Regulation of Proglucagon Transcription by Activated Transcription Factor (ATF) 3 and a Novel Isoform, ATF3b, through the cAMP-response Element/ATF Site of the Proglucagon Gene Promoter*

Received for publication, May 23, 2003, and in revised form, June 16, 2003 Published, JBC Papers in Press, June 18, 2003, DOI 10.1074/jbc.M305456200

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Glucagon, the second major glucose-regulated hormone in the control of glucose homeostasis, functions as a counterregulator to insulin and is specifically produced by the pancreatic α cells. Its excessive biosynthesis and secretion is associated with diabetes mellitus. The expression of the proglucagon gene has been demonstrated to be regulated by a cAMP-dependent pathway through cAMP-response element-binding protein (CREB) and possibly other transcription factors bound to its cAMP-response element (CRE)/activated transcription factor (ATF) site. Elsewhere we have shown that ATF3, a member of the ATF/CREB subfamily of the basic leucine zipper domain proteins, is expressed predominantly in the α cells of the pancreatic islets. In our attempts to further dissect the role of ATF3 proteins in α cells, we identified and characterized a novel alternatively spliced form, ATF3b, and have compared the specific binding ability of ATF3 and ATF3b on the CRE/ATF motif of the proglucagon promoter. Our findings indicate the existence of a novel mechanism by which the transcription of the proglucagon gene is regulated in response to cAMP signals, in addition to CREB and in relation to glucose fluctuations in pancreatic α cells.

Glucagon, the second major hormone in the control of glucose homeostasis, acts as a counterregulator to insulin (1). It is synthesized mainly in the pancreatic α cells in most species. However, proglucagon mRNA is also expressed in the enteroendocrine L cells, where it gives rise to GLP1 and -2, and also in some neurones in the central nervous system (2). Its excessive production and secretion is associated with poorly controlled diabetes mellitus (3). Toward understanding the regulation of glucagon biosynthesis, it has been demonstrated that proglucagon gene expression is regulated by a cAMP-dependent pathway (4–8) through a cAMP-response element (CRE) site bound by one of the bZip proteins, CREB (4, 9), and possibly other factors (4). Moreover, in contrast to the up-regulation of insulin mRNA levels by glucose (10), only minor changes in proglucagon mRNA levels occur in response to glucose in cultured islets; the levels tend to increase during culture irrespective of the glucose concentration (11). In vivo it is increased during prolonged fasting (12). The increase or attenuated decrease of proglucagon mRNA at lower glucose concentrations may be a physiological feature of α cells that supports glucagon secretion at low glucose levels. However, the underlying mechanism is unclear.

ATF3 is a member of the ATF/CREB subfamily of bZip domain proteins (13). It functions by binding to ATF/CRE sites (TGACGTCA, identical to the CRE) or the related activated protein-1 site in DNA (13, 14). Murine ATF3, initially designated as LRG-21 (homologous to rat liver-regenerating factor LRF-1), is composed of 181 aa and contains a bZip domain (residues 88–142) for homo/heterodimer formation and specific DNA binding (15). The aa sequence homology of mouse ATF3 to the rat or human proteins is 99 and 98%, respectively (15). Two C-terminal truncated isoforms, ATF3aZip and ATF3bZip2, which lack the leucine zipper domain, have been isolated from human tissue. These fail to bind to the ATF/CRE sites (16, 17). The corresponding isoforms have not yet been described in rodents.

ATF3 expression is relatively low in most cell types under normal conditions but is strongly induced in response to many environmental changes as an immediate early response gene (18). It appears to function in the regulation of the cellular stress response and in cell proliferation by forming homo- and selective heterodimers with certain other bZip proteins (13, 18). ATF-3 has been implicated in regulating several genes (16, 19–24) including PEPCK (25), that influence glucose homeostasis in transgenic mice (26). Recently, it has been found to be expressed predominantly in α cells within islets. Because the CRE/ATF site of the proglucagon gene promoter has been suggested to be bound by other proteins besides CREB (4), we have further explored the possibility of proglucagon gene expression regulated by ATF3 in α cells. In the present study, we have characterized a novel alternative spliced isoform, ATF3b, compared the specific binding ability of ATF3b with that of ATF3 to the CRE/ATF site, and further have demonstrated a novel element binding protein; bZip, basic leucine zipper; EMSA, electrophoretic mobility shift assay; IBMX, isobutylmethylxanthine; PC, proprotein convertase; PEPCK, phosphoenolpyruvate carboxykinase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.
mechanism by which proglucagon gene expression in pancreatic α cells is regulated by glucose and in response to cAMP signals.

