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ATF-2 stimulates the human insulin promoter through the conserved CRE2 sequence

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Abstract

The insulin promoter contains a number of dissimilar cis-acting regulatory elements that bind a range of tissue specific and ubiquitous transcription factors. Of the regulatory elements within the insulin promoter, the cyclic AMP responsive element (CRE) binds by far the most diverse array of transcription factors. Rodent insulin promoters have a single CRE site, whereas there are four CREs within the human insulin gene, of which CRE2 is the only one conserved between species. The aim of this study was to characterise the human CRE2 site and to investigate the effects of the two principal CRE-associated transcription factors; CREB-1 and ATF-2. Co-transfection of INS-1 pancreatic β-cells with promoter constructs containing the human insulin gene promoter placed upstream of the firefly luciferase reporter gene and expression plasmids for ATF-2 or CREB-1 showed that ATF-2 stimulated transcriptional activity while CREB-1 elicited an inhibitory effect. Mutagenesis of CRE2 diminished the effect of ATF-2 but not that of CREB-1. ATF-2 was shown to bind to the CRE2 site by electrophoretic mobility shift assay and by chromatin immunoprecipitation, while siRNA mediated knockdown of ATF-2 diminished the stimulatory effects of cAMP related signalling on promoter activity. These results suggest that ATF-2 may be a key regulator of the human insulin promoter possibly stimulating activity in response to extracellular signals.

Keywords: Diabetes mellitus; cAMP responsive element; Gene transcription; Insulin gene

1. Introduction

Insulin is synthesised and secreted by β-cells within the pancreatic islets of Langerhans in order to maintain glucose homeostasis. The production of insulin is regulated in part by control of transcription of the insulin gene through the interplay of both β-cell restricted and ubiquitous transcription factors [1]. These bind a diverse range of cis-acting regulatory sequences located within ~300 base pairs from the transcription start site. The principal regulatory elements are: A box sequences binding homeodomain proteins, most importantly PDX-1 [2–7]; C elements binding the basic leucine zipper (bZIP) protein MafA [8–11]; E boxes binding proteins of the basic helix loop helix (bHLH) class of transcription factor with ubiquitous E47 forming a heterodimer with neuroendocrine cell specific NeuroD/β2 [12]; and cyclic AMP responsive elements (CREs) that bind a broad array of closely related members of the bZIP CREB/ATF family [13]. The overall architecture of the insulin promoter is conserved between species, however, there is subtle divergence in the sequences and spacing of regulatory elements resulting in marked variation in transcription rates [14]. The close proximity of regulatory elements that can bind a diverse range of factors permits a multiplicity of outcomes through additive and synergistic interactions between the bound proteins.

Within the insulin promoter, the cyclic AMP responsive element (CRE) is the regulatory element that binds by far the widest range of transcription factors. In addition, the same transcription factors can act as activators, non-activators or repressors by forming homo- or heterodimers, thereby extending the gamut of signals influencing the regulation of gene expression. The permissible interaction of transcription factors

Abbreviations: ATF, activating transcription factor; bHLH, basic helix loop helix; bZIP, basic leucine zipper; CRE, cyclic AMP responsive element; CREB, cAMP responsive element binding protein; CREM, CRE-modulator protein; STAT5A, signal transducer and activator of transcription 5A

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activated by cAMP and diacylglycerol signalling pathways means that CRE sites can act as points of convergence for multiple signals. Another layer of complexity at CRE sites is introduced by the existence of multiple isoforms of the CREB/ATF transcription factors with diverse activating or inhibitory properties [15–18]. Reversible phosphorylation of the CREB/ATF family of transcription factors strongly influences their abilities to bind DNA or interact with other proteins [19] thereby providing another level of control and mediating cross-talk between signalling pathways.

Rodent insulin promoters have a single CRE site at −185, whereas there are four CREs within the human insulin gene: CRE1 at −210; CRE2 at −183; CRE3 at +18 and CRE4 at +61 [20]. Given the importance of CRE sites and their ability to allow the integration of different intracellular signals at the level of transcription, the current studies were undertaken to characterise the human CRE2 site, which is the only one conserved between species [1]. The results show that CRE2 has significant preferential binding for ATF-2 over CREB-1. Furthermore, ATF-2 acts through CRE2 as a strong activator of the insulin promoter whereas CREB-1 behaves as a repressor through pathways that do not involve the CRE2 site.

2. Materials and methods

2.1. Cell culture

The rat insulinoma cell line INS-1 [21] was cultured in RPMI 1640 containing 11.1 mM D-glucose and 2 mM L-glutamine, supplemented with 10% (vol/vol) foetal bovine serum (FBS), 1 mM NaPyruvate, 10 mM HEPES balanced salt solution and 50 μM β-mercaptoethanol (all reagents: Invitrogen, Paisley, UK). Cells were maintained at 37 °C without antibiotics in a humidified atmosphere containing 5% CO_2.

2.2. Transient transfections and treatment

For transient transfections, cells on 6-well plates at ~80% confluence were transfected with plasmids using LipofectAMINE Plus Reagent (Invitrogen) according to the manufacturer's protocol. Each well was incubated for 3 h with 1 ml serum-free transfection cocktail containing 1 μg plasmid DNA and then overnight in 2 ml of normal growth medium. The following day, the medium (11.1 mM glucose) was replaced and the cells were incubated for a further 48 h. Cells were washed with phosphate buffered saline (PBS) and incubated in medium (11.1 mM glucose) lacking foetal bovine serum for 4 h at 37 °C. Cell treatments were carried out during this final incubation and involved either inclusion of 10 μM forskolin in the medium or exposure to UV radiation at the start of the incubation period. UV-irradiated cells were exposed to 254 nm light at 0.6 Joules/cm^2 for 0.5 min using a Biolink BLX-254 UV cross-linker.

