Glucagon-like peptide-1 stimulates human insulin promoter activity in part through cAMP-responsive elements that lie upstream and downstream of the transcription start site

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Abstract

Glucagon-like peptide-1 (GLP-1) is a peptide hormone secreted from the enteroendocrine L-cells of the gut and which acts primarily to potentiate the effects of glucose on insulin secretion from pancreatic β-cells. It also stimulates insulin gene expression, proinsulin biosynthesis and affects the growth and differentiation of the islets of Langerhans. Previous studies on the mechanisms whereby GLP-1 regulates insulin gene transcription have focused on the rat insulin promoter. The aim of this study was to determine whether the human insulin promoter was also responsive to GLP-1, and if so to investigate the possible role of cAMP-responsive elements (CREs) that lie upstream (CRE1 and CRE2) and downstream (CRE3 and CRE4) of the transcription start site. INS-1 pancreatic β-cells were transfected with promoter constructs containing fragments of the insulin gene promoter placed upstream of the firefly luciferase reporter gene. GLP-1 was found to stimulate the human insulin promoter, albeit to a lesser degree than the rat insulin promoter. Mutagenesis of CRE2, CRE3 and CRE4 blocked the stimulatory effect of GLP-1 while mutagenesis of CRE1 had no effect. Analysis of nuclear protein binding to the four CREs showed that, while they share some proteins, each CRE site is unique. Stimulation of transcription by GLP-1 through CRE2, CRE3 and CRE4 resulted in altered protein binding that was different for each of the CRE sites involved. Collectively, these data show that the four human CREs are not simply multiple copies of the rat CRE site and further emphasise that the human insulin promoter is distinct from the rodent promoter.


Introduction

Glucagon-like peptide-1 (GLP-1) is a peptide hormone secreted by the enteroendocrine L-cells of the small intestine in response to food intake (Kieffer & Habener 1999, Drucker 2001). GLP-1 plays an important role in lowering blood glucose levels primarily through its ability to potentiate the stimulatory effects of glucose on insulin secretion from pancreatic β-cells (Holz et al. 1993). It does this through a G-protein-coupled receptor that acts to increase intracellular cAMP levels (Thorens 1992). It also affects blood glucose levels through its inhibitory effects on gastric emptying (Nauck et al. 1997), suppression of appetite (Turton et al. 1996), and inhibition of glucagon secretion from α-cells (Komatsu et al. 1989).

GLP-1 is a particularly important regulator of the pancreatic β-cell with diverse effects on insulin secretion, gene expression, proinsulin biosynthesis and islet cell growth and neogenesis (Drucker et al. 1987, Fehmann & Habener 1992, Edvell & Lindstrom 1999, Xu et al. 1999, Stoffers et al. 2000). It also induces differentiation of pancreatic exocrine cells to an endocrine phenotype (Zhou et al. 1999). Multiple signalling pathways and second messengers are involved in the response of the β-cell to these pleiotropic actions of GLP-1 (Habener 2001).

All of the studies to date on the effects of GLP-1 on insulin gene transcription have focused on the regulation of the rat insulin I promoter. However, since GLP-1 (or its analogues) may have a potentially important role in the treatment of type 2 diabetes (Doyle & Egan 2001), it is important to understand how it affects expression of the human insulin gene. Expression of the insulin gene is regulated by promoter sequences located upstream of
approximately 400 bp from the start site. Although the arrangement of regulatory sequences in the rat insulin I and human insulin promoters is roughly similar (German et al. 1995), there are marked differences in their regulation (Clark & Docherty 1992, Melloul et al. 2002). Given the importance of cAMP in mediating the effects of GLP-1, and, if so, also determining the possible role of cAMP-responsive elements (CREs).

