Foxa2 and MafA regulate islet-specific glucose-6-phosphatase catalytic subunit-related protein gene expression

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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 β TC-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 β TC-3 cells. In contrast, in both HIT and Min6 cells mutation of these four Pdx-1 binding sites resulted in a $\sim 50\%$ 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 glycogenolytic and gluconeogenic pathways, namely the hydrolysis of glucose-6-phosphate 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 $\sim 50\%$ 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 $\sim 15\%$ 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-phosphate 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)

T cells infiltrating islets. Whether interventions directed at IGRP will be helpful in treating or preventing type 1 diabetes is unclear. *In vivo* administration of select IGRP epitope peptides to NOD mice appears to abrogate or delay the disease process (Han *et al.* 2005a). However, recent data suggest that autoimmunity toward IGRP is a secondary event with insulin being the primary autoantigen in NOD mice (Krishnamurthy *et al.* 2006).

Of the relatively few islet β cell-specific gene promoters that have been identified, those sequences controlling transcription of the insulin gene have been the subject of the most detailed analyses (Sander & German 1997, Melloul *et al.* 2002, Hay & Docherty 2006). Four main promoter elements in the insulin promoters of several species have been shown to be critical for activity. These elements have been referred to by numerous names, but under the current nomenclature they are known as the C1, C2, A, and E elements. The C1, C2, and A elements interact with the transcription factors, MafA, Pax6, and Pdx1, respectively, whereas the E elements bind the basic helix-loop-helix proteins BETA2, also known as NeuroD, E12/47, and USF (Sander & German 1997, Melloul *et al.* 2002, Hay & Docherty 2006). We have previously shown using a combination of gel retardation and chromatin immunoprecipitation (ChIP) assays that the IGRP promoter, like the insulin promoter, binds Pax6, Pdx1, BETA2, E12/47, and USF (Martin *et al.* 2003, 2004). To complete this comparison of the cis-acting elements between the two promoters, the possibility that the equivalent of the C1 element existed in the IGRP promoter was investigated. By manually scanning the proximal IGRP promoter region, it was discovered that a sequence closely resembling the C1 element, which binds MafA, is located between -186 and -157 , relative to the transcription start site. We show here that MafA binds the endogenous IGRP gene and stimulates IGRP promoter activity. In addition, a binding site for a member of the Foxa family, formerly known as hepatocyte nuclear factor-3, was previously identified in the IGRP promoter located between -246 and -221 (Bischof *et al.* 2001). This element corresponded to both DNA-protein contacts identified with an *in situ* footprinting technique and a transcriptionally active region detected by a 5' deletion analysis (Bischof *et al.* 2001). We show here that Foxa2 also binds the endogenous IGRP gene and stimulates IGRP promoter activity. Foxa2 has been previously shown to be important for both Pdx1 (Wu *et al.* 1997, Samaras *et al.* 2002) and MafA (Raum *et al.* 2006) expression in islet β cells. These data suggest that the same group of islet-enriched transcription factors, namely Pdx1, Pax6, MafA, BETA2, and Foxa2, directly or indirectly regulate expression of the two major autoantigens in type 1 diabetes.

Materials and methods

Materials

[α - 32 P]dATP (>3000 Ci/mmol) and [3 H] acetic acid, sodium salt (>10 Ci/mmol) were obtained from Amersham and ICN respectively. Antisera raised against Foxa2 (HNF-3 β ; 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 lower-case): for IGRP Foxa SDM: 5'-TATGAAAAATTGCAACgAAACACGATCCAAC-3'. For IGRP Maf SDM: 5'-AAATTGCCCCAGGTCcCATATACTGCACCTC-3'.

Cell culture, transient transfection, CAT, and luciferase assays

Hamster insulinoma tumor (HIT) and β 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 [α - 32 P]dATP using the Klenow fragment of Escherichia coli DNA polymerase I to a specific activity of ~ 2.5 μ Ci/pmol (Sambrook *et al.* 1989).

