Regulation of ATP-sensitive Potassium Channel Subunit Kir6.2 Expression in Rat Intestinal Insulin-producing Progenitor Cells*

We have reported that the combined expression of Pdx-1 (pancreatic duodenal homeobox 1) and Isl-1 (islet 1) enables immature rat enterocytes (IEC-6) to produce and release insulin. A key component regulating the release of insulin is the ATP-sensitive potassium channel subunit Kir6.2. To investigate the regulation of Kir6.2 gene expression, we assessed Kir6.2 expression in IEC-6 cells expressing Pdx-1 and/or Isl-1. We observed that Kir6.2 protein was expressed de novo in IEC-6 cells expressing both Pdx-1 and Isl-1 but not in cells expressing Pdx-1 alone. Next, we analyzed the regions of the Kir6.2 promoter (~1677 to ~45) by performing a luciferase assay and electromechanical mobility shift assay. The results have demonstrated that Kir6.2 promoter possesses two regions regulating the promoter activity: a Foxa2-binding site (~1364 to ~1210) and an Sp1/Sp3-binding site (~1035 to ~939). The additional expression of Isl-1 in IEC-6 cells expressing Pdx-1 attenuated overexpression of Foxa2 protein and enhanced Kir6.2 expression. Finally, knockdown of Isl-1 using the siRNA technique resulted in decreased expression of Kir6.2 protein in a rat pancreatic β-cell line (RIN-5F cells). These results indicate that expression of Kir6.2 in the rat intestine is modulated by Isl-1.

Among the many transcription factors, such as Pdx-1 (pancreatic duodenal homeobox 1) and Isl-1 (islet 1) enables immature rat enterocytes (IEC-6) to produce and release insulin. A key component regulating the release of insulin is the ATP-sensitive potassium channel subunit Kir6.2. To investigate the regulation of Kir6.2 gene expression, we assessed Kir6.2 expression in IEC-6 cells expressing Pdx-1 and/or Isl-1. We observed that Kir6.2 protein was expressed de novo in IEC-6 cells expressing both Pdx-1 and Isl-1 but not in cells expressing Pdx-1 alone. Next, we analyzed the regions of the Kir6.2 promoter (~1677 to ~45) by performing a luciferase assay and electromechanical mobility shift assay. The results have demonstrated that Kir6.2 promoter possesses two regions regulating the promoter activity: a Foxa2-binding site (~1364 to ~1210) and an Sp1/Sp3-binding site (~1035 to ~939). The additional expression of Isl-1 in IEC-6 cells expressing Pdx-1 attenuated overexpression of Foxa2 protein and enhanced Kir6.2 expression. Finally, knockdown of Isl-1 using the siRNA technique resulted in decreased expression of Kir6.2 protein in a rat pancreatic β-cell line (RIN-5F cells). These results indicate that expression of Kir6.2 in the rat intestine is modulated by Isl-1.

Among the many transcription factors, such as Pdx-1 (pancreatic duodenal homeobox 1), Isl-1 (islet 1), Nkx6.1 (NK6 transcription factor-related 1), Pax4 (paired box gene 4), Pax6 (paired box gene 6), and NeuroD (neuropenic differentiation 1) in the differentiation of the pancreatic endocrine cells (1–6), both Pdx-1 and Isl-1 are thought to be the master genes (7, 8). Indeed, we reported that the combined expression of Pdx-1 and Isl-1 caused immature rat enterocytes (IEC-6) (9) to produce and release insulin (10). In that study, we noted that the gene expressions of Gck (glucokinase), Glut2 (glucose transporter 2), and Kir6.2 (ATP-sensitive inward rectifier potassium channel subunit Kir6.2) are continuously expressed. These results prompted us to examine the mechanisms for the expressions of these proteins, which may regulate insulin release in response to glucose. A key component regulating the release of insulin is Kir6.2 (11, 12), which is expressed at particularly high levels in pancreatic β-cells (13). Briefly, an increase in ATP and a decrease in ADP stimulated by glucose metabolism depolarize the β-cells by closing Kir6.2. Membrane depolarization results in the opening of voltage-dependent Ca²⁺ channels and the influx of Ca²⁺ is the main trigger for insulin secretion (14, 15). Thus, insulin secretion in pancreatic β-cells is controlled through metabolic regulation of the electrical activity by Kir6.2 channels that control membrane potential. In this study, we observed no gene expression of Kir6.2 in IEC-6 cells transfected with Pdx-1 alone. However, additional expression of Isl-1 in IEC-6 cells expressing Pdx-1 induced Kir6.2 expression. These results indicate that Isl-1 plays an important role in expression of Kir6.2.

