Binding of activating transcription factor 6 to the A5/Core of the rat insulin II gene promoter does not mediate its transcriptional repression

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Abstract

Pancreatic β-cells have a well-developed endoplasmic reticulum due to their highly specialized secretory function to produce insulin in response to glucose and nutrients. It has been previously reported that overexpression of activating transcription factor 6 (ATF6) reduces insulin gene expression in part via upregulation of small heterodimer partner. In this study, we investigated whether ATF6 directly binds to the insulin gene promoter, and whether its direct binding represses insulin gene promoter activity. A bioinformatics analysis identified a putative ATF6 binding site in the A5/Core region of the rat insulin II gene promoter. Direct binding of ATF6 was confirmed using several approaches. Electrophoretic mobility shift assays in nuclear extracts from MCF7 cells, isolated rat islets and insulin-secreting HIT-T15 cells showed ATF6 binding to the native A5/Core of the rat insulin II gene promoter. Antibody-mediated supershift analyses revealed the presence of both ATF6 isoforms, ATF6α and ATF6β, in the complex. Chromatin immunoprecipitation assays confirmed the binding of ATF6α and ATF6β to a region encompassing the A5/Core of the rat insulin II gene promoter in isolated rat islets. Overexpression of the active (cleaved) fragment of ATF6α, but not ATF6β, inhibited the activity of an insulin promoter–reporter by 50%. However, the inhibitory effect of ATF6α was insensitive to mutational inactivation or deletion of the A5/Core. Therefore, although ATF6 binds directly to the A5/Core of the rat insulin II gene promoter, this direct binding does not appear to contribute to its repressive activity.

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Introduction

Type 2 diabetes (T2D) is characterized by impaired insulin secretion from pancreatic β-cells and peripheral insulin resistance. As the disease progresses, insulin secretion inexorably declines, presumably due to the metabolic perturbations associated with diabetes, such as chronic hyperglycemia and dyslipidemia (Poitout & Robertson 2008). The mechanisms underlying the deterioration of β-cell function are complex and only partly understood. In recent years, endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) have emerged as potentially important contributors to β-cell dysfunction under a variety of stress conditions (reviewed in Eizirik *et al.* (2008)). The pancreatic β-cell is particularly sensitive to ER stress because of its specialized secretory function and highly developed ER. Under conditions of insulin resistance or elevated circulating levels of glucose or fatty acids associated with T2D, the increased demand for insulin biosynthesis overcomes the protein-folding capacity of the ER and triggers the UPR (Scheuner & Kaufman 2008) in an attempt to 1) attenuate global protein synthesis, 2) increase transcription of molecular chaperones and foldases, and 3) activate ER-associated protein degradation (Eizirik *et al.* 2008). When this adaptive response fails to alleviate ER stress, the cell undergoes apoptosis.

The UPR involves the activation of three ER-localized stress sensors: PKR-like kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). Activation of the PERK pathway leads to transient translational attenuation, and both the PERK and IRE1 pathways mediate gene expression changes associated with the UPR via induction of ATF4 and X-box binding protein 1 (XBP1). ATF6 is implicated in

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transcriptional upregulation of molecular chaperones to increase the folding capacity and reduce protein aggregation (Scheuner & Kaufman 2008). In mammalian cells, ATF6 is expressed as two isoforms, ATF6α and ATF6β (Haze *et al.* 2001). Both ATF6α and ATF6β are, respectively, synthetized constitutively as ubiquitous 90 and 110 kDa transmembrane proteins located in the ER (Zhu *et al.* 1997, Haze *et al.* 1999, 2001) interacting with the molecular chaperone binding immunoglobulin protein (BIP) under basal conditions. When unfolded proteins accumulate, BIP dissociates from the ATF6 luminal domain, revealing two ER export signals (Shen *et al.* 2002). This enables ATF6α and ATF6β to translocate to the Golgi compartment (Haze *et al.* 1999, 2001, Chen *et al.* 2002) where they are, respectively, cleaved into 50 and 60 kDa cytosolic basic-leucine zipper (bZIP) transcription factors by the Golgi-resident Site-1 proteases (S1P) and S2P (Ye *et al.* 2000, Haze *et al.* 2001). This regulated intramembrane proteolysis enables ATF6α-p50 and ATF6β-p60 to translocate into the nucleus (Haze *et al.* 1999, 2001) where they directly activate transcription of molecular chaperones and foldases (Haze *et al.* 1999, 2001).

In addition to translation attenuation in response to ER stress, it is reasonable to expect that in highly secretory active endocrine cells, the UPR should also encompass some degree of inhibition of expression of the genes encoding secreted proteins. In the β-cell, insulin is expressed at extremely high levels, up to 100 000 molecules of insulin mRNA under stimulatory glucose conditions (Tillmar *et al.* 2002), and several lines of evidence are consistent with the possibility that the UPR is associated with reduced expression of the insulin gene. First, activation of the IRE1 branch of the UPR under glucotoxic conditions in β-cells is associated with decreased insulin mRNA levels (Lipson *et al.* 2006). Secondly, the ER stress response in insulin-secreting INS1 cells involves early degradation of insulin mRNA transcripts (Pirot *et al.* 2007, Lipson *et al.* 2008). Thirdly, overexpression of the spliced/active form of XBP1 (XBP1s) leads to a decrease in insulin mRNA levels concomitant with decreased mRNA levels of two transcription factors controlling the expression of the insulin gene, pancreas duodenum homeobox-1 (PDX-1) and mammalian homolog of avian MafA/L-Maf (MafA; Allagnat *et al.* 2010). Finally, Seo *et al.* (2008) have demonstrated that ATF6 represses insulin gene transcription in INS1 cells under glucotoxic conditions. This effect is partially mediated by upregulation of small heterodimer partner (SHP) and decreased levels of PDX-1 and MafA. The partial implication of SHP suggests that other mechanisms might be involved by which ATF6 represses insulin gene transcription. This prompted us to examine whether ATF6 directly binds to the insulin gene promoter and whether this contributes to its transcriptional repression.

