *ISL1* gene could cause MODY 7 or type 2 diabetes.[14] *ISL1*$^{+}$/*BETA2*$^{+}$/*PDX1*$^{-}$ brain tumor cells can produce insulin.[17] These imply that ISL1 and BETA2 may play important roles in the production and regulation of insulin. Using luciferase reporter assays, we demonstrated that ISL1 could activate rat insulin I gene promoter in several non-β cell lines such as HeLa cells, BHK cells, and HepG2 cells and, more importantly, in HIT-T15 β cells.

The LIM domain of ISL1 is crucial for the interaction with BETA2

Synergistic transcription is mediated by multiple interactions between activators themselves and by contact with the RNA polymerase II transcriptional apparatus or indirectly through bridging coactivators. The PDX1 and E47/BETA2 heterodimer can bind to the p300 coactivator, which provides a docking and recruitment interface with the general transcriptional machinery,[27] while more than one activation complex may be capable of activating insulin gene transcription through the E2A3/4 mini-enhancer. A LIM-homeodomain protein, LMX1.1, and a bHLH domain protein, E47, could activate the insulin mini-enhancer cooperatively.[28] ISL1 also belongs to a LIM-homeodomain protein family, which is composed of two LIM domains and a homeodomain. It was reported that the LIM domain mediates interactions with other factors[29] and works as a bridge to recruit other proteins to regulate cell fate or cell functions.[30] In our study, ISL1 exhibited a direct interaction with BETA2, which is mediated by LIM1 and LIM2 domains of ISL1 with the bHLH domain of BETA2, revealing the structural basis for the interaction. Mutation of the LIM1 domain (C45G), presumed to disrupt its characteristic zinc-finger structure, had little effect on the interaction between ISL1 and BETA2. The result exhibits a completely different behavior compared with ISL1 and HNF4α.[31] The same mutation, C45G, markedly decreased ISL1 binding to HNF4α. Indeed, the LIM1 domain and homeodomain of ISL1 were involved in the interaction with HNF4α, while the LIM2 domain is dispensable in this interaction. In the interaction between ISL1 and BETA2, the two LIM domains of ISL1 were both required. Mutation on one site in the LIM1 domain would be compensatable by other zinc-finger structures in the LIM2 domain. Some reports revealed that ISL1 could interact with various factors using different domains. In a complex of JAK1, ISL1, and STAT3, the LIM1 and LIM2 domains of ISL1 together strongly mediated the interaction with STAT3 and the homeodomain of ISL1 also interacted with STAT3.[23] However, the homeodomain of ISL1 is dispensable for the interaction with estrogen receptor (ER), consistent with our results. We also showed that LIM1 and LIM2 deletion enhanced the transcriptional activation of the ISL1 protein alone or synergistically with BETA2 by luciferase assays. This might be due to several reasons. First, the LIM domain might inhibit the transcriptional activity of ISL1 by masking the active role of the homeodomain and relieving intramolecular repression. LIM domains have been suggested to be a negative regulatory domain in other LIM-homeodomain proteins.[32] For example, the LIM domains of LMX1.1 inhibit the inherent activity of the LMX1.1 activation domain.[28] Second, the LIM domain may recruit a co-repressor to regulate gene transcription simultaneously. Third, ISL1 might aggregate multiple proteins to assemble a transcriptional activation complex, which may comprise unidentified proteins contributing to the synergic activity between ISL1 without LIM domains and BETA2. However, the detailed mechanism will need further investigation.

BETA2 enhances the binding of ISL1 to A3/4 boxes of rat insulin I promoter

Our results indicated that ISL1 protein is capable of binding to A3/4 boxes of rat insulin I promoter *in vitro* and *in vivo*. EMSA results stated that in the absence of LIM domains, the homeodomain is enough to

mediate the DNA binding activity for ISL1. The probe used for EMSA was a short native oligonucleotide containing only A3/4 sequence, slightly different from previous reported probes that contained four copies in tandem of the TAATGG[33] or a longer fragment containing the E2 and A3/4 regions.[34] Our observation is consistent with previous studies characterizing the homeodomain of rat ISL1.[24,33] Moreover, mutation in the A3/4 box region but not in the E box region of the insulin gene mini-enhancer resulted in remarkable reduction of the promoter activity and affected the synergistic action of ISl1 and BETA2. The A box is the ISL1 binding site while the E box is the bHLH binding site. We found that BETA2 could not bind with the E box of insulin mini-enhancer by EMSA (data not shown). These results suggest further that the activity of the insulin promoter is dependent on the binding of ISL1 to the A box, not to the E box. However, BETA2 could enhance the binding of ISL1 in the insulin promoter. This was triggered presumably by protein–protein interactions, as demonstrated in the interactions between ER and ISL1. ER could enhance ISL1-driven transcriptional regulation in a promoter containing only ISL1 binding sites.[35]

