Abstract

Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP/G6PC2) is a major autoantigen in both mouse and human type 1 diabetes. IGRP is selectively expressed in islet β cells and polymorphisms in the IGRP gene have recently been associated with variations in fasting blood glucose levels and cardiovascular-associated mortality in humans. Chromatin immunoprecipitation (ChiP) assays have shown that the IGRP promoter binds the islet-enriched transcription factors Pax-6 and BETa2. We show here, again using ChiP assays, that the IGRP promoter also binds the islet-enriched transcription factors MafA and Foxa2. Single binding sites for these factors were identified in the proximal IGRP promoter, mutation of which resulted in decreased IGRP fusion gene expression in βTC-3, Hamster insulinoma tumor (HIT), and Min6 cells. ChiP assays have shown that the islet-enriched transcription factor Pdx-1 also binds the IGRP promoter, but mutational analysis of four Pdx-1 binding sites in the proximal IGRP promoter revealed surprisingly little effect of Pdx-1 binding on IGRP fusion gene expression in βTC-3 cells. In contrast, in both HIT and Min6 cells mutation of these four Pdx-1 binding sites resulted in a ~50% reduction in fusion gene expression. These data suggest that the same group of islet-enriched transcription factors, namely Pdx-1, Pax-6, MafA, BETa2, and Foxa2, directly or indirectly regulate expression of the two major autoantigens in type 1 diabetes.

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Introduction

The glucose-6-phosphatase catalytic subunit (G6Pase) is predominantly expressed in liver and kidney and plays a major role in glucose homeostasis by catalyzing the final step in the glycolenolitic and gluconeogenic pathways, namely the hydrolysis of glucose-6-phosphatase to glucose and inorganic phosphate (Van Schaftingen & Gerin 2002). Two G6Pase-related proteins have recently been identified designated the islet-specific G6Pase-related protein (IGRP; Arden et al. 1999, Martin et al. 2001, Wang et al. 2007b) and the ubiquitously expressed G6Pase-related protein (UGRP), also known as G6Pase-β (Guionie et al. 2003, Shieh et al. 2003). The encoded proteins exhibit ~50% sequence identity, exhibit a common nine transmembrane domain topology and are all localized to the endoplasmic reticulum (Martin et al. 2002, Boustead et al. 2004).

UGRP confers low glucose-6-phosphatase activity to a broad range of tissues (Wang et al. 2006, Cheung et al. 2007), whereas the function of IGRP is less certain. IGRP is exclusively expressed in pancreatic islets, principally in β cells (Hutton & Eisenbarth 2003) and is a candidate for the low glucose-6-phosphatase enzyme activity detected in this tissue (Arden et al. 1999, Wang et al. 2007b). A global knockout of the IGRP gene results in a mild metabolic phenotype characterized by a ~15% decrease in fasting blood glucose (Wang et al. 2007b). This observation suggests that IGRP together with glucokinase could create a substrate cycle and therefore modulate β cell glycolytic flux and glucose-stimulated insulin secretion (Newgard et al. 2002). Indeed, recent genetic data also suggest that variations in IGRP expression and/or activity are linked to fasting blood glucose levels and hence cardiovascular-associated mortality in humans (Fradin et al. 2007, Bouatia-Naji et al. 2008, Chen et al. 2008). Unfortunately, attempts to directly demonstrate glucose-6-phosphatase hydrolysis by IGRP in vitro have been mostly unsuccessful (Arden et al. 1999, Martin et al. 2001, Petrolonis et al. 2004, Shieh et al. 2005), raising the question as to whether IGRP requires other cellular factors to exhibit activity or whether it has an unknown catalytic function.

Although its biological function is unclear, several reports have demonstrated that IGRP is an important target of cell-mediated autoimmunity in type 1 diabetes both in mice (Lieberman et al. 2003, Han et al. 2005a,b, Wong et al. 2006) and humans (Yang et al. 2006). In the non-obese diabetic (NOD) mouse model of type 1 diabetes, IGRP is recognized by both CD8 (Lieberman et al. 2003) and CD4 positive (Mukherjee et al. 2005)
Materials and methods

Materials

[\( ^{32}\text{P} \)dATP (\( >3000\) Ci/mmol) and \([^3\text{H}]\) acetic acid, sodium salt (\( >10\) Ci/mmol) were obtained from Amersham and ICN respectively. Antisera raised against Foxa2 (HNF-3\( \beta \); sc-65540) and c-Maf (sc-7866) were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Antisera specific for MafA was a generous gift from Roland Stein (Matsuoka et al. 2003).