Materials and methods

Reagents

RPMI-1640 and fetal bovine serum (FBS) were obtained from Invitrogen. DMSO was obtained from Sigma and thapsigargin was from Calbiochem (EMD Biosciences, San Diego, CA, USA). All other reagents (analytical grade) were from Sigma unless otherwise noted.

Rat islets isolation and cell culture

All procedures were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal; 250–275 g male Wistar rats (Charles River, Saint-Constant, QC, USA) were housed under controlled temperature (21 °C) and a 12 h light:12 h darkness cycle with free access to water and standard laboratory chow. Rats were anesthetized by i.p. injection of a 100 mg/ml ketamine hydrochloride (Bimeda-MTC Animal Health, Inc., Cambridge, ON, USA)/20 mg per ml xylazine (Bayer, Inc.) mixture and islets were isolated by collagenase digestion and dextran density gradient centrifugation as described (Briaud *et al.* 2001). Isolated islets were cultured in RPMI-1640 containing 10% FBS and exposed for 6 h to 2·8 or 16·7 mM glucose in the presence or absence of 1 μM thapsigargin. HIT-T15 cells (passages 74–86; obtained from R P Robertson (Pacific Northwest Diabetes Research Institute, Seattle, WA, USA)) were maintained in RPMI-1640 media containing 10% FBS and 11·1 mM glucose as described (Zhang *et al.* 1989).

Plasmids, transient transfections, and reporter gene studies

All plasmids were subcloned in DH5α bacterial strain and purified with a Qiagen maxiprep kit. The insulin promoter–reporters INS(−327)Luc and INS(−230)Luc encoding *Luciferase* (Luc) under the control of the human insulin gene sequences −327/+30 and −230/+30, respectively, were kindly provided by L K Olson (Michigan State University, East Lansing, MI, USA; Pino *et al.* 2005). The expression vector encoding rat ATF6α-p50 (amino acids 1–377) was generated as described (Thuerauf *et al.* 2004, Vellanki *et al.* 2010). The expression vector coding for rat ATF6β-p60 (amino acids 1–392) was kindly provided by C C Glembotski (San Diego State University, San Diego, CA, USA; Thuerauf *et al.* 2004). A mINS (−327)Luc reporter containing a site-specific mutation of the A5/Core was generated by PCR amplification using the following primer: 5′-CTCTCTCCTGG-TCTAATGT***T***GAAAGTGGCCCAG-3′ (mutated base is

bolded and italicized). Accuracy of mutagenesis was confirmed by sequence analysis on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). For transient transfections, HIT-T15 cells (passages 74–86) were seeded in 12-well plates at a density of 5×10^5 cells/well the day before transfection. Cells were co-transfected with a total of 1·6 μg DNA of either pcDNA3.1, ATF6α-p50 or ATF6β-p60 with INS (−327)Luc, INS(−230)Luc or mINS(−327)Luc, and 4 μl of Lipofectamine 2000. Cells were harvested 48 h later for electrophoretic mobility shift assay (EMSA) or luciferase assay. Dual-Luciferase Reporter assays (Promega) were performed according to the manufacturer's instructions. *Firefly* luciferase activity was normalized by *Renilla* luciferase activity or β-galactosidase activity (absorbance at 450 nm after 30 min incubation with orthonitrophényl-β-D-galactopyrannoside) of internal control plasmids.

RNA extraction and real-time RT-PCR

Total RNA was extracted from aliquots of 150 islets each using the RNeasy Qiagen micro-kit (Qiagen, Inc.), reverse transcribed, and RT-PCR was carried out using the Quantitect SYBR Green PCR Kit (Qiagen, Inc.), as described previously (Hagman *et al.* 2008). To amplify preproinsulin pre-mRNA (Ins2 pre-mRNA), a forward primer was designed against a sequence in exon 2 and a reverse primer designed against a sequence in intron 2, as described (Briaud *et al.* 2001, Iype *et al.* 2005). Primers used for RT-PCR were designed using Primer3 (Rozen & Skaletsky 2000) and are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. Results are expressed as the ratio of target mRNA to β-actin mRNA.

Electrophoretic mobility shift assay

Nuclear extracts of isolated rat islets or HIT-T15 cells were prepared as described previously (Hagman *et al.* 2005). MCF7 nuclear extracts were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Double-stranded oligodeoxynucleotide probes corresponding to the A5/Core of the rat insulin promoter or to the intron 1 (listed in Supplementary Table 1, see section on supplementary data given at the end of this article) were ^{32}P-labeled and column-purified (GE Healthcare, Buckinghamshire, UK). Nuclear extracts (10 μg) were incubated with 60 000 c.p.m. of labeled probe with or without cold competitors in a final volume adjusted to 25 μl with binding buffer (15 mM HEPES, pH 7·5, 60 mM KCL, 5 mM $MgCl_2$, 2 mM EDTA, 12% glycerol, 3·3 mM dithiothreitol, and 100 ng of poly(dI-dC)) at room temperature for 30 min. Binding reactions were resolved on 4·5% acrylamide gels run in 0·5% TBE (44·5 mM Tris, 44·5 mM boric acid, and 1 mM EDTA) for 2 h at 4 °C and visualized by autoradiography. The identity of the protein in the binding complexes was determined by supershift using 5 μg of two antibodies directed against ATF6α ((Zhang *et al.* 2009) and anti-ATF6α kindly provided by Hideo Shinagawa (BioAcademia, Inc., Osaka, Japan) or 5 μg ATF6β (Santa Cruz Biotechnology).

