Chapter 6
CREB Glycosylation Potentiates Apoptosis in Pancreatic Cell Lines by Suppressing Gene Expression of Targets of CREB Regulation

Summary. CREB regulates cell specific processes such as insulin secretion, growth and survival in pancreatic β-cells [1-8]. The survival of pancreatic β-cells depends on the balance between survival and apoptotic signaling cascades that antagonize one another (Fig. 6-1). The fate of the cell depends on the outcome of the “tug of war” between these two forces [9]. Three kinase signaling cascades that promote β-cell survival are the cAMP-dependent protein kinase (PKA), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and mitogen activated protein kinase kinase (MEK)/extracellular signal related kinase (ERK) cascades [4, 10, 11]. The effects of all three kinases signaling cascades are, in part, mediated by activation of CREB. Of particular interest to us were studies showing that CREB phosphorylation by PKA and MEK/ERK pathways leads to expression of insulin receptor substrate 2 (IRS2), a gene critical for β-cell survival [4, 12-14]. We have looked at the effect that O-GlcNAc glycosylation has on CREB activity and the outcomes of increased O-GlcNAc glycosylation on pancreatic β-cell survival. We found that the inhibition of CREB activity by O-GlcNAc glycosylation results in a decrease in the CREB-dependent expression of IRS2, as well as other targets of CREB, and a subsequent increase in the level of programmed cell death observed in these cells.
Glucosamine Treatment Reduces Expression of IRS2 and Other CREB-Dependent Genes. The ability of CREB to regulate pancreatic β-cell survival led us to examine whether CREB glycosylation leads to silencing of IRS2. CREB has been shown to promote islet cell proliferation and viability by regulating the expression of cell cycle
associated factors, cyclin A and anti-apoptotic factors such as cIAP-2, IRS2 and Bcl-2 [4, 13, 15, 16].

We first tested whether activation of the PKA, which has been shown to induce CREB-dependent expression of the luciferase reporter, can also induce expression of endogenous targets of CREB-dependent gene expression. Observation of IRS2 protein levels showed that forskolin (Fsk) treatment increased IRS2 expression, consistent with previous studies, and that pretreatment with GlcN reduced Fsk-induced expression of IRS2 by approximately 21% (Fig. 6-2) [4]. At the same time that decreases in the levels of IRS2 were observed, there was no significant change in the level of CREB present in the cells (Fig. 6-2). Thus the decrease in IRS2 levels are likely as a result of increased protein degradation or a reduction CREB transactivation of the IRS2 gene.

**Figure 6-2.** GlcN treatment of pancreatic HIT-T15 cells reduces basal and Fsk-induced IRS2 levels but has no effect on CREB. Cells were treated in the presence or absence of GlcN (10 mM, 9 h). Fsk (10 μM) was added to the medium 3 h after addition of GlcN, and the cells were incubated for an additional 6 h. IRS2 protein expression was analyzed by immunoblotting of cell lysates using anti-IRS2 (Upstate, Charlottesville, VA; 1:1,000) and anti-CREB (Upstate, Charlottesville, VA; 1:1,000) antibodies and corrected to protein concentration as measured by anti-αtubulin (Sigma-Aldrich, St. Louis, MO; 1:5000). Values were normalized relative to the untreated cells and represent the mean ± SEM (Student’s T-test, * P < 0.03; n = 4).
Previous reports have found that O-GlcNAc glycosylation is capable of affecting protein levels at the level of transcription, translation and degradation [17, 18]. To confirm that IRS2 expression was affected at the level of transcription, relative IRS2 mRNA levels were measured 30 min after Fsk treatment by RT-PCR. IRS2 gene transcription was induced by Fsk treatment and the expression was inhibited by GlcN (Fig. 6-3). In contrast, transcription of IRS1, which along with IRS2 is responsible for transduction of insulin and IGF-1 receptor signaling [19], was unaffected by Fsk activation of cAMP signaling and showed only a slight inhibition in response to GlcN treatment (Fig. 6-3B). The promoter of IRS1 does not contain any full or half-CRE sites and previous studies have shown that the IRS1 gene is not controlled by CREB [4, 20].

