Chapter 4
CREB Glycosylation Represses Transcriptional Activity *In Vitro*

Summary. Analytical studies of recombinant CREB glycosylation revealed that there are at least two major sites of *O*-GlcNAc glycosylation within the Q2 domain of CREB. The specific localization may be indicative of a prescribed function for *O*-GlcNAc glycosylation in modifying the molecular mechanisms that underlie the specific activity of these domains. Recombinant CREB that had been hyper-glycosylated by co-expression with OGT in insect cells and CREB that had been expressed alone were used to test the possible effects of *O*-GlcNAc glycosylation. In this chapter, I will detail initial forays into functional studies of CREB glycosylation using recombinant CREB as a model system. *In vitro* assays of CREB bZIP and Q2 domain function found that *O*-GlcNAc glycosylation specifically disrupts recruitment of the RNA polymerase II initiation complex and inhibits CRE-mediated transcription.

Co-expression of CREB and OGT *In Vitro*. To study the functional implications of CREB glycosylation, we used CREB that had been expressed in insect cells in the absence and presence of OGT. We first determined the glycosylation stoichiometry of these two forms of CREB. GalT labeling has previously been used to measure a ~5.8-fold increase in CREB glycosylation as a result of co-expression with OGT in insect cells (Fig. 3-4). To determine the glycosylation stoichiometry at the identified sites within the Q2 domain, ^{259}TST^{261}, samples were subjected to tryptic digest, and the relative abundance of di-glycosylated, mono-glycosylated and unglycosylated glycoforms of the Q2 domain peptide ^{256}TAPTSTIAPGVVMASSPALPTQPAEEAAR^{284} were quantified

by integration of chromatogram peaks from a $[MH_2]^{2+}$, $[MH_2+GlcNAc]^{2+}$ and $[MH_2+GlcNAc_2]^{2+}$ -selective ion chromatogram (Fig. 4-1A). This analysis suggested that approximately 9.6% of CREB is glycosylated when expressed in insect cells, whereas 55.4% of CREB is glycosylated when co-expressed with OGT in insect cells. It is possible that measurement of the absolute levels of CREB glycosylation by LC-MS may be underestimated, due to changes in the ionization potential for the three glycoforms.