Materials and Methods

Cell culture
INs-1 cells that were directly descended from the original rat insulinoma cell line developed by Asfari et al. (1992) were cultured in RPMI 1640 containing 11·1 mM β-glucose and 2 mM l-glutamine, supplemented with 10% foetal bovine serum (FBS), 1 mM sodium pyruvate, 10 mM Hepes balanced salt solution and 50 µM β-mercaptoethanol. Cells were maintained at 37 °C without antibiotics in a humidified atmosphere containing 95% air and 5% CO₂. Cells were passaged by trypsinisation and subcultured on a weekly basis.

Radioimmunoassays
Total insulin-like immunoreactivity (ILI) in INS-1 cell culture medium was detected with an in-house radioimmunoassay employing guinea pig polyclonal antibodies raised against human (pro)insulin (1011 from Linco Research, Biogenesis, Poole, Dorset, UK). Radioimmunoassays were calibrated using rat insulin (Linco Research) and performed in duplicate. ILI results were calculated as ng/ml per 24 hours.

Plasmid DNA constructs
The rat insulin I promoter (~ 410 to +1bp) construct, pFOXLUC410, and the control vector pFOXLUC were provided by Dr M. German (University of California at San Francisco Medical School, San Francisco, CA, USA). The human insulin promoter construct was generated by PCR amplification of a ~364 to +148 bp fragment of DNA from the plasmid phins300 that contains a Xhol fragment (~5 kb) of λhINS-1 (Bell et al. 1980) cloned into the Sall site of pBR322 plasmid. The primers used had the sequence 5’-CGAGCTCGAAGGCGCA 3’ and 5’-GCCGCCGTGGATGCTTCACGAG-3’.

The amplified fragment was inserted upstream of the firefly luciferase sequences within plasmid pGL3 (Promega) to generate phINS364LUC. The plasmids phINS260LUC and phINS171LUC contained fragments spanning the regions –260 to +148 and –171 to +148 respectively. These were generated by PCR amplification of phins300 using the following primers: 5’-CGAGCTCC TGTGACGAGGGACAG-3’ and 5’-TCCCCCGGGG GAATGCTTCAGACG-3’ for phINS260LUC, and 5’- CGAGCTCATCATTCCCACCAGAC-3’ and 5’-TCCC CCGGGGGAATGCTTCACGAG-3’ for phINS171LUC.

Mutagenesis of CRE3 at position +18 to generate plasmid phINS171m1LUC was achieved by using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) and the following primers: 5’-CCTCCAGGACAGGCTAATCAGAAGAGGCC ATC-3’ and its complementary reverse. Mutagenesis of CRE4 at position +61 to generate plasmid phINS171m2LUC was achieved using the primer 5’-GT TCCAAGGGCCTTTAAATCAGGTCGTCAGG-3’ and its complementary reverse. The DNA construct phINS171m3LUC contained mutations at CRE3 and CRE4 using the above primers.

A further construct (phINS356LUC), containing a ~356 to +14 fragment of the human insulin promoter in the pGL3 plasmid, was obtained from Dr J. Xu, University of Hong Kong, Hong Kong. Sequencing of this construct showed the presence of an A to G substitution at position –189 that was immediately upstream of the CRE2 consensus sequence. This putative error was corrected by mutagenesis as described above using the primer 5’-GCT CTTAGAAGAGGCTGCTGAGACC-3’ and its reverse complement. Similarly, the CRE1 and CRE2 sites were mutated using the following primers and their reverse complements: CRE1 5’-CTGGTTAAGACTCT TTAAACCCTGCTGGTCTGAGAAG-3’ and CRE2 5’-CTGGTTAAGAGGTGCTAAGACCAGGAG TCTCC-3’. Constructs were created that lacked CRE1 (phINS356m1LUC), CRE2 (phINS356m2LUC) and both CRE1 and CRE2 (phINS356m3LUC).

The mutations and integrity of final products were confirmed by DNA sequencing.
the test substances for 4 h at 37 °C in a humidified atmosphere.