MATERIALS AND METHODS

Cell Culture, Islet Isolation, RNA Preparation—αTC1.6 and NIH3T3 cells were grown in 10% fetal calf serum/Dulbecco’s modified Eagle’s medium (Invitrogen) as described (28, 29). Islet isolation and RNA preparation were described previously (30).

Molecular Cloning of ATF3b cDNA—cDNA synthesized by reverse transcription from αTC1.6 total RNA (1 mg) was subjected to PCR as described previously (30) using primer pairs (from 5’ to 3’) for cloning the cDNA sequence of ATF3: 5’-GTTGCTAGCCCTTCTGCACCAGGGTGCT-3’ (underlined sense) and AACCTCGAGGTCGGGAAAGTTCCACCAA (antisense). The products (the expected band and a smaller one) were subcloned into the EcoRI-Xhol sites of the pcDNA3.1 (+) expression vector (Invitrogen). The sequences were determined using an ABI PRISM sequencing kit and compared with the reported ATF3 cDNA and genomic gene sequences. The nucleotide sequence of the cDNA of a novel isoform, ATF3b, has been deposited in GenBankTM accession number (pending).

RT-PCR, Northern and Western Blotting—These procedures were carried out as described previously (31). Using the same primers as described above, total RNA (1 mg) from αTC1.6 and islets was subjected to RT-PCR. 10 mg of total RNA was used for Northern blotting. For Western blotting, whole-cells proteins (50 mg/lane) were separated by Laemmli or Tricine SDS-PAGE and transferred onto an Immobilon-P SQ membrane. Antibodies against the C-terminal region of ATF3 (Santa Cruz Biotechnology, Santa Cruz, CA) and tubulin (Sigma) were purchased commercially.

In Vitro Synthesis, Immunoprecipitation, and N-terminal Sequencing of ATF3b Protein—Labeled ATF3 and ATF3b proteins were synthesized using the TnT-coupled transcription/translation system (Promega) with the plasmids constructed as described above in a mixture containing [35S]methionine or [3H]leucine (Amersham Biosciences). For immunoprecipitation with rabbit anti-ATF3 serum, the [35S]- or [3H]-labeled ATF3 protein was diluted in 0.5 × TRIS buffer (10 mM Tris, 70 mM NaCl, 0.05% Tween 20, pH 7.4) with protease inhibitors (Roche Applied Science). Immunoprecipitation and PAGE analysis was carried out as described previously (31). For the N-terminal sequencing of ATF3b, the [3H]-labeled ATF3b protein immunoprecipitates were separated on a 16.5% Tricine gel and transferred onto an Immobilon-P membrane. The membrane-bound ATF3b protein band was excised and subjected to N-terminal Edman degradation. Tritium radioactivity of aa fractions released on each cycle was counted in a liquid scintillation counter.

EMSA (Electrophoretic Mobility Shift Assay)—ATF3 and ATF3b proteins were synthesized in vitro as described above. DNA probes (normal, 5’-AGCTTGGACGTACCACAAATGAGCCTAG-3’; mutant, 5’-AGCTTGGACGTACCACAAATGAGCCTAG-3’) were derived from the region containing the CRE/ATF site (underlined) of the mouse proglucagon promoter and labeled with γ-[32P]ATP. DNA binding reactions were carried out as follows: aliquots of in vitro translation mixture were incubated in 20 μl of binding buffer (10 mM HEPES-KOH, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, and 10% glycerol) containing 2 μg of poly(dI-dC) (Sigma) and 6-20 × 103 counts per minute of DNA probe at room temperature for 10 min. For supershift assays, 0.2 μg of IgG of rabbit anti-ATF3 or control serum was added and the samples incubated for another 10 min. The binding mixture was then applied onto a 5% polyacrylamide gel (0.5 × Tris borate-EDTA buffer) for electrophoresis (25).