Quantitative chromatin immunoprecipitation assays

Quantitative chromatin immunoprecipitation (ChIP) assays were performed as described previously (Chakrabarti *et al.* 2002) with some modifications. Briefly, 500 islets were crosslinked in 1% formaldehyde for 10 min at room temperature, and the reaction was stopped with 125 mM glycine. After washing in cold PBS, cells where allowed to swell on ice for 10 min in ChIP sonication buffer and 1× complete mini protease inhibitor (Roche Applied Science). The chromatin was fragmented by sonication using Misonix sonicator 3000 (30 s pulse and 30 s cool down (output 4) repeated ten times) to shear DNA into 100–400 bp fragments. Debris was removed by centrifugation, and supernatants were cleared for 1 h at 4 °C with Protein A/G Agarose (Santa Cruz Biotechnology). For each immunoprecipitation, 250 μl aliquots of clarified extracts were diluted with the sonication buffer containing 50 μl Protein A/G Agarose and 10 μg of herring sperm DNA, and then incubated with 5 μg of anti-ATF6α or anti-ATF6β overnight at 4 °C. Immune complexes were successively washed in sonication buffer, high salt buffer, LiCl buffer, and 1× Tris–EDTA. Protein–DNA complexes were eluted twice from Protein A/G in 1% SDS, 0·1 M $NaHCO_3$, supplemented with 2 ng/ml cytomegalovirus (CMV) β-galactosidase control plasmid and reverse-crosslinked at 65 °C for 4 h. DNA and protein were ethanol-precipitated overnight at −20 °C. Precipitated samples were dissolved in proteinase K buffer (0·1 M Tris pH 7·5, 50 mM EDTA, and 5% SDS) and digested for 1 h at 55 °C with proteinase K (Roche Applied Science). DNA was extracted with phenol/$ChCl_3$/isoamyl alcohol (Fisher Scientific, Fair Lawn, NJ, USA) and ethanol-precipitated overnight at −20 °C. Samples were washed in 70% ethanol and then dissolved in 100 μl of 1× Tris/EDTA. Five microliters of each sample were quantified in triplicate by SYBR Green I-based real-time PCR using the primers listed in Supplementary Table 1. Data were expressed as fold-differences relative to control conditions, in which normal rabbit serum was used instead of specific antibody in the ChIP, and normalized to the amount of β-galactosidase recovered from each individual sample at the elution step.

Western blot analyses

Total proteins (10 μg) from isolated rat islets or HIT-T15 cells were subjected to 10% SDS–PAGE as described previously (Hagman *et al.* 2005). Immunoblots were performed with anti-ATF6α (Santa Cruz Biotechnology) and anti-tubulin (Abcam, Inc.) antibodies. Signals were detected using a HRP-labeled anti-rabbit IgG (Bio-Rad) and enhanced chemiluminescence (PerkinElmer Las Canada, Inc., Woodbridge, ON, Canada) on Kodak films (Kodak).

Cell viability assay

Viability of primary islet cells exposed to 16·7 mM glucose ± 1 μM thapsigargin for 6 h was assessed after dispersion of isolated islets. Approximately 150 islets were washed with 1 ml of HBSS–Hepes containing 1 mM EGTA and 5 mM glucose, resuspended in 300 μl, and incubated at 37 °C for 3 min. The islets were then pipetted up and down until loosely dissociated, 1 ml PBS was added, and the dispersed cells were washed again and resuspended in 50 μl of PBS containing 10 μg/ml Hoechst 33 342. Scoring of apoptosis was performed on 20 μl of the stained cells under a fluorescence microscope (Olympus IX71 using a DAPI filter) by counting the cells displaying pyknotic nuclei (~400 cells/condition).

Statistical analysis

Data are expressed as mean ± S.E.M. and were analyzed by one-way ANOVA with Tukey's multiple comparison test or by two-way ANOVA with Bonferroni *post hoc* adjustment for multiple comparisons. $P<0{\cdot}05$ was considered significant.

Results

Thapsigargin inhibits insulin pre-mRNA expression in isolated rat islets

We examined the effects of the ER stress inducer thapsigargin on expression of the ER stress markers BIP, XBP1s, ATF4, and ATF6 and of insulin pre-mRNA in isolated rat islets (Fig. 1). Thapsigargin markedly increased the expression of all ER stress markers examined (Fig. 1A; $P<0{\cdot}05$; $n=4$–5), whereas glucose alone did not affect their expression levels. Under these conditions, we measured insulin gene expression, along with mRNA expression of PDX-1 and MafA (Fig. 1B). Given that the long half-life of mature insulin mRNA species makes it difficult to examine early changes in transcriptional rates, we used a set of primers against the short-lived pre-mRNA species (Ins2 pre-mRNA), as described by Iype *et al.* (2005). As expected, insulin