**Luciferase assays**

Cells were washed once with PBS and lysed with 400 µl Passive Lysis Buffer (Promega). Cell lysis was carried out for 15 min with shaking and cell debris was removed by centrifugation at 13 000 g for 5 min. Then, 10 µl cell extract were added to 350 µl of A buffer (15 mM MgSO₄, 7H₂O, 30 mM glycyglycine, 2 mM Na₂ATP, pH 7-8). To this, 150 µl of G buffer (30 mM glycyglycine, pH 7-8) containing 0.5 mM luciferin were injected and the luminescence read at 560 nm using a Berthold Lumat LB 9501/16. Protein content was measured using the Bio-Rad DC Protein Assay (Bio-Rad) with BSA as a standard.

**Nuclear extracts**

Cells were grown to approximately 80% confluence in 9 cm Petri dishes and treated exactly as for luciferase assays. They were grown in complete RPMI 1640 medium containing 10% foetal bovine serum and 3 mM glucose for 24 h, then washed once with minimal medium before test substances or solvent alone were added. Cells were incubated in minimal medium containing 11.1 mM glucose for 24 h, then washed once with minimal medium and foetal bovine serum. The cells were then pre-incubated in Hepes-balanced buffer containing 3 mM glucose with 0.2% BSA for 1 h followed by stimulation for 45 min in Hepes-balanced buffer containing either 3 or 11.1 mM glucose in the presence or absence of 10 nM GLP-1. The levels of insulin secreted were measured by radioimmunoassay. The data represent the means ± S.E.M. of three independent experiments carried out in triplicate and are expressed as fold increases in insulin secretion compared with 3 mM glucose. Statistical analyses of increases in insulin secretion, which were relevant to specific assays, were calculated. Cells without GLP-1: 11.1 mM glucose compared with 3 mM glucose. Statistical analyses of increases in insulin secretion, which were relevant to specific assays, were calculated. Cells without GLP-1: 11.1 mM glucose compared with 3 mM glucose. Statistical analyses of increases in insulin secretion, which were relevant to specific assays, were calculated.

**Electrophoretic mobility shift assays**

The coding sequences of the 30 bp oligonucleotides of the human insulin gene CRE sites were: CRE1, 5'-TAAGA CTTCTAATGGACCCTGGTCTCGAGG-3'; CRE2, 5'-GGAAGAGGTGCTGAGCAAGGAGATCTT-3'; CRE3, 5'-CCAGGACAGGCTGATCCAAGAGGC CATC-3'; CRE4, 5'-CCAGGGCCTTTGCGTCAG GTGGGCTCAG-3'. The double-stranded CRE oligonucleotides were labelled with T4 polynucleotide kinase (New England Biolabs, Hitchin, UK) and [γ-³²P]ATP (Amersham), followed by purification using Quick Spin Columns (Roche). Nuclear binding assays were carried out in a total volume of 20 µl of 20 mM Hepes, pH 7-9, 70 mM KCl, 0-2 mM EDTA, 0-5 mM dithiothreitol and 10% glycerol. Nuclear extracts (10 µg) were incubated on ice for 15 min with 1 µg poly(dI-dC).poly(dI-dC) as non-specific competitor along with other competitors or antibodies when indicated. Oligonucleotide competition binding assays were carried out with 300 × molar excess of specific competing unlabelled CRE oligonucleotide. Antibody competition assays had the addition of either 1 µl rabbit polyclonal anti-human pancreatic duodenal homeobox-1 (PDX-1) serum (a gift from Dr C. Wright, Vanderbilt University Medical Center, Nashville, TN, USA) or 1 µl rabbit serum as a control. Labelled oligonucleotide corresponding to 60 fmol was added and incubation was carried out at room temperature for 30 min. The reaction products were resolved on 6% non-denaturing polyacrylamide gels run in TGE buffer, pH 8.5 (50 mM Tris, 380 mM glycline, 2 mM EDTA). The gels were dried and subjected to autoradiography at -70 °C.