Fusion gene plasmid construction

The construction of mouse IGRP-chloramphenicol acetyltransferase (CAT) fusion genes, containing the wild-type promoter sequence from \(-306\) to \(+3\), or the same sequence with site-directed mutations (SDMs) of four Pdx-1 binding sites, designated IGRP Quad SDM, has been previously described (Ebert et al. 1999, Martin et al. 2004). A three-step PCR strategy (Martin et al. 2004) was used to create SDMs of the Foxa2 and MafA binding sites in the IGRP promoter. The resulting constructs, designated IGRP Foxa SDM and IGRP Maf SDM, were generated within the context of the \(-306\) to \(+3\) IGRP promoter fragment. The sequences of the sense strand oligonucleotides used for mutagenesis were as follows (mutated nucleotides in bold lowercase): for IGRP Foxa SDM: 5'-TATGAAAAATGGCAAC-gAAACACGATCCAACT-3'. For IGRP Maf SDM: 5'-AAATTGCCAGGTACATCATAGTCGCCACCTC-3'.

Cell culture, transient transfection, CAT, and luciferase assays

Hamster insulinoma tumor (HIT) and \( \beta \)-TC-3 cells were grown in DMEM containing 2-5% (vol/vol) fetal bovine serum and 15% (vol/vol) horse serum, whereas Min6 cells were grown in DMEM containing 15% (vol/vol) fetal bovine serum and HeLa cells were grown in DMEM containing 10% (vol/vol) bovine serum. Cells were transfected using lipofectamine as previously described (Martin et al. 2002). The pcDNA3-Pax6, pCMV4-Pdx-1, pcDNA3-BETA2, pcDNA3-MafA, and pcDNA3-E47 expression vectors were generous gifts from Dr Roland Stein (Qiu et al. 2002, Zhao et al. 2005). The pcDNA3-Foxa2 expression vector has been previously described (Vander Kooi et al. 2005). CAT, luciferase, and protein assays were all performed as previously described (Martin et al. 2004, Vander Kooi et al. 2005).

Gel retardation assay

Labeled probes

Sense and antisense oligonucleotides representing wild-type or mutant Foxa or MafA binding sites...
(see Table 1) were synthesized with BamH I compatible ends and subsequently gel purified, annealed, and labeled with \([\gamma^{32}P]\)dATP using the Klenow fragment of Escherichia coli DNA polymerase I to a specific activity of \(\sim 2.5\ \mu\text{Ci}/\text{pmol}\) (Sambrook et al. 1989).

**Nuclear extract preparation**

The preparation of \(\beta\)TC-3 nuclear extracts through extraction of nuclei with 800 mM (high salt) or 200 mM (low salt) NaCl was as previously described (Martin et al. 2004).

**Binding assays**

Approximately 14 fmol radiolabeled probe (\(\sim 50,000\ \text{c.p.m.}\)) was incubated with the indicated nuclear extract in a final 20 \(\mu\text{l}\) reaction volume. Foxa2 binding reactions contained 1-5 \(\mu\text{g}\) high salt nuclear extract, 20 mM HEPES (pH 7-9), 0-1 mM EDTA, 0-1 mM EGTA, 10% glycerol (v/v), 1 \(\mu\text{M}\) dithiothreitol, 1 \(\mu\text{M}\) poly(const(d-l-dc)) poly(d-l-dc), and 100 mM KCl. MafA binding reactions contained 3-0 \(\mu\text{g}\) low salt nuclear extract, 20 mM HEPES (pH 7-9), 0-1 mM EDTA, 1 mM EGTA, 12-5% glycerol (v/v), 1 \(\mu\text{M}\) dithiothreitol, 1 \(\mu\text{g}\) poly(const(d-l-dc)) poly(d-l-dc), 0-375 \text{mM spermidine}, 0-075 \text{mM spermine}, 100 mM NaCl, and 5 mM MgCl\(_2\). After incubation at room temperature for 20 min, samples were loaded on to a 6\% polyacrylamide gel containing 1 \(\times\) TGE (25 mM Tris Base, 190 mM glycine, 1 mM EDTA), and 2-5% (v/v) glycerol. Samples were electrophoresed for 1.5 h at 100 \text{mA}\) in 1 \(\times\) TGE buffer before the gel was dried and exposed to Kodak X-ray film with intensifying screens. Competition and supershift experiments were performed as previously described (Martin et al. 2004).