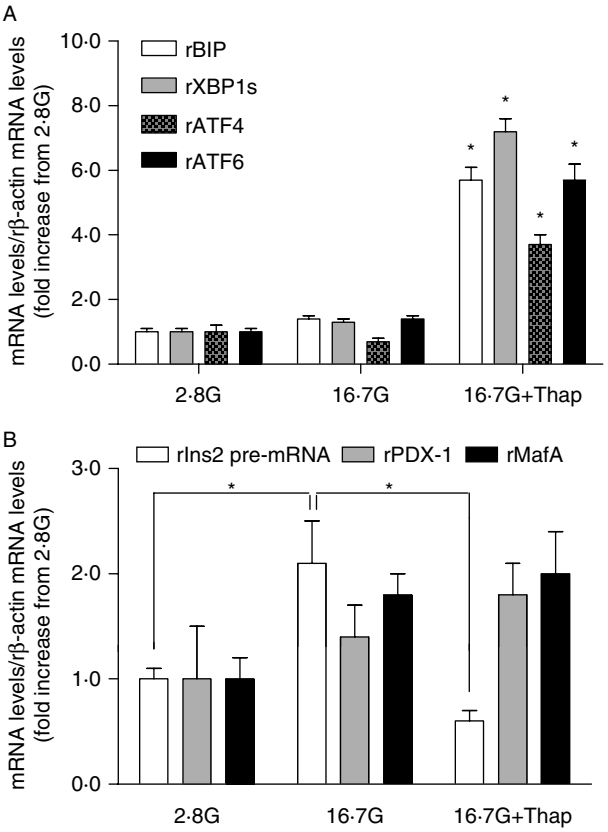

Figure 1 Thapsigargin-induced ER stress in isolated rat islets inhibits insulin pre-mRNA expression. (A) Expression of BIP, XBP1s, ATF4, and ATF6 mRNA in isolated islets exposed to 2·8 and 16·7 mM glucose (2·8 G and 16·7 G) in the presence or absence of 1 μM thapsigargin for 6 h. (B) Expression of insulin pre-mRNA, PDX-1, and MafA mRNA in isolated islets exposed to 2·8 and 16·7 mM glucose (2·8 G and 16·7 G) in the presence or absence of 1 μM thapsigargin for 6 h. Pre-mRNA and mRNA levels were measured by RT-PCR and normalized by β-actin mRNA levels. Data are mean ± S.E.M. of 4–6 independent experiments; *$P<0{\cdot}05$.

pre-mRNA levels were increased after a 6 h exposure to glucose (Fig. 1B; $P<0{\cdot}05$; $n=6$). In contrast, thapsigargin markedly decreased glucose-induced insulin pre-mRNA expression (Fig. 1B; $P<0{\cdot}05$; $n=6$), but not that of PDX-1 or MafA (Fig. 1B; $n=6$). The relatively short (6 h) thapsigargin treatment did not induce detectable apoptosis under these conditions (1·05 ± 0·22-fold increase in percentage of pyknotic nuclei versus control, $n=4$, NS). These results are consistent with the possibility that ER stress in β-cells inhibits insulin gene expression via a direct transcriptional effect.

ATF6α and ATF6β bind to the A5/Core of the insulin gene promoter

A bioinformatics analysis using the Software rVISTA (Loots *et al.* 2002) revealed a putative ATF6 binding site

overlapping with the highly conserved A5/Core at position (−311 TGATGTGG −304) in the rat II and (−316 TGATGTGG −309) in the human promoters (Fig. 2). We first examined by EMSA whether ATF6 can bind to the A5/Core of the insulin gene promoter. Incubation of MCF7 cell nuclear extracts, which express high levels of ATF6, with a radiolabeled DNA probe containing the A5/Core (Fig. 3A) resulted in the formation of a complex whose intensity increased with increasing concentrations of nuclear extracts (Fig. 3A, lanes 1–3) and which was competed by excess cold probe (Fig. 3A, lane 7). Although the addition of ATF6α and ATF6β antisera did not shift the mobility of the complex to a higher molecular weight, the intensity of the band was greatly reduced, indicating that co-incubation of nuclear extracts with two different ATF6α antisera (Fig. 3A, lanes 4 and 5) and with an ATF6β antisera (Fig. 3A, lane 6) inhibited the DNA–protein complex formation, as observed in other studies (Martel *et al.* 2010). This confirmed that the complex contains both isoforms. A complex of similar migration pattern was also detected in nuclear extracts from isolated islets (Fig. 3B, lane 1) albeit of much lower intensity presumably due to the relatively lower levels of expression of ATF6 in islets compared with MCF7 cells. The intensity of the complex was slightly increased in response to thapsigargin (Fig. 3B, lane 2). As in MCF7 extracts, the complex at the A5/Core was reduced in the presence of ATF6α (Fig. 3B, lane 3) or ATF6β (Fig. 3B, lane 4) antibodies and competed in excess of unlabeled probe (Fig. 3B, lane 5). Incubation of islet nuclear extracts with a radiolabeled probe to the intron 1 of the insulin II gene, used as a negative control, yielded a complex of faster mobility (Fig. 3C, lane 1) which was not altered in the presence of ATF6α (Fig. 3C, lane 2) or ATF6β (Fig. 3C, lane 3) antibodies. To circumvent the low levels of endogenous ATF6 in β-cells (Seo *et al.* 2008), we repeated the EMSA analysis using nuclear extracts of HIT-T15 overexpressing ATF6α-p50 (Fig. 3D). Here, again, a complex of similar migration profile was detected (Fig. 3D, lanes 1–3) and its intensity decreased in the presence of ATF6α (Fig. 3D, lane 4) or ATF6β (Fig. 3D, lane 5) antibodies.

We then used ChIP assays to confirm the ability of ATF6α and ATF6β to bind to the endogenous insulin promoter (Fig. 4). Isolated rat islets cultured for 6 h at 2·8 and 16·7 mM glucose in the presence or absence of 1 μM thapsigargin were subjected to chromatin immunoprecipitation and a region of the insulin promoter spanning the A5/Core was amplified by PCR as described in the Materials and methods section. As shown in Fig. 4, a 6 h exposure to glucose and to thapsigargin stimulated the binding of ATF6α (Fig. 4A). However, only thapsigargin, but not glucose, stimulated the binding of ATF6β (Fig. 4B) to the endogenous insulin promoter. The increased binding of ATF6α to the insulin promoter upon high glucose or thapsigargin treatment was associated with cleavage of ATF6α (Fig. 4C).