**Statistical analysis**

Statistical analysis was performed using a Blackwell Science statistical package (Blackwell Scientific Publications, Oxford, UK) and statistical significance was measured by analysis of variance.

**Results**

**Effect of GLP-1 on human insulin promoter activity**

GLP-1 is known to potentiate the effects of glucose on insulin secretion and gene expression. In preliminary experiments the responsiveness of INS-1 β-cells to glucose and GLP-1 was investigated (Fig. 1). INS-1 cells responded to glucose with a significant 2.5-fold increase in insulin secretion when cultured in the presence of 11.1 mM compared with 3 mM glucose. Treatment with
10 nM GLP-1 resulted in significant increases in insulin secretion in both 3 and 11.1 mM glucose. Insulin secretion increased 1.4-fold in the presence of GLP-1 in cells cultured in 3 mM glucose compared with 3 mM glucose alone and by 2.7-fold in cells cultured in GLP-1 and 11 mM glucose compared with 11 mM glucose alone. In addition, Northern blot analysis showed that the GLP-1-stimulated increase in insulin secretion was accompanied by 1.7-fold (P<0.001) and 1.5-fold (P<0.05) increases in the amount of preproinsulin mRNA in low and high glucose respectively (data not shown). Thus, INS-1 cells respond to GLP-1 by increasing insulin secretion in a glucose-dependent manner and were used for all further studies.

More detailed studies on the mechanism of GLP-1 stimulation of the insulin promoter were carried out using luciferase reporter constructs containing the rat insulin I promoter (pFOXLUC410) and a series of human insulin promoter constructs (Fig. 2). Initial experiments using pFOXLUC410, which contained the rat insulin I promoter (−410 to +1bp) in the pFOXLUC vector, showed that GLP-1 stimulated the activity of the rat insulin I promoter in both 3 mM glucose (2-fold increase) and in 11.1 mM glucose (2.4-fold increase) (Fig. 3). The magnitude of this effect on the rat insulin promoter was in keeping with previous studies using a similar rat insulin promoter construct in INS-1 cells (Skoglund et al. 2000, Kemp & Habener 2001, Chepurny et al. 2002). In detailed dose–response experiments (data not shown), a maximal effect of GLP-1 was observed at 10 nM and this concentration was used in all further experiments. The human insulin promoter contains an inhibitory element between −279 and −258 (Boam et al. 1990) and regulatory sequences that lie downstream of the transcription start site (Inada et al. 1999). In detailed dose–response experiments (data not shown), a maximal effect of GLP-1 was observed at 10 nM and this concentration was used in all further experiments. The human insulin promoter contains an inhibitory element between −279 and −258 (Boam et al. 1990) and regulatory sequences that lie downstream of the transcription start site (Inada et al. 1999). When the human insulin promoter construct phINS364LUC (Fig. 2), which contains the
inhibitory and downstream elements, was used, there was a significant stimulation by GLP-1 of promoter activity in 3 mM glucose (1.12-fold increase) and 11.1 mM glucose (1.25-fold increase) (Fig. 3). GLP-1 also stimulated the activity of the human insulin promoter construct phINS260LUC (Fig. 2), which lacked the inhibitory element (1.23- and 1.27-fold in 3 and 11 mM glucose respectively) (Fig. 3). The inhibitory element, which also functions as a glucose response element in transfected islets (Sander et al. 1998), does not therefore appear to affect the responsiveness of the insulin promoter to GLP-1. In general the human insulin promoter constructs were much less responsive to GLP-1 than the rat insulin promoter (Fig. 3). There was no effect of glucose or GLP-1 on the control vectors pFOXLUC or pGL3 (data not shown).