Transient Transfection and Luciferase Activity Assay—A cDNA containing the mouse proglucagon promoter (1453 bp) was PCR with Pfu DNA polymerase (Stratagene) using the primers of GluP5Saci (5’-GTTGACGTACCTCAGCAAACGGCTGTCCT-3’) and Glup3NheI (5’-GTTCGTAGCCCTCGACAGGGGTGCTG-3’). The PCR products harboring the mutant CRE/ATF site was obtained by two steps of PCR: (a) PCR was performed using two sets of primers, the GluP5Saci and GlumP3 (5’-GTTGATTTGGACCAAAATGAGCCTAG-TCT-3’), GlumP5 (5’-GGCTTGGACGTACCACAAATGAGCCTAGG-3’) and the GluP3NheI; (b) the template obtained by combining the diluted PCR products from the first step (a) was subjected to PCR again with the GluP5Saci and Glup3NheI primers. The products were then cloned into the SacI-Xhol sites of pCMV-bgal plasmid (Promega), and the mutant site was confirmed by sequencing. The reporter plasmid (1.0 μg) driven by the proglucagon promoter (normal or mutant) was transfected into αTC1.6 or NIH3T3 cells (3.5 cm well) with effector DNA, 0.5 μg of pcDNA3.1-ATF3, pcDNA3.1-ATF3b, and pcMV-SPORT6 CREB-327 (IMAGE clone; Invitrogen), using LipofectAMINE (Invitrogen). At 24 h after transfection, cells were incubated with media containing 20 μM forskolin/100 μM IBMX (Sigma). Cells were harvested 48 h post-transfection, and luciferase activity was assayed using the dual-luciferase reporter assay system (Promega). For each transfection, 50 ng of Renilla luciferase reporter pRL-TK was included to normalize transfection efficiency. All transfection experiments were performed at least in duplicate.

RESULTS

Cloning and Characterization of a Novel Isoform of ATF3—In the course of cloning the coding sequence of ATF3 using a pair of primers described under “Materials and Methods,” we observed a band of 517 bp in addition to the expected 623-bp band representing ATF3 in both αTC1.6 cells as well as the islets of PC2 null mice (32) and littermate mice (Fig. 2A). Sequencing of the 517-bp cDNA (named ATF3b) reveals that it lacks 106 nucleotides beginning from position 25 of the ATF3 coding sequence. The deleted 106 nucleotides constitute an intron-like DNA fragment (33) that is located in the corresponding exon 2 of the ATF3 gene (Fig. 1A). This deletion generates a novel stop codon at a site corresponding to aa residue position 10 of the ATF3 protein; the novel isoform, ATF3b, then initiates from a downstream in-frame ATG (Fig. 1B). The predicted ATF3b protein (124 aa) is an N-terminal-truncated form lacking the initial 57 aa of ATF3 but preserving the intact bZip domain (Fig. 1, B and C) that is absent in the ATF3b protein and ATF3ΔZip2 isoforms.

Using an antibody specific for the C-terminal region of ATF3, but not that of ATF3ΔZip and ATF3ΔZip2 (17), both ATF3 (upper band) and ATF3b (lower band) were detected in αTC1.6 cells, and both were down-regulated by glucose (Fig. 2B). These bands were also detected in whole pancreas homogenate (data not shown). Moreover, the ATF3b protein could be immunoprecipitated from products translated in vitro using an ATF3 C-terminal-specific antisera (Fig. 2C); additionally, this protein contained the predicted leucine residue at position 5 as determined by N-terminal sequence analysis (Fig. 2D). These
data thus confirm the expression of both ATF3b mRNA and protein in pancreatic α cells in concert with ATF3.

The DNA Binding Properties of ATF3 and ATF3b Homo- and Heterodimers—To test whether these ATF3 proteins are involved in the transcriptional regulation of the proglucagon gene through its CRE/ATF site, we analyzed the binding properties of ATF3 or ATF3b homodimers to the normal and mutant ATF/CRE sites derived from mouse proglucagon promoter (Fig. 3). The EMSA results showed that both ATF3 and ATF3b protein bound to the normal ATF/CRE site (Fig. 3B, lanes 2 and 12) but not to the mutant sequence (lanes 7 and 17). The specificity was verified by supershifting of the DNA-protein complex (lanes 3 and 13) with an ATF3- and ATF3b-specific antiserum but not with a nonspecific antiserum (lanes 4 and 14). Moreover, the formation of the DNA-protein complex was inhibited by addition of an 80-fold excess of unlabeled competitor (lanes 5 and 15). On the other hand, disruption of specific binding by mutation of the CRE/ATF site (lanes 6–10 and 16–20) indicated that the homodimers of ATF3 or ATF3b protein bind directly to the CRE/ATF site of the proglucagon gene promoter and not to the flanking DNA regions.