**Table 1 Oligonucleotides used in gel retardation studies**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>IGRP Foxa WT</td>
<td>TGGACGACGGAGGGTACGTCACGATCGTCC -221</td>
</tr>
<tr>
<td>IGRP Foxa MUT</td>
<td>TGGACGACGGAGGGTACGTCACGATCGTCC -221</td>
</tr>
<tr>
<td>Foxa Consensus</td>
<td>AAGCCAACATTTGATAATCAGC</td>
</tr>
<tr>
<td>IGRP Maf WT</td>
<td>CCTTAATGCTGCAAGGCTCACTGATCACTG -157</td>
</tr>
<tr>
<td>IGRP Maf MUT</td>
<td>CCTTAATGCTGCAAGGCTCACTGATCACTG -157</td>
</tr>
<tr>
<td>Maf Consensus 1</td>
<td>CTGGAGCTGACACAA</td>
</tr>
<tr>
<td>Ins C1 WT</td>
<td>TGGACGACTGACAACTGCACTGCACTG -101</td>
</tr>
<tr>
<td>Maf Consensus 2</td>
<td>CTGGAGCTGACACAA</td>
</tr>
</tbody>
</table>

The sense strand sequence of the wild-type (WT) and mutant (MUT) oligonucleotides used in these studies are shown. The Foxa and Maf binding motifs are boxed and the altered bps in the mutant IGRP sequence are shown in bold lower case letters. The consensus Foxa binding motif is taken from Overdier et al. 1994 and the two consensus Maf binding motifs are taken from Kataoka et al. 1994.

**ChIP assay**

ChIP assays were performed as previously described (Martin et al. 2004) using 10 \(\mu\text{g}\) of each antiserum.

**Statistical analysis**

The transfection data were analyzed for differences from the control values, as specified in the figure legends. Statistical comparisons were calculated using an unpaired Student’s \(t\) test. The level of significance was \(P<0.05\) (two-sided test).

**Results**

MafA binds to the IGRP promoter in vitro

To test the possibility that a C1 element-like sequence in the IGRP promoter interacts with MafA, gel retardation analyses were performed. When a labeled oligonucleotide, designated IGRP Maf WT (Table 1), representing the mouse IGRP sequence between \(-186\) and \(-157\), was incubated with crude \(\beta\)TC-3 nuclear extract, two major protein–DNA complexes, designated A and B, were detected (Fig. 1). To identify the factor(s) present in complexes A and B, gel retardation assays were performed in which \(\beta\)TC-3 cell nuclear extract was pre-incubated with specific antisera. Previous gel retardation experiments had demonstrated Pdx1 binding to an oligonucleotide, designated Site 2, representing the \(-200\) to \(-173\) IGRP promoter sequence (Martin et al. 2004). Since this region partially overlaps the \(-186\) and \(-157\) sequence encompassed by the IGRP Maf WT probe, it was likely that either complex A or B represented Pdx1 binding. Figure 1 shows that the addition of c-Maf antibody selectively altered the mobility of complex B, resulting in the appearance of a supershifted complex, while complex A was unaffected.

Various antisera were tested to definitively determine whether MafA or a related protein was present in complex A. One of these antisera was raised to the c-Maf protein, which, like MafA, is a member of the large Maf family of transcription factors (Aramata et al. 2007). This antiserum was raised to a portion of the c-Maf protein common to all the members of this family and as such is able to cross react with other large Mafs. Figure 1 shows that the addition of c-Maf antibody specifically altered the mobility of complex A, leading to the appearance of a supershifted complex. While this result indicated the binding of a large Maf protein, an antiserum that selectively recognizes MafA (Matsuoka et al. 2003) was used to address the specific presence of this protein. Similar to the effects of the c-Maf
antiserum, the MafA antiserum specifically altered the mobility of complex A, leading to the appearance of a supershifted complex. These results strongly suggest that MafA can interact with the $K_{186}/K_{157}$ region in the IGRP promoter in vitro.