Role of CREs in regulating the human insulin promoter

Further experiments were designed to determine whether CREs within the human insulin promoter might be involved in mediating the effects of GLP-1. There are four CREs within the human insulin promoter located at −210 (CRE1, TGACCGGC), −183 (CRE2, TGACG ACC), +18 (CRE3, TGCATCAG) and in the first intron at +61 (CRE4, TGGCTGCAG) (Fig. 2). CRE2 is the only one conserved between humans and rodents (Philippe & Missotten 1990, Inagaki et al. 1992). The absence of sequences that included CRE1 and CRE2 in phINS171LUC, and CRE3 and CRE4 in phINS356LUC did not diminish the effect of GLP-1 on promoter activity (Fig. 3). This suggests that CREs that lie upstream and downstream of the transcription start site might be involved.

The individual roles of CRE1 and CRE2 were studied by mutating these responsive elements in the construct phINS356LUC as outlined in Fig. 2. Similarly, the roles of CRE3 and CRE4 were investigated by creating a series of mutations in the construct phINS171LUC (Fig. 2). In order to determine if all four CRE sites were transcriptionally active, initial experiments looked at the effect of the mutations on the rates of basal transcription. Mutagenesis of CRE1 (phINS356m1LUC) and of CRE2 (phINS356m2LUC) resulted in a reduction of basal transcription to 0.63 and 0.65 relative to the unmutated construct respectively (Fig. 4A). Construct phINS356m3LUC, in which both CRE1 and CRE2 were mutated, displayed an even greater reduction in basal transcription to 0.4 relative to the unmutated construct respectively (Fig. 4A). Construct phINS356m3LUC, in which both CRE1 and CRE2 were mutated, displayed an even greater reduction in basal transcription to 0.4 relative to the unmutated construct respectively (Fig. 4A). Mutagenesis of CRE3 (phINS356m1LUC) and of CRE4 (phINS356m2LUC) resulted in a reduction of basal transcription to 0.63 and 0.65 relative to the unmutated construct respectively (Fig. 4A). Construct phINS356m3LUC, in which both CRE1 and CRE2 were mutated, displayed an even greater reduction in basal transcription to 0.4 relative to the unmutated construct respectively (Fig. 4A).
show that all four CRE sites in the human insulin promoter are transcriptionally active and that there could be constitutive cAMP/PKA activity in INS-1 cells.

**Role of individual CREs in the human insulin promoter response to GLP-1**

Both constructs phINS356LUC and phINS171LUC showed significant stimulation by GLP-1 (Fig. 5). Mutagenesis of CRE1 (phINS356m1LUC) had no effect on GLP-1 stimulation while mutagenesis of CRE2 (phINS356m2LUC) completely abolished the stimulatory effect of GLP-1 (Fig. 5A). In keeping with these results, the construct, in which both CRE1 and CRE2 were mutated (phINS356m3LUC), was also unresponsive to GLP-1 (Fig. 5A). Mutagenesis of CRE3 (phINS171m1LUC) and of CRE4 (phINS171m2LUC) diminished the stimulatory effect of GLP-1 from a 1.30-fold increase for the unmutated construct to 1.18- and 1.19-fold increases respectively (Fig. 5B), while the
construct phINS171m3LUC, in which both CRE3 and CRE4 were mutated, completely blocked the effects of GLP-1 (Fig. 5B). This would suggest that CRE3 and CRE4 act in a cumulative manner. In summary, the stimulatory effect of GLP-1 on the human insulin promoter is mediated through CRE2, CRE3 and CRE4, while CRE1 is not involved.