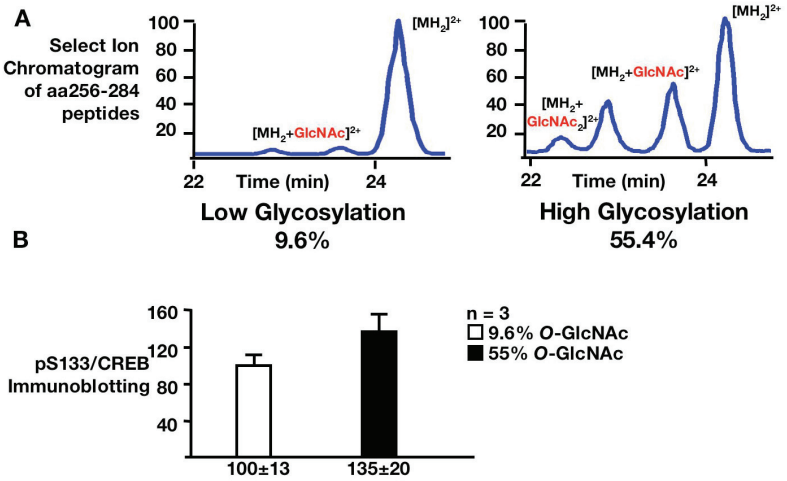

Figure 4-1. Use of recombinant CREB from insect cell expression systems to determine the effect of *O*-GlcNAc glycosylation. Recombinant CREB was expressed in insect cells in the presence or absence of OGT. **(A)** Purified CREB has either ~10% glycosylation stoichiometry in the absence of OGT co-expression, or ~55% glycosylation stoichiometry, when co-expressed with OGT. Absolute *O*-GlcNAc glycosylation stoichiometry was measured by integration of the area beneath the combined di-glycosylated ($[MH_2+GlcNAc_2]^{2+}$ = 1614.9 *m/z*), mono-glycosylated ($[MH_2+GlcNAc]^{2+}$ = 1513.4 *m/z*) and unglycosylated ($[MH_2]^{2+}$ = 1412.6 *m/z*) tryptic CREB peptide selective-ion chromatogram peaks corresponding to the ($M=^{256}TAPTSTIAPGVVMASSPALPTQPAEEAAR^{284}$). These two stocks of purified CREB can be used to compare their relative affinity for DNA, the RNA polymerase II cofactors and their ability to transcribe DNA. *In vitro* analysis provides a controlled environment in which to determine the functional implications of CREB glycosylation. **(B)** The relative levels of phosphoSer133 were measured by immunoblotting and indicate a possible increase in phosphorylation. Samples of purified CREB were resolved by SDS-PAGE and relative phosphoSer133 and CREB levels were measured by immunoblotting (Student's T-test, * P = 0.20, n=3).

However, the relative difference in glycosylation stoichiometry between the two CREB forms measured by LC-MS/MS is consistent with the values measured by GalT labeling, suggesting an accurate measurement.

The relative levels of Ser133 phosphorylation of CREB expressed in the presence and absence of OGT co-expression were also measured, as changes in the level of phosphorylation can have a significant effect on CREB activity *in vitro* [1, 2]. CREB co-expressed with OGT displayed a slightly higher level of glycosylation, but the increase was not statistically significant (Fig. 4-1B).

Glycosylation Has No Effect on the DNA Binding Affinity of CREB *In Vitro*.

Based on the proximity of glycosylation at ^{260}ST^{261} to the bZIP domain, it seemed plausible that glycosylation may disrupt DNA binding by either directly interfering with DNA and/or Mg^{2+} contacts or inducing changes in protein folding that affect DNA binding [3]. The ability of CREB with low (9.6%) and high (55.4%) glycosylation stoichiometry to bind to the canonical CRE element (TGACGTCA) was measured by electrophoretic mobility shift assay (EMSA) (Fig. 4-2). EMSA samples were measured by combination of CREB with either low (9.6%) or high (55.4%) glycosylation stoichiometry were incubated with [^{32}P]-labeled CRE oligonucleotides. DNA-bound CREB and free-DNA were resolved by native gel electrophoresis and analyzed by fluorography. The specificity of the CREB:CRE interaction was affirmed by the addition of excess unlabeled CREB oligonucleotides or Sp1 oligonucleotides. Only the cold CRE oligonucleotides were able to prevent CREB from binding the [^{32}P]-labeled CRE oligonucleotides, through competitive inhibition. No significant difference between CREB with low (9.6%) and high (55.4%) glycosylation stoichiometry was observed in the affinity or specificity for the CRE sequence.