Nuclear extract preparation

The preparation of β 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 (~ 50 000 c.p.m.) was incubated with the indicated nuclear extract in a final 20 μ l reaction volume. Foxa2 binding reactions contained 1.5 μ g high salt nuclear extract, 20 mM HEPES (pH 7.9), 0.1 mM EDTA, 0.1 mM EGTA, 10% glycerol (v/v), 1 mM dithiothreitol, 1 μ g poly(dI-dC) poly(dI-dC), and 100 mM KCl. MafA binding reactions contained 3.0 μ g low salt nuclear extract, 20 mM HEPES (pH 7.9), 0.1 mM EDTA, 1 mM EGTA, 12.5% glycerol (v/v), 1 mM dithiothreitol, 1 μ g poly(dI-dC) poly(dI-dC), 0.375 mM spermidine, 0.075 mM spermine, 100 mM NaCl, and 5 mM MgCl₂. 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 150 V in 1 $\times$ TGE buffer before the gel was dried and exposed to Kodak XB 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

IGRP Foxa WT	-246	TGCAAGCAAACACGATCCAACGTGTC	-221
IGRP Foxa MUT	-246	TGCAACgAAACACGATCCAACGTGTC	-221
Foxa Consensus		AAGCCAACATTT G ATA T A G C	
IGRP Maf WT	-186	CCCTAAATTGCCCCAGGTCAGCATATACTG	-157
IGRP Maf MUT	-186	CCCTAAATTGCCCCAGGTCctCATATACTG	-157
Maf Consensus 1		TGCTGACGTCAGCA	
Ins C1 WT	-126	TGGAACATGCAGCTTCAGCCCTCTG	-101
Maf Consensus 2		TGCTGACTCAGCA	

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 μ 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 β 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 β 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 a control antiserum (IgG) had no effect on the formation or mobility of complexes A or B, whereas addition of antiserum recognizing Pdx1 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

Figure 1 MafA and Pdx1 bind the $-186/-157$ IGRP promoter region *in vitro*. β TC-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.

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 $-186/-157$ 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).

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

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 Maf WT or Ins C1 WT oligonucleotide (Table 1) competitors prior to the addition of β TC-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 $\pm$ S.E.M. of three experiments.

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 Pdx1 binding.

To investigate the functional significance of MafA binding, the AG dinucleotide 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 Maf SDM, was then analyzed by transient transfection of β TC-3, HIT, and Min6 cells. Figure 4A and B show that mutation of the MafA binding site resulted in an ~ 25 – 50% reduction in the level of reporter gene expression in β TC-3 cells as compared with that directed by the wild-type $-306/+3$

Figure 3 Identification of a mutation that selectively disrupts MafA and not Pdx1 binding. A labeled oligonucleotide representing the wild-type –186/–157 IGRP promoter region (IGRP Maf WT) was incubated in the absence or presence of a 100-fold molar excess of the unlabeled IGRP Maf WT or IGRP Maf MUT (Table 1) competitors. β TC-3 nuclear extract was then added and protein binding was 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).

promoter. The magnitude of the effect of the MafA binding site mutation varied between β TC-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 $\sim 50\%$ 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.

Foxa2 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 β TC-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 β TC-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).

Foxa2 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 β TC-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 β TC-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 $\sim 75\%$ 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.

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 $\pm$ S.E.M., each using an independent preparation of each fusion gene plasmid, assayed in triplicate. * $P < 0.05$ versus IGRP WT.

Foxa2 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. βTC-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 immunoprecipitates 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

Figure 5 Foxa2 binds the -246/-221 IGRP promoter region *in vitro*. (A) 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 -246/-221 IGRP promoter region (IGRP Foxa 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. The two major complexes detected were designated X and Y. The arrow indicates a supershifted complex. (B) The labeled oligonucleotide representing the wild-type -246/-221 IGRP promoter region (IGRP Foxa WT) was incubated in the absence or presence of a 100-fold molar excess of the unlabeled IGRP Foxa WT or IGRP Foxa MUT (Table 1) competitors. βTC-3 nuclear extract was then added and protein binding was 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.

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 *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

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 $\pm$ S.E.M., each using an independent preparation of each fusion gene plasmid, assayed in triplicate. * $P < 0.05$ versus IGRP WT.