**The response of individual CREs in the human insulin promoter to forskolin**

GLP-1 is thought to act primarily through a G-protein-coupled receptor leading to increased intracellular cAMP levels (Thorens 1992). The responsiveness of the different CRE sites to cAMP was investigated using forskolin, a stimulator of adenylate cyclase. Although forskolin raises cAMP levels in an unphysiological manner and cannot be used in a qualitative manner, it is widely recognised that increased intracellular levels of cAMP can mimic signalling pathways. Both constructs phINS356LUC and phINS171LUC showed stimulation upon treatment with forskolin (Fig. 6). The contribution of CRE1 and CRE2 sites to forskolin responsiveness was investigated using the phINS356LUC mutant constructs. Loss of CRE2 resulted in a concomitant loss of responsiveness to forskolin, while mutation of CRE1 had only a minor effect (Fig. 6A). Mutagenesis of CRE3 and CRE4 had minimal effect on forskolin stimulation on phINS171LUC, however the double mutation of CRE3 and CRE4 did result in a significant reduction in stimulation (Fig. 6B). The inability of the CRE3 and CRE4 mutations to significantly diminish forskolin effects may reflect the promiscuity of binding specificities for the eight or so members of the bZIP family of transcription factors that are known to recognise CRE-related sequences. It is highly significant, however, that whereas the CRE3 and CRE4 mutations only weakly inhibited forskolin stimulation, they produced significant reductions in the stimulatory effect of GLP-1 as described above. This would suggest that GLP-1 is acting on CRE3 and CRE4 mainly by a mechanism other than through an increase in active cAMP-dependent transcription factors.

The role of cAMP in GLP-1 stimulation of the human insulin promoter was further examined by using H-89, the inhibitor of protein kinase A (PKA). While H-89 (10 µM) completely abolished the stimulatory effect of forskolin on the phINS260LUC construct (P<01), it only partially inhibited the stimulatory effect of GLP-1 on phINS260LUC construct by 48% (P<01) and 39% (P<001) in 3 and 11·1 mM glucose respectively (data not shown), lending further support to the view that GLP-1 can act through cAMP-dependent and non-cAMP-dependent intracellular pathways. These findings are in keeping with the observations that H-89 is ineffective in blocking GLP-1 stimulation of the rat insulin I promoter (Skoglund et al. 2000).

**Nuclear protein binding to individual CRE sites**

Nuclear proteins were purified from INS-1 cells that had been grown in 3 mM glucose for 24 h followed by 4 h in minimal medium containing 11·1 mM glucose. When the proteins were incubated with labelled oligonucleotides containing each of the four human insulin promoter CRE sites and analysed by electrophoretic mobility shift assay, multiple retarded bands were observed. Unique patterns of
protein binding were formed with each CRE site and the various DNA–protein complexes have been assigned arbitrary names (Fig. 7A). Each CRE displayed one or two major bands including (1A and 1D, 2E, 3B and 3D, and 4B and 4D) and a number of minor bands. Antibody competition studies showed the major complex 1D contained PDX-1 (Fig. 7B) and removal of PDX-1 permitted another protein to bind and form the complex 1H. Clearly, the presence of the overlapping PDX-1 binding site affects the binding of proteins and possibly the activity of the CRE1 site. Many complexes were common to several CRE sites. For example, bands corresponding to the CRE1 complex 1A were also observed with CRE3 and CRE4 (3C and 4B respectively). The major band in the CRE2 lane (2E) was present in all the other lanes as 1C, 3D and 4D, while one of the two major complexes created with CRE3 (3B) had analogous forms with CRE2 (2C) and CRE4 (4A). It is important to note, however, that although some proteins could bind to different CRE sites, the relative degree of binding was often completely different. Significantly, all four CREs also bound proteins that were unique for a particular site. The CRE2 lane had two unique minor bands (2A and 2B) that represented high molecular weight DNA–proteins. The unique CRE3 complex 3A also bound a high molecular weight protein and CRE4 had the unique minor complex, 4C. The
DNA–protein complexes and their relative binding to the different CREs were consistently observed with four different nuclear protein preparations. Taken together, these results demonstrate that, while the four CRE sites in the human insulin promoter share some common characteristics, they are distinct from each other.