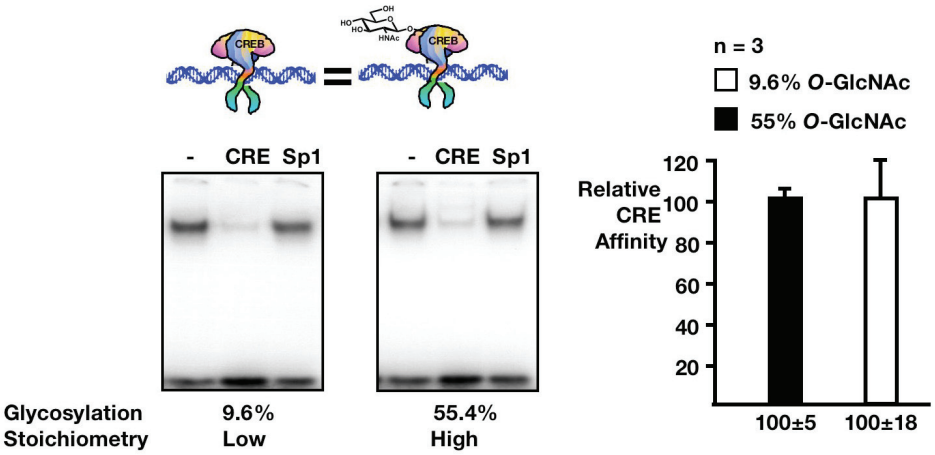

Figure 4-2. *O*-GlcNAc glycosylation does not affect CREB-DNA binding *in vitro*. The affinity of recombinant CREB with low (9.6%) and high (55%) glycosylation stoichiometry was tested by incubating CREB with [^{32}P]-labeled oligonucleotides containing multiple CRE repeats. CREB bound to DNA was identified by electrophoretic mobility shift assay (EMSA). DNA bound to CREB is retarded as it runs through the native gel (see panels). The specificity of CREB binding was confirmed by the inability of an oligonucleotide containing Sp1 binding elements to compete off the [^{32}P]-labeled CRE oligonucleotide (right lane), compared to the loss of the [^{32}P]-labeled CRE oligonucleotide when reactions were incubated with an excess of unlabeled CRE oligonucleotide (middle lane). The relative DNA affinity of CREB with low and high glycosylation stoichiometry was indistinguishable. Relative DNA affinity of CREB with low and high glycosylation stoichiometry was calculated by measuring the density of the EMSA CREB:DNA bands normalized the concentration of CREB in the reaction, as measured by SDS-PAGE and Coomassie staining of reaction aliquots. Values represent the mean ± standard deviation (Student's T-test, * $P = 0.98$, n=3).

Recent studies have highlighted that, in most cases of CREB-dependent transactivation, a full-CRE element does not exist in the promoter region of the target gene [4, 5]. Until recently it had been thought that CREB bound to DNA constitutively, but work by Bernhard Mayr, Ernesto Guzman and Marc Montminy has shown that DNA binding, within cells, is dependent on regions of the Q2 domain [6]. Based on these findings, it may be of interest in the future to perform more comprehensive tests on the effects of glycosylation on DNA-binding using varying half-CRE site sequences and cellular model systems. It has also been shown that the use of chromatin templates, rather than naked DNA, is a better facsimile of the *in vivo* environment and more accurate means for measuring CREB transactivation [2]. Future studies could employ chromatin

templates to confirm that the presence of the nucleosomes does not introduce a glycosylation-dependent effect on DNA binding.

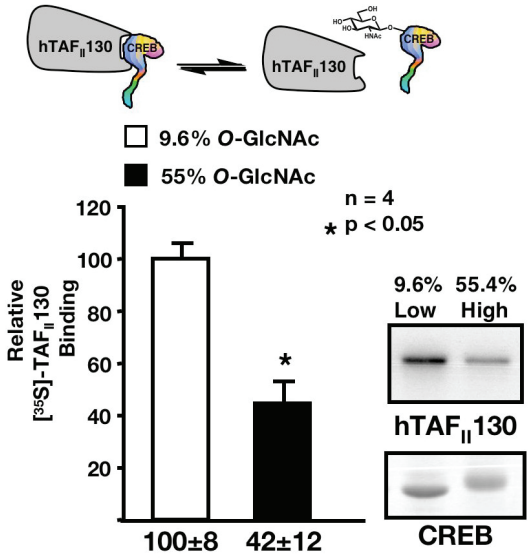

Figure 4-3. *O*-GlcNAc glycosylation reduces the CREB:hTAF$_{II}$130 interaction *in vitro*. The ability of CREB containing low or high levels of *O*-GlcNAc glycosylation to bind to [^{35}S]-hTAF$_{II}$130 was assayed by incubating [^{35}S]-hTAF$_{II}$130 and CREB and then isolating complexes using Ni-NTA affinity chromatography. The relative amounts of CREB and [^{35}S]-hTAF$_{II}$130 were measured by Coomassie staining and fluorography, respectively. Values represent the mean ± standard deviation (Student's T-test, * $P < 0.0001$, n = 14).

Glycosylation Disrupts CREB:hTAF$_{II}$130 Binding *In Vitro*. The location of the major sites of CREB glycosylation within the Q2 domain and the distribution of several secondary sites throughout the Q1 and Q2 domain suggested that glycosylation may affect the ability of these domains to recruit the RNA polymerase II complex [7-9]. The Q2 domain in particular is required for sequestering TFIID via direct contacts with hTAF$_{II}$130 within two specific regions: ^{189}GAIQL^{193} and ^{204}LQTL^{207} [10-12]. To test the effect of glycosylation on hTAF$_{II}$130 recruitment, CREB with low (9.6%) and high (55.4%) glycosylation stoichiometry was bound to Ni-NTA agarose and *in vitro* translation reactions containing [^{35}S]-methionine-labeled hTAF$_{II}$130 were run over agarose. hTAF$_{II}$130 captured by the agarose bound-CREB was visualized by fluorography and quantified by densitometry after normalization for the CREB levels. The increased level of CREB glycosylation had a significant effect on the ability of CREB to associate with hTAF$_{II}$130 (Fig. 4-3). The

roughly 5.8-fold increase in CREB glycosylation resulted in 2.4-fold decrease in the amount of bound $hTAF_{II}130$. Recruitment of the RNA polymerase II complex by the Q2 domain is required for CREB activity, and the inhibition of the interaction by *O*-GlcNAc glycosylation may act to reduce CREB-dependent gene expression.