2.3. Plasmid DNA constructs

The pCRE-LUC reporter plasmid (Stratagene, Amsterdam, The Netherlands) contained four juxtaposed copies of the consensus CRE sequence upstream of a TATA box to drive expression of the firefly luciferase gene. The constructs phINS356LUC, phINS356m1LUC and phINS356m2LUC containing a −356 to +14 fragment of the human insulin promoter in the pGL3 plasmid that was either unmutated or contained mutations in the CRE1 and CRE2 sites respectively have been described previously [22]. More extensive mutation of the CRE1 and CRE2 sites in phINS356m1LUC and phINS356m2LUC was performed using the Quik Change Site-Directed Mutagenesis kit (Stratagene) with the following primers and their reverse complements (mutations are underlined and the CRE region is in italics): CRE1mLF 5′-CTGGTTAAGACTCTAA*TAAAGTTC*TGGTCCTGAGGAAGAGG-3′ and CRE2mLF 5′-CTGAGGAAGAGGTGC*TAAACCTC*AAGGAGATCTTCCCAC-3′ to create phINS356m11LUC and phINS356m22LUC respectively. Plasmid pcDNA3.1 was from Invitrogen and TK-*Renilla* Luciferase (phRL-TK) was from Promega (Southampton, UK). The expression plasmids pEBG2T-ATF2, which contained human ATF-2 cloned into a *Bam*H1 site in the vector pEBG2T, and pCMV5CREB1, which contained human CREB1 cloned as an *Eco*R1/*Bam*H1 fragment in the vector pCMV5, were provided by Philip Cohen, University of Dundee, UK.

2.4. Luciferase reporter assays

Cells were washed once with phosphate buffered saline and lysed with 500 μl Passive Lysis Buffer (Promega) for 15 min with shaking. Cell debris was removed by centrifugation at 13,000×*g* for 5 min at 4 °C and firefly and *Renilla* luciferase activities were determined in supernatants using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

2.5. Nuclear extracts

Nuclear extracts were prepared by the method of Dignam et al. [23] in the presence of protease inhibitors (1.0 mM PMSF, 150 nM aprotinin and 1.5 μM pepstatin A) and protein phosphatase inhibitors (5 mM β-glycerophosphate and 100 μM Na_3VO_4). The protein content was determined using Bio-Rad DC Protein Assay (Bio-Rad, Hemel Hempstead, UK) with bovine serum albumin as standard.

2.6. Electrophoretic mobility shift assays (EMSAs)

Double stranded 30-base pair oligonucleotides were 5′ end-labelled with [γ-^{32}P]ATP (Amersham Biosciences, Chalfont St Giles, UK) and T4 polynucleotide kinase (New England Biolabs, Hitchin, UK), followed by purification using Quick Spin Columns (Roche Diagnostics, Lewes, UK). Nuclear extracts were incubated with labelled oligonucleotide (~25,000 cpm/well) employing EMSA buffers and conditions as described previously [22] except that poly(dI–dC).poly(dI–dC) was omitted from incubations employing purified recombinant transcription factors. Studies on transcription factor binding to the human insulin CRE2 regulatory element used the following oligonucleotides and their reverse complements (mutations are underlined and the CRE motif is in italics): CRE2^{-198} 5′-GGAAGAGGTGC*TGACGAC-CAAGGAGATCTT-3′*$^{-169}$; CRE2m1 5′-GGAAGAGGTGC*TAAAGACC*AAGGAGATCTT-3′; CRE2m2 5′-GGAAGAGGTGC*TGTGGACC*AAGGAGATCTT-3′; CRE2m3 5′-GGAAGAGGTGC*TGATTACC*AAGGAGATCTT-3′; CRE2m4 5′-GGAAGAGGTGC*CAGTTCTT*AAGGAGATCTT-3′ and CRE2m5 5′-GGAAGAGGTGC*CCAGCGCC*AAGGAGATCTT-3′. In addition, 30 bp oligonucleotides and their reverse complements containing either no transcription factor regulatory elements; random oligonucleotide (RO) or the rat somatostatin CRE regulatory element (the CRE motif is in italics) were used as controls: RO 5′-CGAGCACCCTTCACCCTCCAGGCTTAACGG-3′ and SOM-CRE^{-59} 5′-GCCTCCTTGGC*TGACGTCA*GAGAGAGAGTT-3′ $^{-30}$ respectively. The purified recombinant transcription factors were: his-tagged full-length human CREB-1 (Panomics), his-tagged full-length human ATF-2 and his-tagged full-length human CREM-1 (Santa Cruz Biotechnology, Calne, UK). Competing unlabelled oligonucleotides were added 15 min prior to the addition of labelled probe. In the case of supershift experiments, 3 μl of the appropriate antibody or pre-immune serum was incubated with the nuclear extract for 30 min at room temperature before the addition of the labelled probe. The reactions were continued according to the same protocol as all other EMSA incubations.

2.7. Phosphorylation of ATF-2

Recombinant full-length human his-tagged ATF-2 was phosphorylated *in vitro* using [γ-^{32}P]ATP and JNK1α1/SAPK1c (Upstate, Dundee, UK).

2.8. DNA affinity chromatography

INS-1 nuclear extract was incubated with double stranded oligonucleotide encompassing the CRE2 sequence used in the EMSA studies plus an additional