In order to understand better the shared characteristics of the four CRE sites, competition studies were performed, in which the nuclear assays were preincubated with specific competing CRE oligonucleotides (Fig. 8). Formation of the CRE1 complex 1D, and to a lesser degree 1E, was reduced by the CRE2 oligonucleotide, but unaffected by the oligonucleotides for CRE3 and CRE4. Reduced complex 1D assembly suggests that the CRE2 site may have some affinity for PDX-1. The CRE4 oligonucleotide completely abolished the creation of complexes 1A, 1B and 1C while the other CRE oligonucleotides reduced their formation. In keeping with the observation from Fig. 7A that the major CRE2 complex (2E) is common to all CRE sites, its formation, along with that of 2D and 2F, was completely prevented by all four CRE oligonucleotides. On the other hand, the high molecular weight complexes 2A, 2B and 2C were unaffected by the presence of any of the other CRE oligonucleotides. The main CRE3 complexes 3B and 3D were less evident when other CRE oligonucleotides were present, however only the self-competing oligonucleotide completely prevented their creation, suggesting that the proteins in question bind preferentially to CRE3. The major CRE4 complexes 4B and 4F were partly reduced by CRE1 but not by the other CRE oligonucleotides with the CRE3 oligonucleotide increasing formation of complexes 4B and 4C. This could be due to CRE3 sequestering competing proteins that bind to CRE4, e.g. those in the complexes 4D and 4E. It is evident from these competition studies that the binding of nuclear proteins to the four human insulin promoters is a complex and dynamic process.

Figure 8 Competition experiments show shared properties of CRE sites. Nuclear extracts from INS-1 cells that had been incubated for 24 h in 3 mM glucose followed by 4 h in medium containing 11·1 mM glucose were incubated with labelled oligonucleotides containing the human insulin promoter CRE indicated above each gel. DNA–protein complexes were resolved by electrophoretic mobility shift analysis and the complexes were labelled as in Fig. 7. Competing CRE oligonucleotides were added at a 300-fold molar excess 15 min prior to the addition of the labelled CRE oligonucleotide. The identity of the competing CRE oligonucleotide is shown above each lane with ‘0’ representing the absence of any competing oligonucleotide.

Action of GLP-1 and forskolin on the individual CRE sites
Potential variations in transcription factor binding at the individual CRE sites during stimulation by GLP-1 and forskolin were explored by performing binding assays with nuclear extracts from INS-1 cells that had been treated...
with either GLP-1 or forskolin (Fig. 9). For CRE-1, GLP-1 had no effect on the binding of PDX-1 (1D), whereas the major complex 1A and minor complexes 1B and 1C were reduced. Forskolin, on the other hand, led to increased levels of 1B, 1E and 1G along with low levels of a new complex (1I) being produced. The CRE2 site revealed dramatic changes upon treatment with GLP-1 and forskolin. In both cases, the major complex of 2E, and the minor complexes 2D and 2F, were greatly increased, especially with GLP-1. Forskolin, but not GLP-1, also gave rise to a new highly abundant complex (2G). In marked contrast to CRE2, the effect of GLP-1 on CRE3 was the virtual total loss of a major complex, namely 3B. Interestingly, the competition nuclear binding studies showed that the protein in 3B bound more strongly to CRE3 than to the other CREs so this regulatory pathway may be unique to CRE3. Treatment with forskolin led to a less striking reduction of this complex along with the creation of a new one (3H). In a manner similar to CRE3, GLP-1 caused the noticeable loss of a major and a minor CRE4 complex with the levels of 4B and 4C being greatly diminished. Forskolin had no effect on these complexes and increased appreciably the amount of complex 4A. Collectively, these findings show that GLP-1 and forskolin can upregulate transcription through the actions of both identical and different DNA-binding proteins, and that the individual CRE sites of the human insulin promoter respond by diverse mechanisms.