Overall, these results indicate that both isoforms of ATF6 can directly bind to the A5/Core of the rat insulin II gene promoter and prompted us to investigate whether this interaction modulates insulin promoter activity.

Overexpression of ATF6α-p50, but not ATF6β-p60, represses insulin gene promoter activity independently from its binding to the A5/Core

Since ATF6 expression and cleavage are increased, while insulin pre-mRNA levels are decreased, in islets exposed for 6 h to thapsigargin, it is conceivable that binding of ATF6 to the A5/Core represses insulin promoter activity. To test this possibility, HIT-T15 cells were co-transfected with increasing doses of ATF6α-p50 or ATF6β-p60 expression vectors and a human insulin promoter–reporter gene containing 327 bp of the proximal regulatory region (INS(−327)Luc), including the A5/Core. In Fig. 5A, overexpression of ATF6α-p50, confirmed by an increase in the intensity of a 50 kDa band reacting with the anti-ATF6 antibody, dose-dependently decreased human insulin promoter activity, while overexpression of the active form of ATF6β had no effect. We then examined the ability of ATF6α-p50 to repress the activity of a reporter bearing a mutation in the A5/Core (mINS(−327)Luc) or of a truncated reporter devoid of the A5/Core (INS(−230)Luc; Fig. 5B). Surprisingly, the activity of both the mutated and the truncated constructs was inhibited to the same degree as the INS(−327)Luc

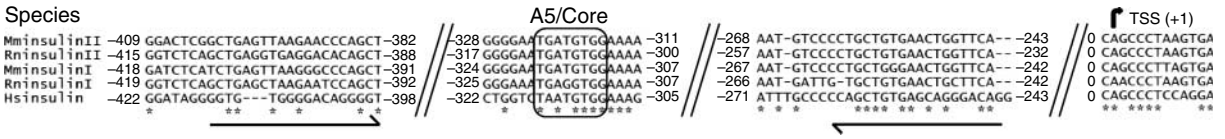

Figure 2 Identification of a putative ATF6 binding site on the A5/Core of the insulin gene promoter region. Alignment of nucleotide sequences of the 5′-flanking region of the insulin I and II genes from mouse, rat, and human. A box indicates a putative ATF6 binding site. The arrow indicates the previously described transcription start site (+1; TSS). Asterisks indicate nucleotide homology across species. Flanking the A5/Core, sequences recognized by forward and reverse primers used for ChIP analysis are underlined (sequences shown in Supplementary Table 1). Bioinformatics analysis shows one putative conserved ATF6 binding site located within the A5/Core.