The Kir6.2 promoter contains several A-box motifs and forkhead/winged helix binding motifs, which are functionally important. Whereas both Pdx-1 and Isl-1 bind to TAAT sites in A-box, forkhead/winged helix proteins, which include the Foxa (forkhead box A) family of transcription factors encoded by the three genes Foxa1 (Hnf3α), Foxa2 (Hnf3β), and Foxa3 (Hnf3γ), bind to the core sequence (AAATA) and regulate hepatic and/or pancreatic gene expression (16–22). Thus, we hypothesized that these transcription factors might induce Kir6.2 expression. To prove our hypothesis, we used cell lines that are parental IEC-6 cells believed to be intestinal progenitor cells differentiating into insulin-producing cells (10) and RIN-5F cells as a pancreatic β-cell line. Through making a comparison between these cell lines, we discovered the transcriptional regulation of Kir6.2 gene expression by Isl-1 in these cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—IEC-6 cells, IEC-6 cells stably overexpressing Pdx-1 (IEC-Pdx cells) or both Isl-1 and Pdx-1 (IEC-1P cells), and rat pancreatic β-cell line (RIN-5F cells) were maintained in culture, as described elsewhere (23). Briefly, cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 5.6 or 22 mMd-glucose, 44 mM NaHCO3, 0.1 g/liter streptomycin, and 107 units/liter penicillin G in T-150 flasks. The cells were washed twice with phosphate-buffered saline before being used for biological analyses.

Preparation of Recombinant Adenoviruses—DNAs encoding for wild type mouse Isl-1 were subcloned into Adeno-X viral DNA vector (BD Biosciences Clontech) and cotransfected into 293 cells. Successful homologous recombination resulted in recombinant viruses encoding Isl-1 (Ad-Isl-1) and the control (Ad-LacZ). Viruses were amplified in 293 cells and used with centrifugation to avoid cell pellet formation. Plaque-forming units (pfu) were assayed using the Adeno-X rapid titrer kit (BD Biosciences Clontech).

Isl-1 RNA Interference Preparation—The double-stranded RNA nucleotides for Isl-1 (Isl-1-iRNA) were obtained from iGENE Therapeutics (Tsukuba, Japan) as follows: Isl-1-iRNA, sense (5'-GCAACUGGC-UUUUCAGAAGGA-AG-3') and antisense (5'-UCCUUCUGAAAA-

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‡ The abbreviations used are: IEC-6, immature rat enterocytes; IEC-Pdx cells, IEC-6 cells overexpressing Pdx-1; IEC-1P cells, IEC-6 cells overexpressing both Isl-1 and Pdx-1; SUR1, sulfonylurea receptor 1 SUR1; pfu, plaque-forming units; RT, reverse transcription; EMSA, electromechanical mobility shift assay.
Ist-1 Induces Kir6.2 Gene Expression

AUGUCCAGUUUGC-AU-3'; Is|l-1-iRNA-2, sense (5’-ACCCACGGAAAACUAUAUCCAG-AG-3’) and antisense (5’-UGGGAUUAUUGUUUGGUUUGGUUG-AU-3’).

Transfection was performed as described elsewhere (23). In brief, RIN-5F cells were transfected with 50 nM Is|l-1-iRNAs using the TransIT-TKO Transfection Reagent (Mirus, Madison, WI). After a 48-h incubation, the cells were washed twice with phosphate-buffered saline and subjected to molecular biological analysis.

**RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Methods**—Total RNA was extracted from IEC-6, IEC-Pd, IEC-IP, and RIN-5F cells as described elsewhere (24). The extracted product was used as a template in reactions containing reverse transcriptase (Invitrogen) and the oligo(dT) primer to synthesize the first strand of the cDNA. RT-PCR analysis was done as described in the literature (25). The first strand cDNA was used in subsequent PCR analysis, with the following oligonucleotide primers being employed for the indicated gene of interest: Glut2, 5’-TTAGCAACTGGGTT-CTGCAAT-3’ (sense primer) and 5’-GGTGTAGTCTCTACATCATG-3’ (antisense primer); Gck, 5’-GGTGTAGGAGGCTCAGGAA-3’ (sense primer) and 5’-GGTGAACGGCTCTGAG-3’ (antisense primer); Kir6.2, 5’-TCGGGAACACCGTTAAAGTG-3’ (sense primer) and 5’-GACCCGTTAACCTTCTAGT-3’ (antisense primer); SUR1, 5’-ACTGGTATGGTGGAACTCCTG-3’ (sense primer) and 5’-GCTTCACTACCATCGTCTGGA-3’ (antisense primer).

The thermal cycle profile was as follows: a single 1-min denaturing step at 94 °C was followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. The identities of the PCR products were confirmed by agarose gel electrophoresis and nucleotide sequence analysis.

**Western Blot Analysis**—Relative amounts of Is|l-1 and Kir6.2 proteins in the cells were assessed using Western blot analysis, as described elsewhere (25). Specifically, 20 μg of cell lysate was separated by standard SDS-PAGE and then transferred to a polyvinylidine difluoride membrane. Specific antibodies against Isl-1 (25), Foxa2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and Kir6.2 (Chemicom International, Temecula, CA) were used to probe the blot. Specific antibody against Nucleoporin P62 (BD Biosciences) was also used as a control.

The antibody-protein complex was detected using an enhanced chemiluminescence kit (PerkinElmer Life Sciences).

**Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)**—After preparation of nuclear extracts (23), we performed EMSA (24). In brief, the double-stranded probes were end-labeled with [γ-32P]ATP (Amersham Biosciences) using T4 kinase (TAKARA, Shiga, Japan). One μl of radiolabeled probe (activity 50,000–100,000 cpm/μl) was added to the mixture, and the samples were incubated for 20 min at room temperature. Three μg of nuclear protein was used for each reaction. For the cold competition experiments, a 50-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture. DNA-protein complexes were separated from unbound probe on native 4–6% polyacrylamide gels in 0.25 M Tris–HCl, pH 8.3, 10% glycerol, 0.5% acrylamide, and 0.05% SDS. The separated complexes were visualized by autoradiography and quantitated as described previously (24).

**Effects of Pdx-1 and/or Isl-1 on islet β-cell-specific transcripts.** A, mRNAs for Glut2, Gck, Kir6.2, and SUR1 were measured by RT-PCR in RIN-5F, IEC-6, IEC-Pd, and IEC-IP cells. Each experiment was repeated three times. B, whole-cell lysates were analyzed by Western blot analysis for Kir6.2 protein in IEC-6, IEC-Pd, IEC-IP, and RIN-5F cells. Each experiment was repeated three times. C, IEC-Pd cells were transfected with recombinant adenosine expressing either LacZ or Isl-1 at the indicated titration for 24 h. The transfected cells were incubated for 7 days. Whole-cell lysates were analyzed by Western blot analysis for Isl-1, Kir6.2, or Nucleoporin P62 protein. Each experiment was repeated three times. D, RIN-5F cells were transfected with control iRNA or Isl-1-iRNAs at 50 nM for 48 h. Whole-cell lysates were analyzed by Western blot analysis for Isl-1, Kir6.2, or Nucleoporin P62 protein.

Kir6.2-1.6-kb plasmid using the QuikChange site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) according to the manufacturer’s instructions. The mutated oligonucleotides are as follows: 5’-GGGGAATCCTGGCTTAAGAGACG-3’ (underline letters represent mutated nucleotides).

**Statistical Analysis**—Results are expressed as mean ± S.E., unless otherwise stated. Scheffé’s multiple comparison test was used to determine the significance of any differences among three or more groups, and the unpaired Student’s t test was used to determine the significance of any differences between two groups. p < 0.05 was considered significant.