![Image of Figure 6-3](image.png)

**Figure 6-3.** CREB-dependent gene expression is suppressed by glucosamine in HIT-T15 cells. Fsk-induced transcription of CREB-target genes, IRS2 and NR4A2, are specifically inhibited by GlcN treatment. **(A)** HIT-T15 β-cells were incubated in the presence or absence of GlcN (10 mM) 3 h prior to the addition of Fsk (10 μM). 60 min after the addition of Fsk, RNA was harvested from cells and used in first strand cDNA synthesis using oligo dT_{12-18} primers (Invitrogen). cDNA was used in semi-quantitative PCR to determine the relative levels of IRS1, IRS2, DUSP1 and NR4A2 transcripts normalized to level of β-actin transcripts. **(B)** GlcN reduced IRS2 and NR4A2 expression on an order equivalent to that observed by IRS2 immunoblotting shown in Figure 6-2. DUSP1, which is also controlled by CREB, was not affected by GlcN. The relative intensity of PCR products was quantified with an Alpha Imager 3400 (Alpha Innotech Corp., San Leandro CA) using software AlphaEaseFC software version 4.0.1 (Alpha Innotech Corp.). Values represent the mean ± SEM (Student’s T-test, * P < 0.04; n = 5).
IRS1 is thought to play a larger role in the transduction of insulin signaling and, unlike IRS2, knockout of IRS1 has a minimal effect on pancreatic β-cell survival [19].

In addition to IRS2 transcription, we looked at the effects of GlcN on the transcription of other targets of CREB activity. Gene-targets were chosen based on the reported CREB:DNA occupancy reported in genome-wide ChIP studies [20]. Transcription of the steroid nuclear hormone receptor NR4A2 and the dual-specificity phosphatase 1 (DUSP1, also known as MAPK kinase phosphatase 1), both identified as targets of CREB, was induced robustly by Fsk treatment (Fig. 6-3A) [4, 20, 21]. The Fsk-induced transcription of Nr4A2 was inhibited by GlcN (Fig. 6-3B). NR4A2, like IRS2, has been implicated in the prevention of apoptosis, and the reduction of Nr4A2-induced expression by GlcN could reduce a cell’s ability to withstand increases in pro-apoptotic stress [4, 21-23]. Surprisingly, no changes in the level of Fsk-induced transcription of DUSP1 were observed in response to GlcN treatment (Fig. 6-3B). DUSP1, like IRS2 and Nr4A2, promotes cell survival [24]. DUSP1 inhibits apoptotic pathways through dephosphorylation/deactivation of p38 MAPK and c-Jun NH2-terminal kinase (JNK) (Fig. 6-1) [25, 26]. In fact, DUSP1 over expression has been linked to pancreatic cancer and may contribute to tumorigenicity through suppression of MAPK and JNK signaling [27]. It may be that DUSP1 expression is also abnormally expressed in the pancreatic β-cell lines HIT-T15 and MIN6, used in these studies.

These findings support the role of CREB in the regulation of anti-apoptotic genes IRS2, NR4A2 and DUSP1 and suggest that glycosylation of CREB can reduce the level of cAMP-induced expression of IRS2 and Nr4A2. The different effect of GlcN treatment on IRS2 and Nr4A2 compared with DUSP1 suggests that O-GlcNAc glycosylation may
enable differential regulation of specific genes and may depend on the occupancy of OGT at particular promoters or require additional cofactors to mediate the down-regulation of CREB-target gene expression.

**OGT Associates Directly With RNA Polymerase II-Dependent Gene Promoters.** A large number of proteins involved in gene expression are O-GlcNAc glycosylated (Table 2-1), and the fact that OGT is concentrated in the nucleus suggests an active role for OGT in the regulation of gene expression. Studies have found that OGT can interact with a number of co-repressor complexes, and these interactions would have the potential to confer specificity to OGT activity through directed localization of OGT. Chromatin immunoprecipitation using an anti-OGT antibody was used to investigate whether OGT associates specifically with CREB-regulated genes.