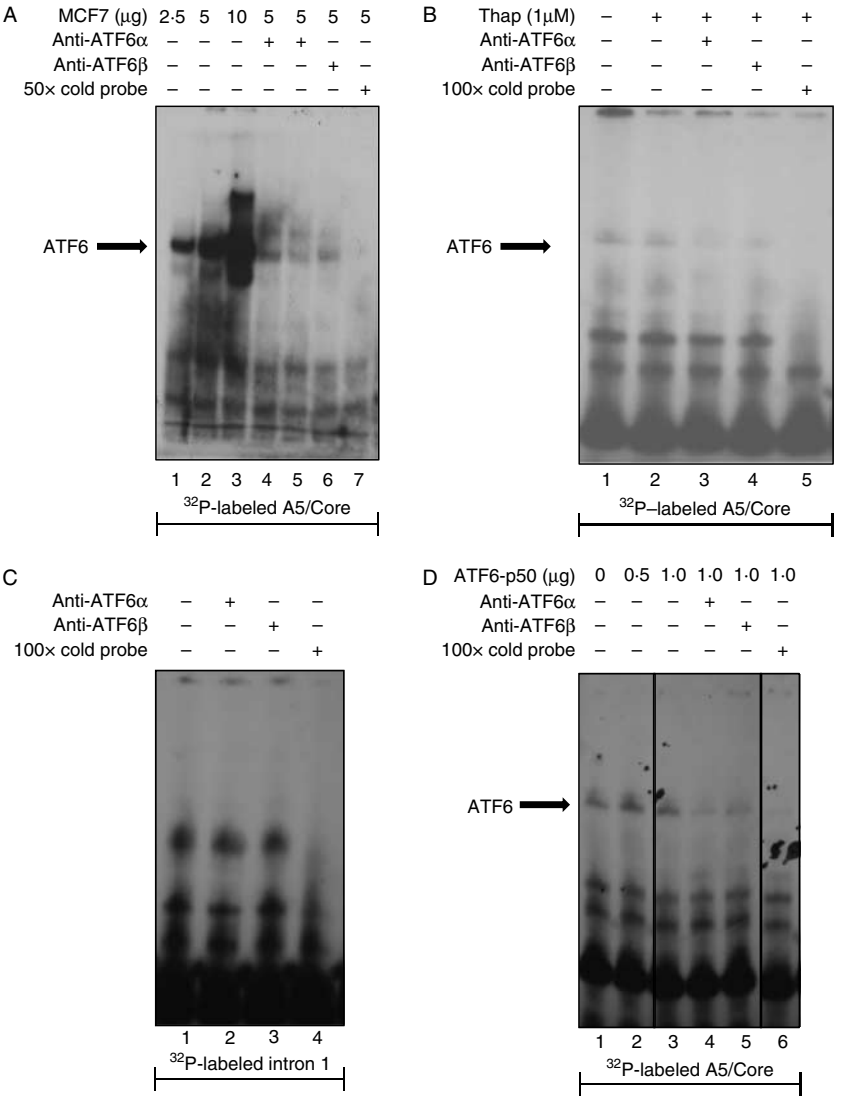

Figure 3 ATF6α and ATF6β bind to the A5/Core of the rat insulin II gene promoter. Nuclear extracts from MCF7 cells, isolated rat islets, and HIT-T15 cells were tested by EMSA for their ability to bind to DNA probe containing the A5/Core. (A) EMSA of ^{32}P-labeled A5/Core probe. Increasing concentrations of nuclear extracts (2·5, 5, and 10 μg) isolated from MCF7 cells (lanes 1–3). Two different anti-ATF6α antibodies were added to lanes 4 and 5, and anti-ATF6β was added to lane 6. Competition was done with 50-fold molar excess of unlabeled A5/Core probe (lane 7). (B) EMSA of ^{32}P-labeled A5/Core probe. Nuclear extracts were isolated from rat islets exposed for 6 h to 11·1 mM glucose in the absence or presence of 1 μM thapsigargin (lanes 1 and 2). Anti-ATF6α and anti-ATF6β antibodies were added, respectively, to lanes 3 and 4. Competition was done with 100-fold molar excess of unlabeled A5/Core probe (lane 5). (C) EMSA of ^{32}P-labeled intron 1 probe. Nuclear extracts were isolated from rat islets exposed for 6 h to 11·1 mM glucose in the presence of 1 μM thapsigargin. Anti-ATF6α and anti-ATF6β antibodies were added, respectively, to lanes 2 and 3. Competition was done with 100-fold molar excess of unlabeled intron 1 probe (lane 4). (D) EMSA of ^{32}P-labeled A5/Core probe. Nuclear extracts isolated from immortalized pancreatic β-cells HIT-T15 transfected with increasing amount of ATF6-p50 (0, 0·5, and 1·0 μg; lanes 1–3). Anti-ATF6α and anti-ATF6β antibodies were added, respectively, to lanes 4 and 5. Competition was done with 100-fold molar excess of unlabeled A5/Core probe (lane 6). EMSA probe sequences are indicated in Supplementary Table 1. Data shown are representative gels of at least three independent experiments.

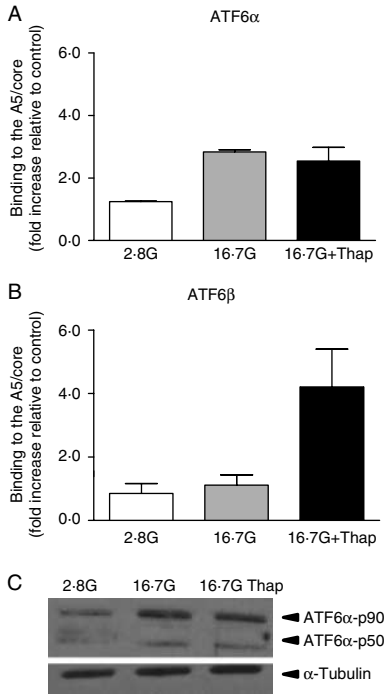

Figure 4 Binding of ATF6 to the endogenous rat insulin II gene promoter, as assessed by ChIP analysis. Isolated rat islets were exposed to 2·8 or 16·7 mM glucose (2·8 G and 16·7 G) in the presence or absence of 1 μM thapsigargin for 6 h. Chromatin was immunoprecipitated with ATF6α antiserum (A), ATF6β antiserum (B), or normal rabbit serum. Data are expressed as the fold increase in the immunoprecipitated sample relative to the control and normalized to the amount of β-galactosidase recovered at the elution step. Data are mean±S.E.M. of 2–5 separate experiments. (C) Representative immunoblot from three independent experiments probed for antibodies against cleaved (ATF6α-p50) and uncleaved (ATF6α-p90) ATF6α and α-tubulin.

reporter upon overexpression of ATF6α-p50. Overall, these data suggest that the A5/Core is not required for ATF6α repression of human insulin gene promoter activity.

Discussion

This study was designed to determine whether ATF6, a transcription factor involved in the UPR and ER stress, binds to the insulin gene promoter. We found that both isoforms of ATF6 can indeed bind to the A5/Core of the insulin gene promoter in response to the ER stress inducer thapsigargin and that ATF6α represses the insulin promoter, but that direct binding does not contribute to this repressing activity.

The pancreatic β-cell has a high protein-folding load: proinsulin represents up to 20% of the total mRNA and 30–50% of the total protein synthesis in the β-cell (Schuit *et al.* 1988, 1991, Van Lommel *et al.* 2006). This renders β-cells particularly susceptible to metabolic stress due to their highly specialized secretory function to produce insulin in response to glucose and nutrients (Poitout *et al.* 2004). Several recent studies have provided evidence in favor of the involvement of ER stress in β-cell dysfunction and T2D (reviewed in Eizirik *et al.* (2008) and Scheuner & Kaufman (2008)). We observed that thapsigargin, an ER stress inducer, impairs insulin gene pre-mRNA expression in isolated islets, suggesting that the UPR in β-cells encompasses transcriptional repression of the insulin gene in addition to the classical translational inhibition as shown in previous studies (Lipson *et al.* 2006, 2008, Pirot *et al.* 2007, Seo *et al.* 2008, Allagnat *et al.* 2010). This appears to occur both via IRE1-mediated insulin mRNA degradation (Pirot *et al.* 2007, Lipson *et al.* 2008) and transcriptional inhibition (Seo *et al.* (2008), Allagnat *et al.* (2010), and our results). It is unlikely that the observed impairment of insulin gene expression in response to thapsigargin merely results from β-cell death, since cell viability was unchanged under the experimental conditions.