Competition experiments, in which a varying molar excess of unlabeled DNA was included with the labeled probe, were used to compare the affinity of MafA binding with the $-186/-157$ IGRP promoter region and the rat insulin II C1 element. Figure 2A shows that both the IGRP Maf WT oligonucleotide and an oligonucleotide representing the rat insulin II C1 element competed effectively for the formation of the MafA–DNA complex. However, quantitation of the results of several experiments showed that MafA binds the $-186/-157$ IGRP promoter region with approximately five to tenfold higher affinity than the rat insulin II C1 element (Fig. 2B). By contrast, we have previously shown that Pax-6 binds the rat insulin I C2 element with an approximately fivefold higher affinity than the $-270/-246$ Pax-6 binding site in the IGRP promoter (Martin et al. 2004).

**Figure 1** MafA and Pdx1 bind the $-186/-157$ IGRP promoter region in vitro. βTC-3 nuclear extract was incubated in the absence (−) or presence of the indicated anti-serum for 10 min on ice. A labeled oligonucleotide representing the wild-type $-186/-157$ IGRP promoter region (Maf WT; Table 1) was then added and incubation continued for 20 min at room temperature. Protein binding was then analyzed using the gel retardation assay as described in Materials and methods. In the representative autoradiograph shown, only the retarded complexes are visible and not the free probe, which was present in excess.

**Figure 2** Comparison of MafA binding to the $-186/-157$ IGRP promoter region and rat insulin II promoter C1 element in vitro. (A) A labeled oligonucleotide representing the wild-type $-186/-157$ IGRP promoter region (IGRP Maf WT; Table 1) was incubated in the absence (−) or presence of the indicated molar excess of the unlabeled IGRP MafA WT or Ins C1 WT oligonucleotide (Table 1) competitors prior to the addition of βTC-3 cell nuclear extract. Protein binding was then analyzed using the gel retardation assay as described in Materials and methods. In the representative autoradiograph shown only the retarded complexes are visible and not the free probe, which was present in excess. The MafA and Pdx1 complexes are indicated (see Fig. 1). (B) Protein binding was quantified by using a Packard Instant Imager to count $^{32}$P associated with the retarded complex. The data represent the mean ± S.E.M. of three experiments.

**MafA binding is required for maximal IGRP promoter activity**

Determining the contribution of MafA binding to IGRP promoter activity required the identification of a mutation that would specifically affect the binding of MafA while leaving Pdx1 binding to the adjacent promoter sequence unaltered. Gel retardation assays were used to identify such a mutant. It was previously established that mutation of the dinucleotide AG pair at the 3′ end of the insulin C1 element disrupts MafA binding (Harrington & Sharma 2001). Figure 3 shows that while a 100-fold molar excess of the unlabeled IGRP Maf WT oligonucleotide competed effectively for the formation of the MafA–DNA complex an oligonucleotide, designated IGRP Maf MUT, which contains a mutation in the analogous dinucleotide AG pair in the IGRP Maf element (Table 1), failed to compete with the labeled probe for formation of the MafA–DNA complex. Both the IGRP Maf WT and MUT oligonucleotides compete equally effectively for formation of the Pdx1–DNA complex (Fig. 3). This indicates that the AG mutation specifically disrupts MafA and not Pdx-1 binding.

To investigate the functional significance of MafA binding, the AG dinucleotide was mutated, using site-directed mutagenesis, in the context of the $-306/+3$ IGRP promoter region. A fusion gene containing this mutation, designated IGRP Maf SDM, was then analyzed by transient transfection of βTC-3, HIT, and Min6 cells. Figure 4A and B show that mutation of the MafA binding site resulted in an $\sim 25–50\%$ reduction in the level of reporter gene expression in βTC-3 cells as compared with that directed by the wild-type $-306/+3$.
promoter. The magnitude of the effect of the MafA binding site mutation varied between βTC-3 cells that had been cultured for different passages (Fig. 4A and B). Figure 4C and D show that mutation of the MafA binding site resulted in an ~50% reduction in the level of reporter gene expression in both HIT and Min6 cells respectively. These results demonstrate that MafA is important for IGRP promoter activity in multiple islet-derived cell lines.

**Fossa2 binds to the IGRP promoter in vitro**

We next examined Foxa2 binding to a previously identified site in the IGRP promoter (Ref. (Bischof et al. 2001); Table 1). When a labeled oligonucleotide, designated IGRP Foxa WT (Table 1), representing the mouse IGRP sequence between −246 and −221, was incubated with crude βTC-3 nuclear extract, two major protein–DNA complexes, designated X and Y, were detected (Fig. 5A). To identify the factor(s) present in complexes X and Y, gel retardation assays were performed in which βTC-3 cell nuclear extract was pre-incubated with specific antisera. Figure 5A shows that the addition of a control antiserum (IgG) had no effect on the mobility of complex X or Y, whereas addition of antiserum recognizing Foxa2 specifically altered the mobility of complex Y leading to the appearance of a supershifted complex, while complex X was unaffected.