*IRS1, IRS2* and *NR4A2* promoter DNA were all recovered by chromatin immunoprecipitation (ChIP) using an OGT-specific antibody in MIN6 cells and pancreatic islets, whereas only *IRS2* and *NR4A2* promoter DNA were isolated by ChIP using a CREB antibody (Fig. 6-4A). These findings suggest that the *IRS2* and *NR4A2* genes are direct targets for regulation by OGT and CREB in pancreatic β-cells. Interestingly, DUSP1 was isolated by both CREB and OGT-ChIP experiments in isolated pancreatic islets but was only isolated by CREB-ChIP experiments in MIN6 cells (Fig. 6-4A). This difference could reflect changes that result from the immortalization of the pancreatic β-cell lines, as noted earlier dysfunction in the regulation of DUSP1 expression is linked to pancreatic cancer cell tumorigenicity [27, 28].
When multiple primer sets were used to test the specificity of the CREB and OGT interaction with the IRS2 gene, ChIP analysis showed that CREB was bound precisely to a region of the promoter roughly 200 bp upstream of the initial ATG. While OGT was enriched within the promoter region of IRS2, it was also bound to DNA further downstream, well within the coding region, suggesting a less stringent means of localization for OGT and associated corepressors (Fig. 6-4B). Similar experiments have been performed on a genome-wide scale to identify transcription factor, such as CREB,
and other DNA-binding factor localization and specific targeting of a transcription factor within a gene [20, 29-32].

**Glucosamine Stimulates Apoptotic Signaling.** IRS2 has been shown to promote islet-cell survival through a cAMP-dependent pathway that potentiates insulin/IGF-1 signaling through the phosphatidylinositol 3-kinase (PI3K)/Akt kinase and ERK1/2 signaling pathways [5, 11, 33]. Consistent with a role for IRS2 in β-cell survival, the addition of GlcN promoted β-cell apoptosis, as demonstrated by enhanced caspase-3/7 activity (Fig. 6-5). GlcN-induced apoptosis is of the same magnitude as that produced by camptothecin (0.2 mg/ml), which causes apoptosis via irreversible DNA-double strand breaks during replication and transcription [34]. Co-treatment of cells with GlcN and camptothecin showed that activation of caspase-3/7 by GlcN and camptothecin was additive. To examine the contribution of the O-GlcNAc modification to the apoptotic effects of GlcN, we used small interfering RNA (siRNA) to silence the expression of OGT. Introduction of OGT siRNA into MIN6 cells produced a significant reduction in OGT expression (Fig. 6-6). Cells transfected with the scramble siRNA control displayed increased caspase-3/7 activity.

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**Figure 6-5.** O-GlcNAc glycosylation of CREB attenuates β-cell survival. GlcN treatment of pancreatic MIN-6 β-cells induces apoptotic cell death. Cells were treated with GlcN (10 mM) and/or camptothecin, which induces apoptosis via DNA damage, (0.2 μg/mL) for 18 h, and apoptosis was quantified by measuring caspase-3/7 activity (Promega, Madison, WI). Values were normalized relative to the untreated GlcN control and represent the mean ± SEM (Student’s T-test, * and **P < 0.05; n = 4).
activity in response to treatment with GlcN and Fsk (Fig. 6-6). In contrast, no change in GlcN-induced apoptosis was observed upon reduction of OGT expression. The relative induction of caspase-3/7 activity, caused by GlcN treatment, observed in cells treated with the scrambled siRNA was muted when compared to the levels previously observed (Fig. 6-5). It may be the case that the electroporation method used to shuttle the siRNA into the cell also increases caspase-3/7 activity and as a result reduces the magnitude of the GlcN-induced apoptosis. The observation that introduction of OGT siRNA into β-cells increases the overall level of apoptosis is consistent with previous studies, which demonstrate that OGT is required for cell survival and that hypo-glycosylation may also be deleterious to cell survival [35].

We suggest that the functions of OGT may be finely balanced, such that under normal conditions OGT contributes to the survival of
β-cells, but conditions leading to enhanced OGT activity can have deleterious effects through a mechanism involving the glycosylation of CREB.