ATF6 is a member of the ATF/cAMP-response element binding (CREB) bZIP DNA-binding protein family (Hai *et al.* 1989). It regulates gene expression of a number of ER chaperones, such as BIP, glucose-regulated protein 94 and protein disulfide isomerase, among others (Okada *et al.* 2002), by interacting with nuclear factor-Y (NF-Y) and subsequent binding to a consensus ER stress response element, $CCAATN_9$ CCACG (Yoshida *et al.* 1998, Haze *et al.* 1999). ATF6 can also bind to a consensus UPR element (UPRE; Yoshida *et al.* 2001) (G)(G)TGACGTG(G/A), where the nucleotides in parentheses are more or less conserved (Wang *et al.* 2000). A bioinformatics analysis of the rat insulin II promoter revealed that the sequence −311 TGATGTGG −304 was similar to an UPRE and could therefore possibly bind ATF6. The insulin promoter is a highly conserved region spanning ∼400 bp upstream of the transcription start site. Expression of the insulin gene, essentially restricted to the pancreatic β-cells, is tightly regulated by several transcription factors. The coordinated and synergistic activation of insulin gene expression is mainly controlled by PDX-1, MafA and BETA2/NeuroD, which bind, respectively, to the AT-rich A3 box, C1 and E1 *cis*-acting DNA elements on the insulin gene promoter (reviewed in Poitout *et al.* (2004)). Farther upstream, a region containing the A5 element resembles a consensus PDX-1 binding site and is part of the highly conserved enhancer core sequence (German *et al.* 1995), which binds a NF complex enriched in β-cells (Ohlsson & Edlund 1986). PDX-1, MafA, and an A2-like binding factor have been reported to bind to the A5/Core (Pino *et al.* 2005). The putative UPRE identified on the rat insulin II promoter maps to the A5/Core. In fact, we demonstrated binding of ATF6α and ATF6β to the A5/Core using several

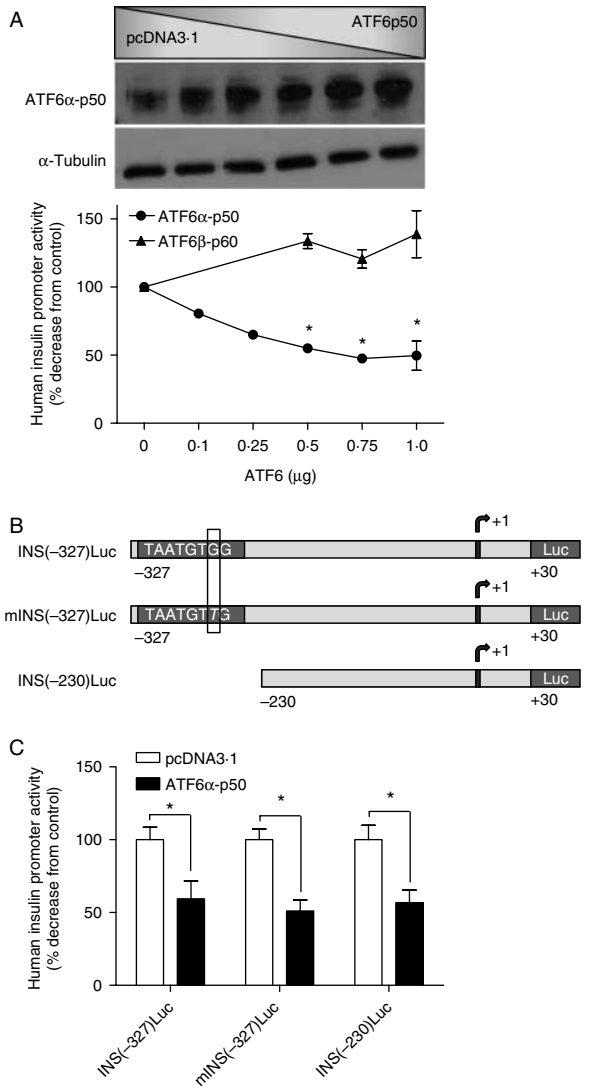

Figure 5 Overexpression of ATF6α-p50, but not ATF6β-p60, represses human insulin promoter activity. (A) HIT-T15 cells were co-transfected with the INS(−327)Luc with increasing amounts of the ATF6α-p50 or ATF6β-p60 expression vector or an empty vector (pcDNA3.1). Total DNA amount was identical amongst conditions. *Firefly* luciferase activity was corrected with *Renilla* or β-galactosidase activity. (B) Schematic representation of the different constructs used to assess the role of the A5/Core in INS(−327)Luc, INS(−230)Luc, and mINS(−327)Luc containing a site-specific mutation of the A5/Core. (C) HIT-T15 cells were co-transfected with 0·5 μg ATF6α-p50 or the empty expression vector (pcDNA3.1), and INS(−327)Luc, mINS(−327)Luc, or INS(−230)Luc. Transfection efficacy was corrected by normalizing *Firefly* luciferase activity to *Renilla* activity. Data are mean±S.E.M. of 3–4 separate experiments; *$P<0·05$.