Glycosylation Reduces CREB-Dependent Transcription *In Vitro*. Based on the observation that *O*-GlcNAc glycosylation reduces CREB's ability to recruit $hTAF_{II}130$ and, by extension, RNA polymerase II, we next tested the effect of glycosylation on CREB-mediated transcription *in vitro*. Recombinant CREB with low (9.6%) or high (55.4%) glycosylation stoichiometry was combined with HeLa nuclear extracts and assayed for its ability to initiate transcription using a linear DNA template driven by a minimal promoter containing four full cAMP response elements and a TATA box. Consistent with the ability of *O*-GlcNAc to prevent $hTAF_{II}130$ binding, increased *O*-GlcNAc glycosylation leads to a significant decrease in the level of transcription mediated by CREB (Fig. 4-4). The level of inhibition was on the same order of magnitude as observed with the reduction in $hTAF_{II}130$ binding. When compared to the 4- to 5-fold increase in CREB activity mediated by phosphorylation of Ser133, the attenuation of CREB activity by *O*-GlcNAc glycosylation cannot be expected to completely suppress phosphoSer133-dependent activity, but may act to regulate the magnitude of phosphoSer133-dependent activation [2, 13].

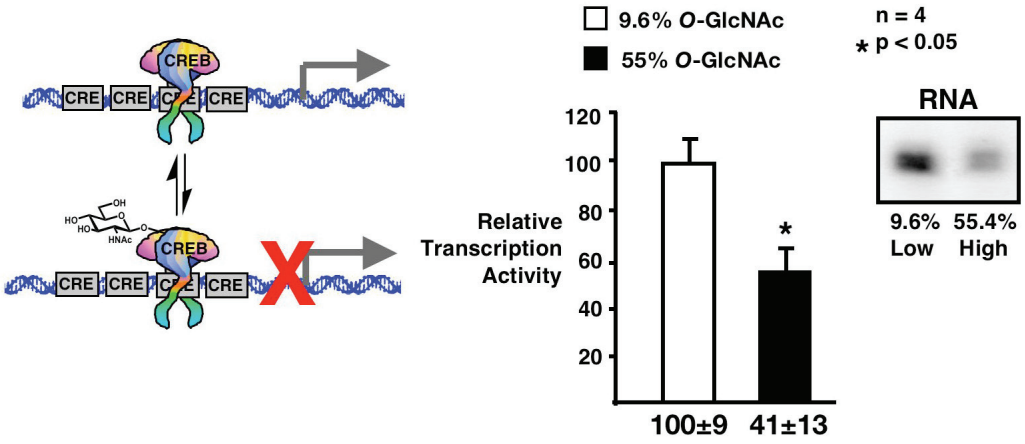

Figure 4-4. *O*-GlcNAc glycosylation reduces basal CREB transactivation *in vitro*. Recombinant CREB with high and low glycosylation stoichiometry was tested for its ability to initiate transcription using a linear DNA template driven by a 4xCRE promoter, HeLa nuclear extracts (Promega, Madison, WI) and [α-^{32}P]rGTP (Amersham Biosciences, Cambridge, MA). Relative activity was measured by resolving reactions by SDS-PAGE and measuring the relative amounts RNA transcript and CREB in each reaction by fluorography and Coomassie staining, respectively. Values represent the mean ± standard deviation (Student's T-test, * $P < 0.05$, n = 4).

Glycosylation Inhibits CREB Transactivation Via Inhibition of the Q2 Domain *In Vitro*.