To compare the relative binding ability of ATF3 and ATF3b homodimers, we initially quantified the amount of ATF3 and ATF3b protein required for binding. Compared with ATF3, ~10-fold less ATF3b protein was synthesized in vitro from the same amount of cDNA template based on normalized methionine radioactivity (ATF3/ATF3b = 10.3, p < 0.0005, n = 3) (Fig. 4A). The low production of the ATF3b protein may reflect the presence of an atypical Kozak sequence for initiation of translation (34), because the transcriptional efficiency of the ATF3b plasmid is likely to be similar to that of the ATF3 plasmid in this system. However, comparative binding results based on

32P radioactivity of the respective bands indicated that the binding ability of the ATF3b homodimer is ~15-fold higher than that of ATF3 homodimer normalized to the same protein amount (ATF3/ATF3b = 15.5, p < 0.001, n = 3) (Fig. 4B). We also tested the binding ability of ATF3 and ATF3b heterodimers. As shown in Fig. 4C, such heterodimers can be formed and show a higher binding ability with the CRE/ATF site than the homodimers based on densitometric analysis (ATF3b heterodimer versus homodimer = 2.53, p < 0.003, n = 3) (Fig. 4C).

ATF3 and ATF3b Stimulate Proglucagon Transcription and This Action Is Potentiated by cAMP—To assess the role of ATF3 proteins in proglucagon gene transcription, transient transfection assays were performed in both αTC1.6 and NIH3T3 cells. The results indicated that the transcription of the proglucagon gene was stimulated by transfection of ATF3 or ATF3b along with the normal proglucagon gene promoter-luciferase construct (p < 0.05, n = 4) in both αTC1.6 and NIH3T3 cells (Fig. 5B). By contrast, the stimulatory activity was significantly reduced when these were transfected with a promoter construct containing a mutation in the CRE/ATF site (Fig. 5B, p < 0.0005, n = 3). The results demonstrate that the stimulatory effect of the ATF3 proteins is dependent on the CRE/ATF site. Moreover, the stimulatory activity was dose-dependent on the amount of transfected DNA (data not shown), potentiated by elevated cAMP levels (Fig. 5B, p < 0.05, n = 4), and paralleled the increased amounts of ATF3 and ATF3b protein (Fig. 5C). These observations suggest alterations in the levels of ATF3 proteins contribute to the regulation of proglucagon gene transcription. Note that the effects of ATF3 and ATF3b cotransfection lie between the effects of ATF3 or ATF3b transfection alone. This presumably reflects additivity in the binding ability of heterodimers with the CRE/ATF site. In these experiments, the basal activity of the proglucagon promoter was lower in NIH3T3 versus αTC1cells, which may reflect the lack of some important transcription factors for basal proglucagon gene transcription in these non-glucagon-producing NIH3T3 cells. It should be noted also that, in agreement with the transfection results described above, the increased proglucagon mRNA level

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in αTC1.6 cells paralleled the increased level of ATF3 proteins in αTC1.6 cells treated with forskolin/IBMX (Fig. 5D).

ATF3 Proteins Have Effects Similar to Those of CREB Protein on Proglucagon Gene Transcription in Pancreatic α Cells—The effects of ATF3 proteins on proglucagon gene transcription were compared with those of CREB-327, a cAMP signal effector (4, 7, 9). As shown in Fig. 6, in non-glucagon-producing NIH3T3 cells (lower panel), CREB-327 showed a greater stimulation of proglucagon gene transcription as compared with the ATF3 proteins. However, in αTC1.6 cells (upper panel), the effects of ATF3 proteins were similar under normal culture conditions (columns 2 or 3 or 4 versus 5, p > 0.05, n = 3) and were potentiated more than that of CREB-327 in response to forskolin/IBMX (columns 11 or 12 versus 13, p < 0.01, n = 3), although ATF3 alone exhibited the highest activity among the three proteins (column 11). Compared with that in NIH3T3 cells (lower panel, column 13 versus 5 = 4.54, p < 0.01, n = 3), the attenuated effect of CREB-327 in response to the cAMP signal in αTC1.6 cells (upper panel, column 13 versus 5 = 2.69, p < 0.05, n = 3) may reflect the fact that pancreatic α cells express at high levels a large number of transcription factors, including the bZip proteins,2 some of which may associate with and attenuate the activity of CREB-327 as described previously (9). Another possibility may be related to the high basal cAMP-dependent protein kinase activity and its reduced responsiveness to added exogenous cAMP in the original αTC1 cells from which the αTC1.6 cells were cloned (35). Without forskolin/IBMX, no significant synergistic effects of CREB and ATF3 proteins were observed in either cell type (Fig. 6, column 5).
The effects of ATF3 proteins on proglucagon gene transcription were compared with those of CREB-327, a cAMP signal effector. In non-beta cells (upper panel), the effect of CREB-327 in response to the cAMP signal in TC1.6 cells (Fig. 2B). The results of EMSA (Figs. 3 and 4C) and transient transfection assays (Fig. 5) clearly indicate that the homo- and heterodimers of ATF3 and ATF3b directly bind to the specific CRE/ATF element and stimulate proglucagon gene expression. This role of the ATF3 proteins is indirectly supported by the observations of higher plasma glucagon concentrations in ATF3 transgenic mice (25) and the elevated levels of proglucagon mRNA in the islets of PC2 null mice (1) that is also accompanied by a significant increase of ATF3 protein (data not shown).