approaches and cell types. EMSA performed with oligonucleotides to the A5/Core of the rat insulin II gene promoter confirmed the formation of a DNA–protein complex with MCF7 cells, insulin-secreting HIT-T15 cells and isolated rat islets. The weaker signal intensity observed with islet nuclear extracts might be due to the poor stability and solubility of the protein (Fonseca *et al.* 2010) and/or to its low levels of endogenous expression in β-cells (Seo *et al.* 2008). ChIP assays confirmed the recruitment of ATF6α and ATF6β to the endogenous A5/Core within the rat insulin II gene promoter in response to thapsigargin in isolated rat islets, with a stronger enrichment for ATF6β. This is consistent with the role of ATF6β acting as a negative regulator of ATF6α expression (Thuerauf *et al.* 2007), but can also be explained by the different characteristics of the two isoforms. ATF6β is 10–15 times more expressed than ATF6α and has a longer half-life (Thuerauf *et al.* 2004), both of which could account for the differences in enrichment in ChIP assays. In contrast to thapsigargin, glucose stimulated only the binding of ATF6α, consistent with previous observations by Seo *et al.* (2008) who showed activation of ATF6α under glucotoxic conditions in INS1 cells. The increased binding of ATF6α to the endogenous promoter suggests that this isoform is more rapidly activated by glucose than ATF6β, consistent with the known differences in the activation kinetics of the two isoforms (Thuerauf *et al.* 2004). Our observations however reveal an apparent paradox: on the one hand, high glucose induces cleavage of ATF6α and its binding to the insulin promoter, and ATF6α represses insulin gene expression. On the other hand, high glucose increases insulin gene expression. The reasons for this discrepancy are unknown, although we speculate that the repression of the insulin promoter by endogenous ATF6α might be overridden by other transcriptional activators (e.g. PDX-1 and MafA) under high glucose conditions.

We observed that the activity of a human insulin promoter–reporter construct containing the A5/Core was reduced by overexpressing an active form of ATF6α, confirming previous observations (Seo *et al.* 2008). This repressive effect seems to be specific to ATF6α-p50 since overexpression of ATF6β-p60 did not alter insulin gene promoter activity. To determine the role of the A5/Core, we mutated the G flanking the TGATGT core, which is critical for ATF6 binding (Wang *et al.* 2000). This, however, did not prevent the ability of overexpressed active ATF6α to repress insulin promoter activity. In contrast to the rat insulin II and mouse insulin I and II genes in which the consensus sequence TGATGTG of the A5/Core is well conserved, the human A5/Core has a one nucleotide difference (T**A**ATGTGG) which introduces a putative PDX-1 binding site (TAAT). The proximity of the introduced mutation to the PDX-1 binding site (TAATGT***T***G) could explain the repressive activity observed in the mutated plasmid. On the other hand, the fact that a truncated reporter that does not contain the A5/Core was repressed by overexpressed ATF6α to the same

extent as the A5/Core-containing construct suggests other possibilities. First, given the limitations of bioinformatics predictions of transcription factor binding sites, it is possible that other ATF6 binding sites may be present in the −230/+30 region of the human insulin promoter. For example, the rat insulin II promoter contains a CRE (Crowe & Tsai 1989) that might bind ATF6 (Hai *et al.* 1989). Secondly, ATF6α repression of the insulin gene might be indirect and involve either induction of other transcriptional repressors or competition with other factors at the same binding sites. In fact, exposure of isolated islets to thapsigargin increased SHP mRNA expression (Supplementary Figure 1), consistent with the possibility that ATF6 indirectly affects the insulin gene by stimulating SHP expression, as shown in glucotoxic conditions in INS1 cells (Seo *et al.* 2008). Also, thapsigargin moderately increased mRNA expression levels of the transcription factor CREB (Supplementary Figure 1, see section on supplementary data given at the end of this article), which might compete for binding with ATF6 at the CRE (contained within the shorter −230/+30 construct (Pino *et al.* 2005)), as shown in hepatocytes (Seo *et al.* 2010). Thirdly, it has been previously reported that overexpression of ATF6 or XBP1s occurs concomitantly with a decrease in PDX-1 and MafA expression levels in INS1 cells and in dispersed islets (Seo *et al.* 2008, Allagnat *et al.* 2010). Therefore, it is conceivable that the levels of these transcription factors were affected by thapsigargin in isolated rat islets. However, Pdx-1 and MafA mRNA levels were not altered in isolated rat islets exposed for 6 h to thapsigargin (Fig. 1B), suggesting that ATF6 repression of the insulin gene does not involved a titration of PDX-1 and MafA transcription factors at that time point, although protein levels were not directly measured. Finally, ATF6 is known to regulate gene expression by interacting with partners such as serum response factor, NF-Y, and BIP (Zhu *et al.* 1997, Yoshida *et al.* 2001, Shen *et al.* 2002), which can affect its transcriptional activity.

A question arising from these observations is what is the functional importance of ATF6α repression of the insulin gene under conditions of ER stress? First, it is interesting to note that in most cases ATF6α acts as a transcriptional activator (Yamamoto *et al.* 2007). In this context, however, it appears that ATF6α acts as a repressor of the insulin gene, although the contribution, if any, of its direct binding to the insulin promoter remains to be demonstrated (Crowe & Tsai 1989).

In conclusion, we propose that the early repression of insulin gene transcription by the ATF6 branch of the UPR might represent a protective mechanism that contributes to reducing the protein load to the ER. Our results show that ATF6 binds to the A5/Core of the rat insulin II gene promoter and therefore represents a novel transcription factor of the insulin gene. However, the binding of ATF6α does not appear to contribute to its repressive activity, and its functional importance remains to be ascertained. Further studies are needed to determine the mechanisms and the physiological relevance of the repression of insulin gene expression by ATF6α.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.10.1530/JME-11-0016.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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