The specificity of this interaction was examined by testing the ability of Foxa2 to bind to an altered version of this element that contained two point mutations at positions implicated in protein binding by the in situ footprinting technique (Bischof et al. 2001); Table 1). Figure 5B shows that while a 100-fold molar excess of the unlabeled IGRP Foxa WT oligonucleotide competed effectively for the formation of both complexes X and Y, an oligonucleotide, designated IGRP Foxa MUT, which contains a mutation in the two bases implicated in protein binding by the in situ footprinting (Bischof et al. 2001); Table 1), failed to compete with the labeled probe for formation of the Foxa2–DNA complex (complex Y) while competition for formation of complex X was unaffected. This indicates that the mutation specifically disrupts Foxa2 and that complex X either represents a nonspecific interaction or binding to another region of the probe.

A similar competition analysis previously indicated that these mutations only partially disrupt Foxa2 binding (Bischof et al. 2001). Subsequently, however, it was discovered that the formation of a non-specific complex that co-migrates with the Foxa2 complex had complicated the interpretation of this initial result. In the experiment shown in Fig. 5 modified gel retardation conditions were used which did not allow the formation of this non-specific complex, making it possible to demonstrate that the described mutations are more disruptive than previously thought (Bischof et al. 2001).

**Fossa2 binding is required for maximal IGRP promoter activity**

To investigate the functional significance of these observations, the Foxa2 binding site was mutated using site-directed mutagenesis in the context of the −306 to +3 IGRP promoter region. A fusion gene containing this mutation, designated IGRP Foxa2 SDM, which is identical to that described in the Foxa MUT oligonucleotide, was then analyzed by transient transfection of TC3, HIT, and Min6 cells. Figure 6A and B show that mutation of the Foxa2 binding site resulted in an ~25–75% reduction in the level of reporter gene expression in βTC-3 cells as compared with that directed by the wild-type −306/+3 promoter. As seen with the effect of the MafA binding site mutation, the magnitude of the effect of the Foxa2 binding site mutation varied between βTC-3 cells that had been cultured for different passages (Fig. 6A and B). Figure 6C and D show that mutation of the Foxa2 binding site resulted in an ~75% reduction in the level of reporter gene expression in both HIT and Min6 cells respectively. These results demonstrate that Foxa2 is important for IGRP promoter activity in multiple islet-derived cell lines.
**Foa2 and a Maf family member interact with the IGRP promoter in situ**

The gel retardation studies demonstrate that MafA and Foxa2 are capable of specifically binding to the IGRP promoter in vitro. To determine whether these interactions occur on the IGRP promoter inside intact cells, a ChIP assay was performed. TC-3 cells were treated with formaldehyde to preserve DNA–protein interactions and chromatin was subsequently isolated from these cells. The isolated chromatin was subjected to sonication to produce genomic fragments that were ~500 bps in size and this material served as the input for immunoprecipitation experiments. Antisera raised to c-Maf and Foxa2, the same antibodies described in the gel retardation experiments, were incubated with the chromatin and the resulting immune complexes were precipitated by centrifugation. The presence of the IGRP promoter was then assessed by isolating genomic DNA from the immunopellets and performing a PCR with primers that specifically recognize the IGRP promoter. Figure 7 shows that the IGRP promoter is clearly enriched in the c-Maf and Foxa2

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**Figure 4** Disruption of MafA binding reduces IGRP promoter activity. Two separate batches of (A and B) βTC-3 cells, (C) HIT cells, and (D) Min6 cells were transiently co-transfected, as described in Materials and methods, using a lipofectamine solution containing various IGRP–CAT fusion genes (2 µg) and an expression vector encoding firefly luciferase (0.5 µg). The IGRP–CAT fusion genes represented either the wild-type promoter sequence, located between −306 and +3 (IGRP WT), or the same sequence with a site-directed mutation (SDM) in the Maf A binding site (IGRP Maf SDM). The mutation was identical to that used in the gel retardation analysis (Fig.3). Following transfection, cells were incubated for 18–20 h in serum-containing medium. The cells were then harvested and both CAT and luciferase activity was assayed as described in Materials and methods. Results are presented as the ratio of CAT: firefly luciferase activity, expressed as a percentage relative to the value obtained with the IGRP WT fusion gene, and represent the mean of three experiments ± S.E.M., each using an independent preparation of each fusion gene plasmid, assayed in triplicate. *P<0.05 versus IGRP WT.
immunopellets compared with the IgG control. A similar result was seen using the MafA-specific antibody (data not shown). To test the specificity of this interaction, the immunopellets were also analyzed for the presence of a target not predicted to associate with either of these transcription factors. In this instance, the control target chosen was IGRP exon 5, which is ~6 kbps downstream from the transcription start site. As expected, the PCR analyses show that this genomic region is not enriched in the various immunopellets (Fig. 7), confirming that c-Maf and Foxa2 associate specifically with the IGRP promoter.