The expression of ISL1 changes in different diabetes animal models

Although the functions of ISL1 in pancreatic development have been extensively studied, we have little knowledge on the regulation of ISL1 in physiological or pathological conditions in the pancreatic islet. Glucose is one of the most important factors stimulating insulin gene expression in pancreatic β cells. Some insulin gene activators, such as PDX1[36] and MafA,[37] were reported to be elevated in high glucose concentration *in vitro* and *in vivo*.[38,39] We found that the mRNA expression level of Isl1 was also up-regulated in type 2 diabetes *db/db* mice with hyperglycemia and hyperinsulinemia, which would serve to guide future research on the relationship between ISL1 and type 2 diabetes.

DEX treatment suppresses ISL1 expression

Glucocorticoids are likely to contribute to the pathophysiology of insulin resistance with the metabolic syndrome, insulin sensitivity, and the inhibition of insulin secretion from pancreatic β cells.[40] DEX suppresses insulin expression in pancreatic β cells via a mechanism involving down-regulation of PDX1 and BETA2 and induction of C/EBPβ and HES1.[25] Here, we showed that DEX reduced the mRNA levels of insulin and Isl1 in time- and dose-dependent manners in pancreatic RIN cells, indicating that Isl1 gene expression is also reduced by glucocorticoids. However, it remains to be investigated whether glucocorticoids suppress insulin gene expression by reducing Isl1 expression. Furthermore, the regulation of Isl1 might be mediated by glucocorticoids through a glucocorticoid receptor binding element lying 5′ upstream regulation sequence of Isl1 gene promoter (data not shown). This mechanism can be seen in other insulin gene activators. For example, glucocorticoids reduced Pdx1 gene expression by affecting a glucocorticoid receptor binding element on its enhancer.[41]

In conclusion, our results provide a novel insight into understanding the precise molecular mechanism of insulin activation by protein–protein interaction complex. We showed that ISL1 and BETA2, which are co-expressed in pancreatic β cells, interact and synergize to activate insulin gene transcription. In delineating the domains of each factor required for their optimal synergy, we have shown an interaction between the LIM domain and the bHLH domain. We have extended knowledge of the crucial role of the homeodomain of ISL1 that critically modulates responsiveness for binding with the insulin promoter. We also began to explore the function of Isl1 in pathological conditions (diabetic mice and DEX treatment), but the roles of ISL1 and BETA2 in the modulation of insulin gene activity in the diabetes or other disease conditions require further investigation.

Insulin gene expression is a complicated regulatory network involving more than 40 nuclear factors. Our results indicate that ISL1 acts as an activator of insulin gene promoter within this network. They also provide insights into the structure–function relationship of ISL1 domains, which has been scarcely studied up to now. We also extend knowledge of the role of ISL1 on mature pancreatic islets after birth. It remains a possibility that ISL1 plays a more important or extensive role than activation of insulin gene transcription in pancreatic islet cells.

Materials and Methods

Plasmid constructs

The plasmid constructs pcDNA3-ISL1, pCMV-FLAG-ISL1, pCMV-BETA2, pRIP1-luc, and pFOX-luc were previously described.[19] The mutant pcDNA3M-ISL1-C45G was a kind gift from Dr. B. Laine.[31] Plasmids pFOXluc.prl.5mC1 and pFOXluc.prl.5mEF1 were generous gifts from Dr. Michael S. German.[34] Plasmids encoding BETA2 proteins with truncations and/or deletions [BETA2 (1–158), Δ49–155, Δ49–96, Δ113–128, and BETA2 (1–138)] were generous gifts from Dr. Andrew B. Leiter.[22] DNA sequences encoding ISL1-LIM1, LIM2, HD, and C-terminus fragments were generated by PCR and subcloned into the BamHI-XhoI sites of the pGEX 4T-2 vector, for the GST pull-down experiment. Plasmid pGEX4T-2 ISL1C45G was obtained by site-directed mutagenesis using the QuikChange™ kit (Stratagene) to introduce the mutation C45G. The sequences encoding ISL1-LIM1-LIM2, ISL1-LIM2-HD-C-terminal, and HD-C-terminal fragments were produced by PCR and inserted into the HindIII-XbaI sites of pcDNA3 vector for luciferase assay. All the PCR primers and the conditions are listed in Table 1, and the generated constructs were verified by DNA sequencing.