**Figure 9** Effect of GLP-1 and forskolin on proteins binding to individual CRE sites. Labelled oligonucleotides containing the human insulin promoter CRE indicated above each gel were incubated with nuclear extracts from INS-1 cells that had been cultured in medium containing low glucose (3 mM) and foetal bovine serum for 24 h, followed by washing with serum-free medium and cultured for 4 h in serum-free 11.1 mM glucose medium. During incubation in 11.1 mM glucose, the cells either received no treatment (lane N), were incubated in the presence of 10 nM GLP-1 (lane G) or were incubated in the presence of 10 μM forskolin (lane F). DNA–protein complexes were resolved by electrophoretic mobility shift analysis and are marked as above.
Discussion

A major finding of this study was that GLP-1 stimulates the activity of the human insulin promoter and that this effect is mediated through CRE2, CRE3 and CRE4 while CRE1 does not participate. The use of forskolin to raise intracellular cAMP levels suggested that CRE2, CRE3 and CRE4 function as CREs but that CRE1 does not. The most likely explanation for this latter observation is that the binding of PDX-1 at the overlapping A3 site results in CRE1 being permanently occupied, thereby preventing other transcription factors from binding. Another major finding of this study was that, although the four CREs of the human insulin promoter share some nuclear protein-binding properties (Inagaki et al. 1992, Inada et al. 1999), each CRE site is unique. In addition, the CRE sites involved in stimulation by GLP-1 and forskolin do so by different mechanisms. Changes in DNA–protein complex formation brought about by raised levels of cAMP through administration of forskolin were most obvious at CRE2 where large increases in several complexes were observed. Furthermore, all of the proteins responsible for GLP-1 stimulation at CRE2 were the same as those involved in the cAMP response. These findings strongly suggest that GLP-1 stimulates CRE2 through the cAMP signalling pathways. Further studies are underway to identify the proteins involved: potential candidates being CRE-binding protein (CREB), CRE modulator (CREM), activating transcription factor-1 (ATF-1) or other PKA- and cAMP-associated factors. Preliminary results using CRE2 oligonucleotide trapping along with Western blotting show that the transcription factor ATF-2 appears to bind preferentially in comparison with CREB-1.

CRE3 and CRE4, on the other hand, appear to respond to GLP-1 in an alternative manner. At these CREs, GLP-1 stimulation is accompanied by a large reduction in GLP-1 stimulation is accompanied by a large reduction in to GLP-1 in an alternative manner. At these CREs, GLP-1 response that reflects subtle differences in the organisation and regulation of cis-acting elements in the insulin promoters from different species (Clark & Docherty 1992, Melloul et al. 2002). The reduced responsiveness may also reflect the difficulties in studying the human insulin promoter in rodent cell lines. There are no available human β-cell lines. NES2Y, a human β-cell-like line derived from a patient with persistent hypoglycaemia of infancy, that was used in our previous studies (Macfarlane et al. 1997), stopped expressing insulin beyond about passage eight, the lowest passage now available. This cell is therefore of limited use for this or related studies on the human insulin promoter. On the other hand, the reliance on rodent β-cell lines may not be a significant problem as evidenced by the fact that the human insulin promoter functions well in rodent cells. It will drive β-cell-specific expression in transgenic mice (Itier et al. 1996) and previous studies on the mapping of regulatory elements in the human insulin gene have utilised available rodent (and hamster) β-cell lines (Boam et al. 1990, Tomonari et al. 1996). On balance therefore it is likely that the human insulin promoter is less responsive to GLP-1.

GLP-1 and its long-lasting analogues may have a role in the treatment of type 2 diabetes. Thus subcutaneous

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administration of GLP-1 increased insulin secretion, inhibited gastric emptying and lowered glucagon levels in type 2 diabetic patients (Willms et al. 1996). It has the further therapeutic advantage that its administration is associated with low incidence of hypoglycaemia without any risk of weight gain (Zander et al. 2002, Knop et al. 2003). If GLP-1 is to find use as a therapeutic agent in the treatment of type 2 diabetes, then more data are required on exactly how GLP-1 regulates expression of the human gene. Most of the previous studies have focused on rodent insulin genes. The present study represents the first to document GLP-1 responsive sequences within the human insulin gene and is, therefore, of relevance to enable a better understanding of how GLP-1 mediates its effects on glucose homeostasis in people with diabetes.

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**References**


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