In summary, the ChIP experiments extend the genomic region is not enriched in the various immunopellets (Fig. 7), confirming that c-Maf and Foxa2 associate specifically with the IGRP promoter. In summary, the ChIP experiments extend the in vitro binding studies by demonstrating that a Maf family member and Foxa2 associate with the IGRP promoter inside intact cells.

Pdx1 binding is required for maximal IGRP promoter activity in HIT and Min6 cells

Previously published data showed that mutation of four Pdx1 binding sites in the IGRP promoter had little effect on IGRP fusion gene expression in βTC-3 cells (Martin et al. 2004). However, ChIP assays show that this factor associates with the IGRP promoter in both βTC-3 (Martin et al. 2004) and NIT-1 (Keller et al. 2007) cells. While MafA and Foxa2 binding are important for IGRP promoter activity in βTC-3, HIT, and Min6 cells, we investigated whether Pdx1 binding was selectively important for IGRP promoter activity in HIT and Min6 cells. A fusion gene containing a mutation of all four Pdx1 binding sites, designated IGRP Quad SDM, was analyzed by transient transfection. Figure 8 shows that mutation of the Pdx1 binding sites resulted in an ~50% reduction in the level of reporter gene expression as compared with that directed by the wild-type −306/+3 promoter in HIT and Min6 cells, respectively, whereas this mutation has little effect in βTC-3 cells.

Islet-enriched factors synergistically induce IGRP fusion gene expression

Previously published data and that shown in Figs 1–8 demonstrate that Pax6, BETA2, Foxa2, MafA, and Pdx1 are important for IGRP gene expression. Co-transfection experiments were performed in cervix-derived HeLa cells, in which neither of these islet-enriched transcription factors nor the IGRP gene are likely to be endogenously expressed. Several other groups have previously used this strategy to investigate the mechanism by which islet-enriched factors activate gene expression (Qiu et al. 2002, Docherty et al. 2005). Expression vectors for Pax6, Pdx1, Foxa2, MafA, BETA2, and E47, a ubiquitously expressed binding partner for BETA2 (Qiu et al. 2002), were co-transfected with various −306/+3 IGRP fusion genes. Since cryptic transcription factor binding sites have been reported in the commonly used pGL2 and pGL3 luciferase vectors (Thirunavukkarasu et al. 2000), we first compared fusion genes in which the −306/+3 IGRP promoter fragment was ligated into either the pGL2, pGL3 or pCAT (An) vectors. Supplemental Fig. 1, which can be viewed online at http://jme.endocrinology-journals.org/content/vol41/issue5/, shows that co-transfection with the transcription factor expression vectors strongly induced pGL2 and pGL3 expression, regardless of the presence of the IGRP promoter. By contrast, there was no effect on CAT expression in the absence of the IGRP promoter. The −306/+3 IGRP–CAT fusion gene was therefore used in subsequent experiments.

Figure 9A shows that, individually, with the exception of BETA2, none of these transcription factors was able to fully activate the IGRP–CAT reporter gene. However, when co-transfected, all of these factors result in at least a doubling of expression compared with each other (Supporting Fig. 1). In addition, we found that co-transfection of MafA and Foxa2 resulted in a higher expression than either alone (Supporting Fig. 1).
factors markedly induced IGRP fusion gene expression, whereas together they acted synergistically to strongly induce expression. We next investigated the individual contributions of each factor to this synergistic induction by assessing the effect of subtracting each one on the induction of IGRP fusion gene expression. The individual absence of Pdx1, Pax6, BETα2, and MafA resulted in a marked reduction in IGRP fusion gene expression (Fig. 9B).