Cell cultures, transfection, and luciferase assays

Monolayer cultures of pancreatic islet β cells (HIT-T15, RIN) were maintained as described previously.[26,42] Non-β

Table 1. The sequences of PCR primers

Genes	Primer sequence (5′–3′)	Restriction sites (5′–3′)
ISL1-LIM1	F, CGGGATCCTGTGTTGGTTGCGGCAATC R, CCCTCGAGCTAACACTCCGCACATTTCAAAC	BamHI-XhoI
ISL1-LIM2	F, CGGGATCCTGCGCCAAATGCAGCATTG R, CCCTCGAGCTAGCAGGCTACACAGCGGAAACA	
ISL1-HD	F, GCAAGCTTATGGATGTGGTGGAGAGA R, CTCTAGAAGTCCTCATGCCTCAATAGG	
ISL1-C-terminus	F, CAGGATCCATCATGATGAAGCAGCTCCAAC R, GCTCTAGAAGTCCTCATGCCTCAAT	
ISL1-LIM1-LIM2	F, TCAAGCTTATGGGAGACATGGGCGAT R, CCCTCGAGCTAGCAGGCTACACAGCGGAAACA	HindIII-XbaI
ISL1-LIM2-HD	F, CAAGCTTATGGATGGAAAAACCTACTG R, CTCTAGAAGTCCTCATGCCTCAATAGG	
ISL1-HD	F, GCAAGCTTATGGATGTGGTGGAGAGA R, GCTCTAGAAGTCCTCATGCCTCAATAGG	

cells (BHK-21, HeLa, HepG2, and 293T) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin–streptomycin. All transfections were performed in 24-well plates using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. The total amount of DNA was kept constant using pcDNA3 vector. Forty-eight hours after transfection, the cells were harvested and luciferase and renilla activities were measured using the dual luciferase kit (Promega). The firefly luciferase data for each sample were normalized based on transfection efficiency measured by renilla luciferase activity. Each assay was performed in triplicate and repeated at least three times.

Immunoprecipitation and Western blotting

HIT-T15 cells were transfected with pcDNA3-ISL1 and pCMV-BETA2 expression plasmids. The cells were harvested after 48 h and lysed in immunoprecipitation cell lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100] with 1:100 diluted protease inhibitor cocktail (Calbiochem). The cell lysate was incubated with antiserum against BETA2 (sc-1084, Santa Cruz Biotechnology) overnight at 4 °C. Immune complexes were then captured using protein G-agarose (Roche, Indianapolis, IN). After four washes, bound proteins were released from the protein G-agarose by boiling for 5 min in 2×SDS loading buffer and followed by immunoblot analysis using ISL1 polyclonal antibody (Cat. No. ARP32336, Avivasysbio) or BETA2 antibody (Cat. No. sc-1084, Santa Cruz Biotechnology). Detection was performed using enhanced chemiluminescence detection reagents (Amersham Biosciences) after incubation with a horseradish-peroxidase-conjugated secondary antibody (Cat. No. ZB-2301, ZSGB-BIO).

GST pull-down assay

To detect protein interactions *in vitro* between ISL1 and BETA2, we performed pull-down reactions. GST or GST fused with full-length ISL1 or various truncated ISL1 proteins were prepared under the manufacturer's conditions (Amersham Biosciences). [^{35}S]methionine (1000 Ci/mmol; Amersham Biosciences)-labeled wild-type BETA2 or a series of deletions were synthesized using the TNT Quick Coupled Transcription/Translation System (Promega). Labeled BETA2 proteins were incubated for 2 h in binding buffer (40 mM Hepes, pH 7.2, 50 mM Na-acetate, pH 7.0, 200 mM NaCl, 2 mM EDTA, 5 mM dithiothreitol, 0.5% Nonidet P-40, protease inhibitors, and 2 μg bovine serum albumin/ml) with GST-ISL1 fusion proteins coupled to glutathione-Sepharose beads (Pharmacia). After four washes in GST binding buffer, beads were boiled in SDS sample buffer to elute bound protein, which was subsequently resolved by SDS-PAGE and analyzed by autoradiography.