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, BETA2, 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, BETA2, 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 7 The IGRP promoter binds Foxa2 and a Maf family member *in situ*. Foxa2 and Maf binding to the IGRP promoter was analyzed *in situ* using the chromatin immunoprecipitation (ChIP) assay. Chromatin from formaldehyde-treated β TC-3 cells was immunoprecipitated using anti-Foxa2 or anti-c-Maf antibodies or, as a control, using IgG. The presence of the IGRP promoter and exon 5 in the chromatin preparation prior to immunoprecipitation (1:100 input) and in the immunoprecipitates was then assayed using PCR as described in Materials and methods. The autoradiograph shown is representative of three different experiments. Both the IGRP promoter and exon 5 are amplified with similar efficiencies in the chromatin preparation (Martin *et al.* 2003). MW, molecular weight.

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 $-306/+3$ *IGRP* fusion gene (Fig. 10A), surprisingly this induction was not impaired comparing the wild-type fusion gene with the $-306/+3$ *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 $-306/+3$ *IGRP* Quad SDM fusion gene expression is not dependent on Pdx1. Taken together, these data indicate that Pdx1 must be stimulating wild-type $-306/+3$ *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.

Figure 8 Disruption of Pdx1 binding reduces *IGRP* promoter activity in HIT and Min6 but has little effect in β TC-3 cells. (A) β TC-3 cells, (B) HIT cells, and (C) Min6 cells were transiently cotransfected, as described in Materials and methods, using a lipofectamine solution containing various *IGRP*-CAT fusion genes (2 μ g) and an expression vector encoding firefly luciferase (0.5 μ g). 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 four Pdx-1 binding sites (*IGRP* Quad SDM). 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 $\pm$ s.e.m., each using an independent preparation of each fusion gene plasmid, assayed in triplicate. * $P < 0.05$ versus *IGRP* WT.

Figure 9 Islet-enriched transcription factors synergistically activate *IGRP* fusion gene transcription in HeLa cells. HeLa cells were transiently co-transfected, as described in Materials and methods, using a lipofectamine solution containing the $-306/+3$ *IGRP*-CAT fusion gene (2 μ g) and expression vectors (0.1 μ g each) encoding the indicated transcription factors or the matching empty vector pCMV4/pcDNA3 controls. (A) Shows the effect of adding individual factors, whereas (B) shows the effect of removing individual factors. Following transfection, cells were incubated for 18–20 h in serum-free medium. The cells were then harvested and both CAT activity and the protein concentration of the cell lysate were assayed as described in Materials and methods. Results are presented as the ratio of CAT activities, corrected for the protein concentration in the cell lysate, relative to that obtained in the presence of all factors, expressed as a percentage, and represent the mean $\pm$ s.e.m. of three experiments, each using an independent preparation of the fusion gene plasmid with each condition assayed in triplicate.

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 β TC-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 β TC-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 β TC-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-3 α , 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

Figure 10 Pdx1 does not directly regulate *IGRP* fusion gene expression in HeLa cells. HeLa cells were transiently co-transfected, as described in Fig. 8, with various *IGRP*-CAT fusion genes (2 µg) and expression vectors (0.1 µg) encoding Pax6, MafA, BETA2, and E47 and either pCMV4-Pdx1 (+ All), or pCMV4 alone (- Pdx-1). The *IGRP*-CAT fusion genes represented either the wild-type (WT) promoter sequence, located between -306 and +3, or the same sequence with a site-directed mutation (SDM) in the four Pdx1 binding sites (Quad SDM). Following transfection, cells were incubated for 18–20 h in serum-free medium. The cells were then harvested and both CAT activity and the protein concentration of the cell lysate were assayed as described in Materials and methods. Results are presented as the ratio of CAT activities, corrected for the protein concentration in the cell lysate, relative to that obtained with the WT fusion gene in the presence of all factors, expressed as a percentage, and represent the mean $\pm$ S.E.M. of three experiments, each using an independent preparation of each fusion gene plasmids, with each condition assayed in triplicate.

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 K_{ATP} 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 (Petrotonis *et al.* 2004) and the work presented here suggests that MafA is a potential candidate for the mediator of this response.

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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