This result confirms the synergistic nature of the interactions between Pax6, BETα2, MafA, and Pdx1. Surprisingly, the absence of Foxa2 resulted in increased expression (Fig. 9B). This suggests that, for reasons that are unclear, Foxa2 appears to repress IGRP fusion gene expression in HeLa cells, even though mutation of the Foxa2 binding site in the IGRP promoter clearly results in reduced expression in βTC-3, Min6, and HIT cells (Fig. 6).

Figure 6 Disruption of Foxa2 binding reduces IGRP promoter activity. Two separate batches of (A and B) βTC-3 cells, (C) HIT cells and (D) Min6 cells were transiently co-transfected, as described in Materials and methods, using a lipofectamine solution containing various IGRP–CAT fusion genes (2 μg) and an expression vector encoding firefly luciferase (0.5 μg). The IGRP–CAT fusion genes represented either the wild-type promoter sequence, located between −306 and +3 (IGRP WT), or the same sequence with a site-directed mutation (SDM) in the Foxa2 binding site (IGRP Foxa SDM). The mutation was identical to that used in the gel retardation analysis (Fig. 4B). Following transfection, cells were incubated for 18–20 h in serum-containing medium. The cells were then harvested and both CAT and luciferase activities were assayed as described in Materials and methods. Results are presented as the ratio of CAT: firefly luciferase activity, expressed as a percentage relative to the value obtained with the −306 WT fusion gene, and represent the mean of three experiments ± S.E.M., each using an independent preparation of each fusion gene plasmid, assayed in triplicate. *P<0.05 versus IGRP WT.
Pdx1 does not directly regulate IGRP fusion gene expression in HeLa cells

While Pdx1 acts synergistically with Pax6, MafA, BETA2, and E47 to induce expression of the wild-type K306/C3 IGRP fusion gene (Fig. 10A), surprisingly this induction was not impaired comparing the wild-type fusion gene with the K306/C3 IGRP Quad SDM fusion gene, which contains combined mutations of all four Pdx1 binding sites (Fig. 10B). This result suggested that perhaps Pdx1 stimulated IGRP fusion gene expression through a DNA binding-independent mechanism in HeLa cells. However, Fig. 10C shows that this is also not the case because the induction of K306/+3 IGRP Quad SDM fusion gene expression is not dependent on Pdx1. Taken together, these data indicate that Pdx1 must be stimulating wild-type K306/+3 IGRP fusion gene expression in HeLa cells by displacing an endogenous repressor rather than directly stimulating expression itself. This observation reveals a limitation in the analysis of islet-enriched transcription factors in non-islet derived cell lines and highlights the importance of control experiments to demonstrate the involvement of DNA binding.
Discussion

We have been interested in understanding the molecular mechanisms that determine the islet-specific expression of the IGRP gene (Wang et al. 2008). A 306 bp IGRP promoter region upstream of the transcription start site is sufficient to drive expression of reporter genes specifically in cell-derived cell lines in situ and newborn pancreatic β islets in vivo (Frigeri et al. 2004). The results of a 5′ deletion analysis, coupled with in situ footprinting, indicated that multiple cis-acting elements within this 306 bp region contribute to IGRP promoter activity (Bischof et al. 2001). Subsequent studies demonstrated the importance of two conserved E-Box motifs (Martin et al. 2003) and two non-consensus Pax6 binding sites (Martin et al. 2004) for IGRP promoter activity. The results presented here show that the IGRP promoter also binds the islet-enriched transcription factors Foxa2 and MafA in intact cells (Fig. 7) and that mutation of the binding sites for MafA (Figs 1–3) and Foxa2 (Fig. 5) identified in the proximal IGRP promoter results in decreased IGRP fusion gene expression in βTC-3, HIT, and Min6 cells (Figs 4 and 6). In addition, while mutation of the four Pdx1 binding sites in the IGRP promoter had little effect on fusion gene expression in βTC-3 cells, it resulted in reduced expression in both HIT and Min6 cells (Fig. 8). Why Pdx1 binding would be selectively important for IGRP fusion gene expression in HIT and Min6 but not βTC-3 cells is currently unknown. However, these results suggest that the same islet-enriched transcription factors regulate, directly or indirectly, the promoter activity of the two major autoantigens in type 1 diabetes, namely insulin and IGRP.