ChIP assay

293T cells were transfected with 4 μg of pRIP1-luc reporter plasmid, 2 μg of pCMV-FLAG-ISL1, and/or 2 μg of pCMV-BETA2 plasmid. Forty-eight hours after transfection, ChIP assay was performed as described previously.[43] The anti-FLAG M2 antibody (Cat. No. F-3165, Sigma) was used to precipitate DNA associated to FLAG-ISL1. Both the input DNA and immunoprecipitated DNA were detected by real-time PCR using SYBR® Green Real-Time PCR Master Mix (TOYOBO, Japan). The primers for pRIP1-luc were as follows: forward (F), 5′-GGAAATGAGGTGGAAAATG-3′; reverse (R), 5′-GGTAGGTAGGCAGATGGC-3′ (247 bp fragment). The data obtained were normalized to the corresponding DNA input control.

Electrophoretic mobility shift assays

Nuclear extracts from 293T cells transfected with pcDNA3-ISL1, pcDNA3-ISL1-LIM2-HD, and pcDNA3-ISL1-HD, respectively, were prepared as described previously.[44] A double-stranded oligonucleotide was designed to contain the A3/4 box from the rat insulin I promoter and end-labeled with [α-^{32}P]dATP by Klenow enzyme. The sequence of the sense strand of this oligonucleotide was 5′-CTTGTTATTATTCTAATTACCCT-3′ (A3/4 box underlined). The binding reactions were performed at 4 °C (20 μl final volume) with 10 μg of nuclear protein and 0.25 ng (20 kcpm) of radiolabeled, double-stranded oligonucleotide in a buffer containing 20 mM Hepes (pH 7.9), 10% glycerol, 20 mM KCl, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 μg of salmon sperm DNA. Antibody analyses were performed by preincubation of nuclear extract protein with specific ISL1 polyclonal antibody (Cat. No. AB-210, Avivasysbio) for 20 min at 4 °C prior to addition of the radiolabeled probe. Samples were electrophoresed on 6% nondenaturing polyacrylamide gels at 150 V for 2.5 h in 0.5×TBE buffer. Gels were then dried and visualized by autoradiography.

Real-time RT-PCR

Total RNA was extracted from cultured monolayer cells or murine pancreases using TRIzol reagents (Invitrogen)

according to the manufacturer's instruction. Total RNA (2 μg) was reverse transcribed using the Superscript first-strand synthesis system (Promega). Real-time PCR reactions were performed in triplicate using the SYBR® Green Real-Time PCR Master Mix (TOYOBO) and the ABI 7300 Real-Time PCR System. Transcript levels were normalized to 18S RNA levels. Primers used for amplification were as follows: insulin (140 bp fragment): F, 5′-AGGACCCACAAGTG-GAACAACT-3′; R, 5′-CAACGCCAAGGTCTGAAGGT-3′; Isl1 (123 bp fragment): F, 5′-CTGCTTTTCAGCAACTGG-TCA-3′; R, 5′-TAGGACTGGCTACCATGCTGT-3′; 18S RNA (151 bp fragment): F, 5′-GTAACCCGTTGAACCC-CATT-3′; R, 5′-CCATCCAATCGGTAGTAGCG-3′.

Type 1 and type 2 diabetes animal models

The animal experiments were performed in accordance with the ethical principles and guidelines for scientific experiments on animals of the Swiss Academy of Medical Sciences (1995). STZ-induced diabetic mice [male C57BALB/c mice at the age of 8 to 10 weeks] were established as described previously.[45] The mice with stable hyperglycemia (blood glucose levels >20 mmol/L) were used as the type 1 diabetes animal model. *db/db* mice at age of 14–16 weeks were used as the type 2 diabetes animal model, which were homozygous for the *db* gene and, thus, exhibited an obese, diabetic phenotype. *db/w* mice that were heterozygous for the *db* gene exhibited a nondiabetic, normal phenotype and were used as controls to *db/db* mice. The type 1 and type 2 diabetes mice in the postprandial state were anesthetized with 5 mg/100 g body weight of sodium pentobarbital and pancreatic tissue was removed; RNA was prepared from these tissues and used for real–time RT-PCR assay.

Statistical analysis

The data were expressed as means±SD. Comparisons between groups were analyzed by Student's *t* test or ANOVA, and the Student–Newman–Keuls method was used to estimate the level of significance. $p<0.05$ was considered statistically significant.

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