Finally, we tested whether blocking CREB glycosylation would overcome the apoptotic effects of GlcN. Cells transiently expressing either wild-type CREB or the AAA mutant form were monitored for caspase activity following treatment with GlcN and Fsk. Cells were monitored to ensure that expression of recombinant CREB did not lead to transcriptional squelching of CREB activity (Fig. 5-11), a phenomena where in over expression of a transcription activator inhibits the activators activity, presumably by competition for cofactor and RNA polymerase II constituents between the DNA bound factor and an excess unbound activator [36]. While GlcN treatment led to increased caspase-3/7 of pancreatic β-cells expressing wild-type CREB, prevention of glycosylation via the AAA mutation reduced the level of caspase-3/7 activity (Fig. 6-7). These results provide direct evidence that O-
GlcNAc glycosylation of CREB contributes to GlcN-induced pancreatic β-cell death and that inhibition of CREB glycosylation may play a protective role.

**Conclusions**

We have demonstrated that O-GlcNAc glycosylation of CREB represses CREB-dependent transcription in pancreatic cells. Glycosylation functionally opposes phosphorylation and provides a novel mechanism for the regulation of CREB activity. One important consequence of CREB glycosylation may be to down-regulate the IRS2 gene, thereby reducing the strength of insulin and IGF-1 anti-apoptotic signaling and thus promoting the death of pancreatic β-cells (Fig. 6-8). We also observed a reduction in the expression of the NR4A2 gene in response to GlcN treatment of pancreatic cells, but no effect on the DUSP1 gene, which is also controlled by CREB. These observations suggest that O-GlcNAc glycosylation may enable CREB to regulate different programs of gene expression by moderating the effect of CREB phosphorylation through the targeting of OGT to specific CREB-bound promoters.

Studies have shown that OGT associates with the co-repressors mSin3a, N-CoR and SMRT, suggesting that OGT may play a role in creating a silencing environment through histone deacetylation and O-GlcNAc glycosylation [37, 38]. The make-up of repressor complexes with which OGT is associated may dictate its targeting. As OGT is recruited directly to the NR4A2, IRS1 and IRS2 promoters, the targeting of OGT and O-GlcNAc modifications to particular promoter regions might provide a mechanism to silence specific genes in response to increased flux through the hexosamine biosynthesis pathway and other stimuli. In the future it would be interesting to undertake comprehensive study of the targeting of OGT to promoters and its effects on CREB-
dependent gene expression in order to determine which select programs of CREB-dependent gene expression are targeted by OGT, or whether that O-GlcNAc glycosylation affects the entire CREB regulon.

**Figure 6-8.** CREB glycosylation and the regulation of β-cell survival. Increasing CREB glycosylation via GlcN treatment moderates the phosphoSer133-dependent CREB transactivation by forskolin. OGT associates directly with CREB at promoters to inhibit CREB-dependent gene expression. This inhibition may result in an increase in apoptosis as the expression of CREB-dependent anti-apoptotic genes (IRS2 and Nr4A2) are reduced.

**Role for CREB Glycosylation Beyond Pancreatic β-Cells.** CREB-dependent gene expression is important in a number of cell types, including neurons, where it is activated in response to the consolidation of long-term memory as well as pro-survival signaling. In neurons, as in pancreatic β-cells, the insulin/IGF-1 signaling pathway has been
implicated in the inhibition of apoptosis, and both cell types highly express OGT [39-41]. Given the importance of CREB and insulin/IGF-1 signaling in neuronal cell survival and the prevalence of OGT in both cell types, future experiments could address the possibility that OGT acts in a similar manner in neurons to moderate CREB-dependent gene expression and enhance cellular apoptosis (Fig. 6-9).

Regulation of gene expression involves complex interactions among histones, transcription factors, coactivators and corepressors. An emerging concept is that transcription factors may be modified by multiple post-translational modifications simultaneously, coordinating distinct biological programs. Our studies demonstrate for the first time that O-GlcNAc glycosylation of CREB modulates its ability to activate gene expression. The levels of global O-GlcNAc glycosylation reflect the net effect of metabolic flux through the hexosamine biosynthesis pathway, and may thus function as a new integrating signal to coordinately regulate distinct patterns of gene expression.
Bibliography