In conclusion, these studies demonstrate for the first time that the transcription factor CREB is covalently modified by *O*-GlcNAc. *O*-GlcNAc glycosylation disrupts the binding interaction between CREB and hTAF$_{II}$130, thereby repressing the transcriptional activity of CREB *in vitro* (Fig. 4-5). These results provide a potential link between *O*-GlcNAc and key cellular processes that are regulated by CREB activity such as learning and memory, cell survival and growth and metabolic regulation [14, 15].

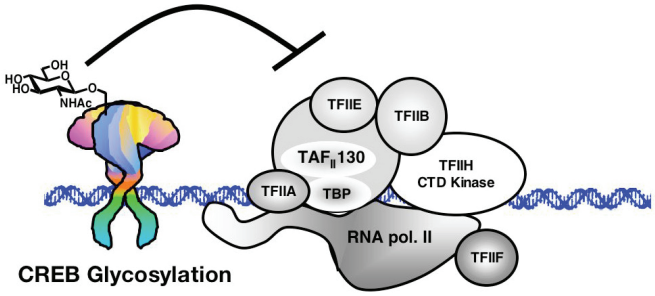

Figure 4-5. *In vitro* CREB glycosylation suppresses transcription by disrupting CREB recruitment of the RNA Polymerase II initiation complex.

Bibliography

1. Hagiwara, M., et al., *Coupling of Hormonal Stimulation and Transcription via the Cyclic AMPR-Responsive Factor CREB is Rate Limited by Nuclear Entry of Protein Kinase A*. Molecular and Cellular Biology, 1993. **13**(8): p. 4852-4859.

2. Asahara, H., et al., *Chromatin-Dependent Cooperativity between Constitutive and Inducible Activation Domains in CREB*. Molecular and Cellular Biology, 2001. **21**: p. 7892-7900.

3. Moll, J.R., et al., *Magnesium is required for specific DNA binding of the CREB b-ZIP domain*. Nucleic Acids Research, 2002. **30**(5): p. 1240-1246.

4. Impey, S., et al., *Defining the CREB Regulon: A Genome-Wide Analysis of Transcription Factor Regulatory Regions*. Cell, 2004. **119**: p. 1041-1054.

5. Euskirchen, G., et al., *CREB binds to Multiple Loci on Human Chromosome 22*. Molecular and Cellular Biology, 2004. **24**(9): p. 3804-3814.

6. Mayr, B., E. Guzman, and M. Montminy, *Glutamine Rich and bZIP Domains Stabilize CREB Binding to Chromatin*. Journal of Biological Chemistry, 2005. **280**(15): p. 15103-15110.

7. Quinn, P.G., *Distinct domains of CREB mediate basal and cAMP-regulated gene transcription*. The Journal of Biological Chemistry, 1993. **268**: p. 16999-17009.

8. Feliniski, E.A., et al., *Recruitment of an RNA polymerase II complex is mediated by the constitutive activation domain in CREB, independently of CREB phosphorylation*. Molecular and Cellular Biology, 2001. **21**(4): p. 1001-1010.

9. Feliniski, E.A. and P.G. Quinn, *The CREB constitutive activation domain interacts with TATA-binding protein-associated factor 110 (TAF110) through specific hydrophobic residues in of three subdomains required for both activation and TAF110 binding*. The Journal of Biological Chemistry, 1999. **274**: p. 11672-11678.

10. Feliniski, E.A. and P.G. Quinn, *The coactivator dTAFII110/hTAFII135 is sufficient to recruit a polymerase complex and activate basal transcription mediated by CREB*. Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(23): p. 13078-13083.

11. Saluja, D., M.F. Vassallo, and N. Tanese, *Distinct subdomains of human TAFII130 are required for interactions with glutamine-rich transcriptional activators*. Molecular and Cellular Biology, 1998. **18**: p. 5734-5743.

12. Tanese, N., et al., Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**: p. 13611-13616.

13. Cardinaux, J.-R., et al., *Recruitment of CREB Binding Protein is Sufficient for CREB-Mediated Gene Activation*. Molecular and Cellular Biology, 2000. **20**(5): p. 1546-1552.

14. Lonze, B.E. and D.D. Ginty, *Function and regulation of CREB family transcription factors in the nervous system*. Neuron, 2002. **35**: p. 605-623.

15. Mayr, B. and M. Montminy, *Transcriptional Regulation by the Phosphorylation-Dependent Factor CREB*. Nature Reviews, 2001. **2**: p. 599-609.