**RESULTS**

**Is|l-1 Induces Expression of Kir6.2**—We have already reported that immature intestinal stem cells can differentiate into insulin-producing cells upon expression of the transcription factors Pdx-1 and Is|l-1 (10). The process of differentiation leading to the formation of pancreatic β-cells requires activation of specific genes for insulin secretion. We examined whether expression of these genes is observed in IEC-6 cells
protein content was also reduced to at least 50% (Fig. 1, lane 1), the expression of Kir6.2 decreased expression to 30% of the control, and the expression of Kir6.2, Isl-1-iRNAs reduced Isl-1 antibodies. As shown in Fig. 1D by Western blot analysis using both anti-Isl-1 and anti-Kir6.2 with Isl-1-iRNAs for 2 days, and whole-cell lysate was analyzed protein expression of Kir6.2. These cells were treated with Ad-Isl-1 (12.5 ρg of each of the several Kir6.2 5'-flanking fragments cloned upstream of the firefly luciferase reporter gene and 0.2 ρg of pTK Renilla luciferase vector or with 0.6 ρg of the promoterless pGL3 basic vector and 0.2 ρg of pTK Renilla luciferase vector. Results for the same procedure performed in RIN-5F cells. The results are presented as the mean ± S.E. of triplicate assays.

**Fig. 2.** Characterization of promoter activity of 5'-flanking region of Kir6.2. Nucleotide number 1 corresponds to the translation initiation site. A, IEC-Pd cells and IEC-IP cells were co-transfected either with 0.6 ρg of each of the several Kir6.2 5'-flanking fragments cloned upstream of the firefly luciferase reporter gene and 0.2 ρg of pTK Renilla luciferase vector or with 0.6 ρg of the promoterless pGL3 basic vector and 0.2 ρg of pTK Renilla luciferase vector. B, results for the same procedure performed in RIN-5F cells. The results are presented as the mean ± S.E. of triplicate assays.

**Relative Luciferase Activity (fold activation of control)**

* p < 0.01.

expressing Pdx-1 and/or Isl-1 by RT-PCR. IEC-6 cells expressing both Pdx-1 and Isl-1 (IEC-IP cells) clearly expressed de novo mRNAs of both Kir6.2 and Glut2 (Fig. 1A), whereas Gck was faintly expressed in these cells. mRNA expression of sulfonylurea receptor 1 (SUR1) was much stronger in these cells than in IEC cells expressing Pdx-1 alone (IEC-Pd) (Fig. 1A). RT-PCR product was verified as corresponding to each mRNA by nucleotide sequencing. To confirm expression of Kir6.2 protein, Western blot analysis was performed. As in RIN-5F cells, we observed a 45-kDa band of Kir6.2 (26) in IEC-IP cells but not in other cells (Fig. 1B). Next, to study the effect of Isl-1 on the expression of the Kir6.2 gene, IEC-Pd cells were transduced with Ad-Isl-1 (12.5 × 10⁸ pfu or 25 × 10⁸ pfu/ml), and expression of Kir6.2 was studied after 7-day infection. Consistent with our previous results (10), Isl-1 (43-kDa molecular mass) was identified. In this experiment, de novo expression of the Kir6.2 gene was found, whereas no signals were identified with Ad-LacZ (Fig. 1C). Identical results were observed in IEC-6 cells transduced with Ad-Isl-1 (data not shown). These studies clearly show that additional expression of Isl-1 in IEC-6 and IEC-IP cells is associated with de novo production of Kir6.2 protein. To investigate the effects of endogenous Isl-1 on expression of Kir6.2, we knocked down Isl-1 expression in RIN-5F cells, using a 25-nucleotide RNA duplex (Isl-1-iRNAs), and analyzed protein expression of Kir6.2. These cells were treated with Isl-1-iRNAs for 2 days, and whole-cell lysate was analyzed by Western blot analysis using both anti-Isl-1 and anti-Kir6.2 antibodies. As shown in Fig. 1D, Isl-1-iRNAs reduced Isl-1 expression to 30% of the control, and the expression of Kir6.2 protein content was also reduced to at least 50% (Fig. 1D, lanes 2 and 3). In contrast, no change was observed in nucleoporin P62 in the presence of Isl-1-iRNAs (Fig. 1D, lane 1). The decreased expression of Isl-1 thus causes a reduction of Kir6.2 protein content.