In the pancreatic α cell, despite the fact that the CREB and ATF3 proteins specifically act on the same CRE/ATF motif of the proglucagon promoter, their effects may be exerted in different physiological states. Thus, the CREB protein can be rapidly phosphorylated by cAMP-dependent protein kinase and may be adapted to meet the increased need for elevated glucagon production and/or secretion in stressful situations, such as hypoglycemia and sudden muscular activity, through activation of adenylyl cyclase. However, the ATF3 proteins do not have consensus phosphorylation sites for PKA (15). Thus, the ATF3 proteins may provide a more prolonged glucagon response by acting through increasing amounts of active protein. Because the levels of the ATF3 proteins can be up-regulated both by decreasing glucose concentration and elevated cAMP, regulation of proglucagon gene expression by ATF3 proteins may cover a broader spectrum of physiological demands, including fasting and hypoglycemia, in addition to stress responses. The increased proglucagon mRNA level seen in islets cultured for 6 h at reduced levels of glucose from 5 to 2 mM (11) and in the pancreas of 4-day fasting rats (12) may be partially because of positive contributions of increased ATF3 proteins to the expression of the proglucagon gene. Overall, we have demonstrated the existence of a novel mechanism for regulation of the transcription of the proglucagon gene by glucose and cAMP signals in addition to the CREB protein in pancreatic α cells.

Although high ATF3 levels are present in α cells, whether this mechanism is dominant compared with the role of CREB or how specific it might be for α cells as compared with two other cell types, such as the enteroglucagon cell and the others (36) and ourselves. With forskolin/IBMX, some combination of CREB/ATF3 proteins (upper panel, column 14 versus 13, p < 0.05, n = 3) showed enhanced effects compared with that of each alone. This result may reflect partial overlapped positive effects of ATF3 and CREB proteins in response to cAMP signal. It should be noted that although the double banding pattern of ATF3b expression seen in Fig. 5C suggests phosphorylation, this could not be demonstrated (data not shown). Therefore, it is more likely that the lower band arises from initiation at an internal ATG codon (see Fig. 1).
recently been demonstrated in enteroendocrine L cells. However, regulation of proglucagon gene expression by lithium via this pathway has not been shown in pancreatic α cells (27).

In summary, in the present study we have characterized the specific binding ability of ATF3 and ATF3b with the CRE/ATF motif of the proglucagon gene and demonstrated a novel mechanism in addition to CREB by which the transcription of this gene may be regulated in response to cAMP signals and to glucose fluctuations in pancreatic α cells. Because pancreatic α cells play an important physiological role in responding to low glucose levels, a stressful condition for many cell types, the α cells appear to be equipped with a multifaceted mechanism to support this feature. The higher levels of bZip proteins, including ATF3 and ATF3b, in α cells may be a part of this mechanism. Further dissection of the interactions of ATF3 proteins with some of the other bZip protein family members may extend our understanding of this aspect of proglucagon gene expression.

Acknowledgments—We thank Raymond Carroll, Paul Gardner, Jeffrey Stein, and Margaret Milewski for technical assistance and Rosie Ricks for expert assistance in preparing this manuscript. Our thanks also to Graeme Bell and Louis Philipson for encouragement.

REFERENCES
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doi: 10.1074/jbc.M305456200 originally published online June 18, 2003

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