The Foxa proteins, of which there are three isoforms Foxa1-3, formerly designated HNF-3α, β, and γ, have previously been studied in the context of the pancreas. Promoter analyses, for example, have implicated Foxa1 in the regulation of glucagon gene expression and, accordingly, mice that are deficient in this factor exhibit impaired glucagon synthesis and are hypoglycemic (Kaestner et al. 1999, Gauthier et al. 2002). Similar types of analyses have shown that Foxa2 regulates the expression of Pdx1 (Wu et al. 1997, Samaras et al. 2002). Because Foxa2 is required for the formation of the foregut endoderm, a global knockout approach could not be used to assess the role of this transcription factor in pancreas function (Ang & Rossant 1994). To circumvent these early embryonic defects, a conditional allele was introduced into mice that allowed the inactivation of Foxa2 later in development and specifically in pancreatic β cells (Sund et al. 2001). Interestingly, like Foxa1 knockout mice (Kaestner et al. 1999), the conditional Foxa2 knockout mice are severely hypoglycemic (Sund et al. 2001). In this case however, the mice exhibited hyperinsulinemia owing to dysregulation of the insulin secretory pathway (Sund et al. 2001). Further investigations revealed this phenotype could be at least partly explained by decreased expression of the K_{ATP} channel subunits, Sur1, and Kir6.2 (Sund et al. 2001, Lantz et al. 2004), Pdx-1 (Lee et al. 2002) and altered insulin secretory vesicle docking (Gao et al. 2007). These results are generally consistent with what was observed when a dominant negative form of Foxa2 was overexpressed in INS-1 cells (Wang et al. 2002). In these experiments, the
expression of various genes involved in the insulin secretory pathway, as well as other previously identified targets of Foxa2, were measured under conditions where Foxa2 was functionally impaired. Consistent with the in vivo experiments, the KATP channel subunits were down regulated, various genes that play a positive role in insulin secretion were upregulated and there was a leftward shift in glucose-stimulated insulin secretion (Wang et al. 2002). However, in contrast to the in vivo experiments (Lee et al. 2002), Pdx1 expression was unaltered (Wang et al. 2002). Overall, these data suggest that Foxa2 has a negative impact on insulin secretion. Although these studies did not report whether IGRP expression was altered, a positive role for Foxa2 in IGRP gene expression would be consistent with this concept given that IGRP appears to negatively effect stimulus-secretion coupling (Wang et al. 2007b).

While Maf family proteins have long been known to play a role in lens development (Reza & Yasuda 2004), the role of MafA in the context of the pancreas has only recently been investigated. The impetus for these studies was the identification of MafA as the factor binding the C1 element, previously referred to as Ripe3b1, in the insulin promoter (Kataoka et al. 2002, Olbrot et al. 2002, Matsuoka et al. 2003). Using the C1 element in conjunction with DNA affinity chromatography, two different groups independently purified the basic-leucine zipper protein MafA (Olbrot et al. 2002, Matsuoka et al. 2003). In addition, a third group identified the C1 element-binding protein as MafA by recognizing that this element bears similarity to a Maf recognition element, a consensus sequence recognized by members of the Maf family (Kataoka et al. 2002). The C1 element contributes to basal insulin gene transcription and is also believed to mediate a response to glucose, an effect that is coincident with an increase in binding activity at this element (Sharma & Stein 1994). The mechanism by which glucose regulates gene transcription through MafA is unclear. Glucose does induce MafA mRNA expression (Kataoka et al. 2002) and protein stability (Han et al. 2007), however, complex serine, threonine (Rocques et al. 2007), and tyrosine (Matsuoka et al. 2001) phosphorylation mechanisms are also potentially involved. Interestingly, like insulin, IGRP mRNA levels increase in response to high glucose (Petrolonis et al. 2004) and the work presented here suggests that MafA is a potential candidate for the mediator of this response.

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Although MafA is expressed during pancreas development (Matsuoka et al. 2004, Nishimura et al. 2006), global deletion of the MafA gene does not appear to affect this process (Zhang et al. 2005), possibly due to compensation by MafB (Artner et al. 2007). Nevertheless, adult MafA knockout mice do exhibit glucose intolerance, consistent with the exclusive expression of MafA in pancreatic islet β cells (Zhang et al. 2005). Interestingly, in contrast to the correlation seen with Foxa2, the stimulatory action of MafA on IGRP fusion gene expression appears inconsistent with this phenotype given that IGRP appears to negatively
effect stimulus–secretion coupling (Wang et al. 2007b). However, the expression of multiple genes are likely altered in these animals (Wang et al. 2007a) such that the contribution of altered expression of individual factors could be negated.

Declaration of interest
The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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