Luciferase Reporter Assay Using Kir6.2 Promoter—We cloned the 5'-flanking region of the mouse Kir6.2 gene (−1677/−45) and fused it to luciferase reporter gene to investigate the mechanism of the new expression of Kir6.2 in IEC-IP cells. First, we compared the promoter activity of IEC-Pd and IEC-IP cells. When we analyzed the promoter activities, the basal luciferase activities were adjusted by measurement of the activity obtained using pGL3-Basic vector alone in each cell line. Upon analysis of the regulatory regions of Kir6.2 promoter activity, the luciferase activity was significantly increased with a truncation from −1364 to −1210 in IEC-Pd cells, whereas no significant change was observed in IEC-IP cells (Fig. 2A). We also identified another regulatory element of Kir6.2 promoter, from −1035 to −939, in both IEC-Pd and IEC-IP cells, since promoter activities were significantly decreased with the truncation from −1035 to −939 in these cells. Thus, we found that Kir6.2 promoter contains two regions that regulate Kir6.2 promoter activity, one extending from −1364 to −1210 and the other from −1035 to −939 (Fig. 2A).

To confirm whether identical findings would be observed in pancreatic β-cells, we measured the promoter activity in RIN-5F cells, one of the rat pancreatic β-cell lines. As expected, the promoter activities in RIN-5F cells were similar to that in IEC-IP cells (Fig. 2B). Taken together, these results suggest that the 5'-flanking region of the mouse Kir6.2 gene (−1364/−1210) may work as a suppressive element in the absence of...
FIG. 3. Localization of the core regulatory region within the −1035−939 of the mouse Kir6.2 promoter. A, the region at −1035−939 of mouse Kir6.2 promoter was divided into the five fragments shown (i.e., 1 (−1035−1006), 2 (−1018−989), 3 (−1003−974), 4 (−985−956), and 5 (−969−940)). Potential transcription factor binding sites are underlined. B, 3 μg of nuclear extract of RIN-5F cells was incubated with each radiolabeled probe. To test specificity, lanes 2, 4, 6, 8, and 10 include a 50-fold molar excess of each oligonucleotide. C, gel shift competition analysis using fragment 4 wild type (4-WT) and its mutants was performed. Radiolabeled probe spanning the −985−956 region of the Kir6.2 promoter was incubated with 3 μg of nuclear extract of RIN-5F. Unlabeled mutant probes (m1, m2, and m3) were used as competitors in a 50-fold molar excess. D, nuclear extracts of RIN-5F were incubated with 2 μg of polyclonal Sp1, Sp3, and Foxa2 antibodies before the addition of the fragment 4 wild type radiolabeled probes.
Isl-1 expression. On the other hand, unknown transcription factor(s) that functions as an activator(s) may bind to the region (−1035 to −939) of the Kir6.2 promoter.

Identification of Nuclear Transcription Factors Binding to the Kir6.2 Gene Promoter GC-box (−985 to −956)—In order to identify which transcription factor(s) binds to the promoter region from −1035 to −939 of Kir6.2 promoter, EMSA was performed. The Kir6.2 promoter region from −1035 to −939 was divided into five fragments (Fig. 3A), and EMSA was performed using nuclear extract from RIN-5F cells. Two specific DNA-protein complexes (Fig. 3B, lane 7) were identified using oligonucleotides from −985 to −956 (probe 4). Corresponding cold oligonucleotides completely abrogated this complex formation (Fig. 3B, lane 8). Next, to confirm whether nuclear proteins from RIN-5F cells specifically bind to the Kir6.2 promoter from −985 to −956, we constructed three modifications (mut1, mut2, and mut3) of the core sequences of GC-box (−985 to −956) as a competitor (Fig. 3C). [γ-32P]ATP-labeled oligonucleotides of the Kir6.2 promoter from −985 to −956 formed a DNA-protein complex in the presence of wild type, mut1, or mut2 oligonucleotide as a competitor (Fig. 3C, lanes 3 and 4), whereas mut3 oligonucleotide was ineffective for use as a competitor (Fig. 3C, lane 5). Since this sequence contains some regions recognized by Maf-A, Pax4, and Pax6, which are all key transcription factors in the development of pancreatic β-cells (4, 27), we performed EMSA with anti-MafA, Pax4, and Pax6 antibodies. However, we failed to find any interaction (data not shown). It is well known that Sp family members contain conserved zinc finger DNA binding domains and bind to the GC-box of several genes (28). Next, we performed a supershift assay using anti-Sp1 and anti-Sp3 antibodies (Fig. 3D). In the supershift assay, the addition of anti-Sp1 antibody reduced the intensity of the Sp1 band and supershifted its complex (Fig. 3D, lane 3). When anti-Sp3 antibody was used, the Sp3 band completely disappeared, and its complex supershifted (Fig. 3D, lane 4). On the other hand, the addition of anti-Foxa2 antibody had no effect (Fig. 3D, lane 5). Identical results were obtained using nuclear extracts from IEC-6, IEC-Pd, and IEC-IP cells (data not shown).

To study the effects of Sp1 and Sp3 on the promoter activity of Kir6.2, we overexpressed each of these proteins in RIN-5F cells and measured the luciferase activities (Fig. 4A). We found that the promoter activity paralleled the expression level of Sp1, whereas overexpression of Sp3 decreased this activity in RIN-5F cells. Thus, Sp1 increases the promoter activities of Kir6.2, and Sp3 decreases it. Next, we measured luciferase activity using reporter vectors containing mutated regions of Kir6.2 promoter (Fig. 4B). In both IEC-IP and RIN-5F cells, the luciferase activity was 50% compared with the wild type promoter when investigated using mutant promoter. Taken together, these results demonstrate that both Sp1 and Sp3 bind to Kir6.2 promoter from −985 to −956 and regulate its activity.

Identification of Nuclear Transcription Factors Binding to the Kir6.2 Gene Promoter (−1364 to −1210)—Since both Sp1 and Sp3 are ubiquitously expressed in IEC-Pd, IEC-IP, and RIN-5F cells, there were no differences in Kir6.2 promoter activity (−985 to −956) among these cells (data not shown). Therefore, this element is not a region specifically associated with the effect of Isl-1. Thus, we hypothesized that the promoter region (−1364 to −1210) of the Kir6.2 gene is a crucial regulatory element affected by Isl-1 expression. To test this hypothesis, the Kir6.2 promoter (−1364 to −1210) was divided
into five fragments (Fig. 5A), and EMSA was performed using nuclear extracts from IEC-Pd cells (Fig. 5B). Two specific binding proteins were observed (Fig. 5B, lanes 3 and 5). The Kir6.2 promoter (−1364 to −1210) possesses several binding elements for Foxa2, which is one of the key transcription factors that regulate the Kir6.2 gene (29, 30). Thus, we performed supershift assay using [γ-32P]ATP-labeled oligonucleotides of the Kir6.2 promoter regions from −1338 to −1294 (probe 2) and from −1310 to −1266 (probe 3). As expected, when anti-Foxa2 antibody was added to the nuclear extracts from IEC-Pd cells, the complexes were supershifted (Fig. 5C, lanes 3 and 6). Similar findings were observed in IEC-6, IEC-IP, and RIN-5F cells (data not shown). Thus, Foxa2 binds to the Kir6.2 promoter region (−1364 to −1210) and might regulate expression of this gene.

Effects of Foxa2 on Kir6.2 Promoter (−1364 to −1210)—We demonstrated that Kir6.2 promoter activity does not change with the truncation of the promoter (−1364/−1210) in cells expressing Isl-1, such as IEC-IP and RIN-5F cells. Indeed, the activity increases with this truncation in IEC-Pd cells. To investigate its effect on Kir6.2 promoter activity, we measured the effect of Foxa2 on luciferase activity in RIN-5F cells (Fig. 6). We transfected RIN-5F cells with pGL3-Basic or pGL3-Kir6.2 reporter construct along with Foxa2 expression vector. We found that Foxa2 increased Kir6.2 promoter activity in a dose-dependent manner, in accordance with a previous report (29). However, Foxa2 similarly increased the basic promoter activity in a dose-dependent manner. Thus, to adjust the effect of Foxa2 on Kir6.2 promoter activity, we calculated the ratio between the luciferase activities of Kir6.2 promoter and promoterless reporter construct in the presence of the same dose of Foxa2 expression vector (Fig. 6A). The relative luciferase activity reciprocally decreased, to 50, 32, and 28% of basal level, in the presence of 0.4, 0.8, and 1.6 μg of Foxa2 vector, respectively.

Foa2 Protein Levels in the Presence of Isl-1—How does Isl-1 influence the expression of Foxa2? To study this issue, we measured the amount of Foxa2 protein by Western blot analysis in IEC-6, IEC-Pd, and IEC-IP cells. Our data showed that

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**Fig. 5. Localization of the core regulatory region within −1364/−1210 of mouse Kir6.2 promoter.** A, the −1364/−1210 region of the mouse Kir6.2 promoter was divided into the five fragments shown (i.e. 1 (−1366/−1322), 2 (−1338/−1294), 3 (−1310/−1266), 4 (−1282/−1239), and 5 (−1255/−1209)). Potential transcription factor binding sites are underlined. B, 3 μg of nuclear extract of IEC-Pd cells was incubated with each radiolabeled probe. To test specificity, lanes 2, 4, 6, 8, and 10 include a 50-fold molar excess of each oligonucleotide. C, 3 μg of nuclear extract of IEC-Pd cells was incubated with 2 μg of polyclonal Foxa2 antibodies before the addition of the indicated radiolabeled probes.
the amount of Foxa2 protein was lower in IEC-IP cells than in parent IEC-6 and IEC-Pd cells (Fig. 6D). Next, to identify the effect of Isl-1 on Foxa2 protein level, we transduced IEC-Pd cells with the Ad-Isl-1 virus. The cells were transduced with 12.5, 25, or 50 × 10⁶ pfu/ml of Ad-Isl-1 and harvested after 48 h of infection. The amount of Foxa2 protein was decreased to 38, 23, and 18% of basal level, respectively (Fig. 6C). These results indicate that Isl-1 attenuates overexpression of Foxa2 in intestinal progenitor cells.

**DISCUSSION**

Differentiation of pancreatic cells from progenitor cells requires the actions of both intrinsic and extrinsic influences, such as transcription and growth factors. In this study, we used the cell line IEC-6, derived from rat immature intestinal crypt cells (9), to test whether these cells can be induced to express Kir6.2. Our previous studies showed that when IEC-6 cells acquire the ability to express the transcription factor Pdx-1, they undergo differentiation into entero-endocrine cells (31). In IEC-Pd cells, NeuroD, Pax6, and Nkx6.1 were expressed but not Isl-1. After transfection with Isl-1 to IEC-6 cells expressing Pdx-1 (IEC-Pd cells), insulin release into the culture medium was observed. These previous data prompted us to investigate whether the specific regulatory factors for insulin secretion, such as Gck, Glut2, SUR1, and Kir6.2, would also be observed in IEC-IP cells. We detected the de novo expression of mRNAs of Kir6.2, SUR1, and Glut2. These results suggest that the additional expression of Isl-1 enables IEC-Pd cells to express Kir6.2, SUR1, and Glut2 through the regulation of their promoter activities.

In this study, focusing on the regulation of Kir6.2 gene expression, we found that additional expression of Isl-1 enabled de novo Kir6.2 gene expression through attenuating Foxa2 in IEC cells overexpressing Pdx-1. Both Pdx-1 and Isl-1 are homeobox proteins, which bind TAAT sites in the A-box, whereas Isl-1 is also a class of LIM/homeodomain transcription factors with important roles in determining cell lineage and pattern formation during differentiation to pancreas or brain cells (32). Since the LIM domain has been demonstrated to be a protein-protein interaction motif that is crucially involved in these processes, we suspect that the LIM domain of Isl-1 may regulate Foxa2 gene expression in both IEC-IP and RIN-5F cells.

Foxa2 encodes a transcription factor (forkhead box A2) that has been postulated to play a central role in β-cell development due to its ability to bind to the Kir6.2 promoter (29). Lantz et al. (29) demonstrated that Foxa2 deficiency resulted in excessive insulin release in response to amino acids and complete loss of glucose-stimulated insulin secretion. They also showed that co-transfection of Foxa2 expression vector resulted in stimulation of luciferase activity from the Kir6.2 promoter construct containing a 1.73-kb promoter sequence (−551+1186) and that Foxa2 acted as a transcriptional activator of Kir6.2 in β-cells. However, our results revealed that Foxa2 can serve as a suppressor in the Kir6.2 promoter from −1324 to −1149 (Fig. 3). To assess this discrepancy, we transfected pancreatic β-cells (RIN-5F cells) with Kir6.2 promoter/luciferase (pGL3-Kir6.2) or reporter constructs without the promoter (pGL3-Basic) along with a Foxa2 expression plasmid (Fig. 6A). Interestingly, more than 15-fold activation was observed with the transfection of both pGL3-Basic and Foxa2 vector (1.6 μg/plate), whereas only 7-fold activation was observed with the transfection of both pGL3-Kir6.2 and Foxa2. No significant activity was observed in these cells transfected with both pGL3-Basic and Foxa2 vector (data not shown) (i.e. Foxa2 enabled pGL3-Basic vector to activate luciferase activity by itself). Thus, the difference between the luciferase activities of pGL3-Basic and pGL3-Kir6.2 revealed that Foxa2 overexpression suppresses Kir6.2 luciferase activity. Lantz et al. (29) did not report the luciferase activity of promoterless vector with Foxa2 expression. Another possibility is that the discrepancy may arise from the nature of the cells studied. We used intestinal cells, such as IEC-6, IEC-Pd, and IEC-IP cells, which express intrinsic Foxa2 (Fig. 6B), whereas Lantz et al. transfected baby hamster kidney cells, which might not express intrinsic Foxa2, with Foxa2 expression vector; thus, the transfection with Foxa2 might have enabled induction of Kir6.2 gene activity in these cells. We need to study this discrepancy in more detail. Nevertheless, it is known that mRNA levels of both SUR1 and Kir6.2 are reduced by 75% in Foxa2 knockout mice (30). Furthermore, the requirement of Foxa2 for maintenance of Kir6.2 expression has already been confirmed by Northern blot analysis in insulinoma-1 cells overexpressing a dominant negative mutant Foxa2 (33). These results indicate that Foxa2 is an essential transcriptional regulator of Kir6.2 gene. Although the expression of Foxa2 in intestinal cells (IEC-IP cells) newly expressing Kir6.2 was lower than that in IEC-Pd or IEC-6 cells (Fig. 6B), this expression may be sufficient to enhance Kir6.2.
gene expression in intestinal cells. Moreover, we showed that IEC-Pd cells transduced with Ad-Isl-1 for 7 days newly expressed Kir6.2 protein by Western blot analysis (Fig. 2). At the same time, the overexpression of Isl-1 significantly reduced Foxa2 protein level (Fig. 6C), suggesting that weak Foxa2 expression may be enough to regulate Kir6.2 gene expression.

Kir6.2 promoter does not contain any putative TATA or CCAAT box, but it has a GC-box that is a putative binding site for the Sp family of transcription factors (34, 35). Sp1 is a sequence-specific ubiquitously expressed zinc finger transcription factor that supports constitutive basal expression of a variety of eukaryotic genes that lack a functional TATA box. The Sp family is one of the most studied transcription factors. Five other, Sp1-related proteins, Sp2–Sp6, have been described (36). Consistent with the previous data (37), EMSA showed that full-length and truncated forms of Sp3 and Sp1 can interact specifically with the GC-box in the Kir6.2 promoter. Sp3 slightly suppresses Kir6.2 promoter activity, whereas Sp1 stimulates that activity in a dose-dependent manner. Additionally, we also confirmed that there was no difference in Sp1 and Sp3 concentrations among these cells, suggesting that Isl-1 did not influence Sp1 and Sp3 expression (data not shown). Our results suggest that Sp1 is an important mediator of the basal transcriptional activity of the Kir6.2 gene.

This is the first demonstration that the expression of Isl-1 activates Kir6.2 gene expression in rat intestinal progenitor cell lines. Our data demonstrate that the expression of Pdx-1 is not sufficient for activation of Kir6.2 gene expression in these cells, although it may activate insulin, Gck, and pancreatic polypeptide promoter (38). The gut constitutes a potentially interesting tissue for gene therapy for targeting the gut epithelium to enhance production of both insulin and Kir6.2 for potential application in new therapies for diabetes.

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