

Dynamic Glycosylation of the Transcription Factor CREB: A Potential Role in Gene Regulation

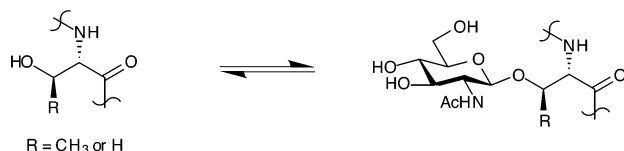
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With the recent sequencing of various genomes, increasing attention has focused on understanding how the complexity of higher organisms is encoded in a surprisingly small number of genes. In this regard, protein posttranslational modifications have emerged as an important determinant of the complexity and dynamic control of biological systems. The covalent attachment of phosphate,¹ lipid,² and other groups³ to protein side chains regulates many vital processes, including cell cycle progression, transcription, and programmed cell death.⁴

As part of a broader program to explore the functional significance of protein posttranslational modifications in the brain, we are studying the dynamic modification of serine and threonine residues by β -*N*-acetylglucosamine (*O*-GlcNAc glycosylation).



Previous studies have suggested that *O*-GlcNAc glycosylation may regulate transcription factors and other proteins in the nucleus.⁵ The addition of *O*-GlcNAc to RNA polymerase II, for example, modulates the structure of the enzyme^{3b} and appears to inhibit transcriptional elongation by preventing phosphorylation of the C-terminal domain.⁶ In addition, the *O*-GlcNAc glycosyltransferase (OGT) enzyme that catalyzes the covalent attachment of GlcNAc to substrates *in vivo* has been shown to promote gene silencing through an association with the transcriptional repressor mSin3A.⁷ Despite these intriguing observations, however, the extent of *O*-GlcNAc glycosylation in the nucleus and its impact on gene regulation are only beginning to be understood.

We report here that CREB (cyclic AMP-responsive element-binding protein), a transcription factor essential for long-term memory,⁸ is covalently modified by *O*-GlcNAc. Our studies demonstrate that *O*-GlcNAc glycosylation impairs the ability of CREB to associate with TAF_{II}130 and to activate transcription. These findings have important implications for the role of *O*-GlcNAc glycosylation in gene regulation, and they provide the first link between *O*-GlcNAc and information storage processes in the brain.

We determined that CREB was covalently modified with an *O*-linked GlcNAc sugar by radiolabeling rat brain nuclear extracts with β 1,4-galactosyltransferase (GalT). GalT catalyzes the transfer of galactose from [³H]UDP-galactose to the C4 hydroxyl group of terminal GlcNAc residues.⁹ Following radiolabeling, CREB was immunoprecipitated and resolved by SDS-PAGE (Figure 1A). Detection of the tritium label by fluorography revealed that CREB was GlcNAc glycosylated. Treatment of CREB with PNGase F (an enzyme that cleaves *N*-linked carbohydrates⁹) had no effect, confirming that the GlcNAc moiety was linked to a serine or threonine residue.

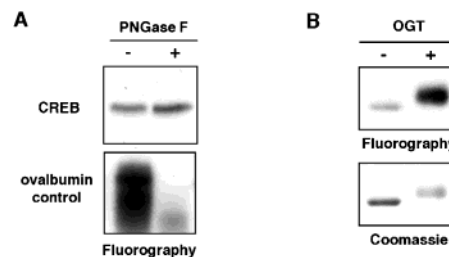


Figure 1. (A) Incorporation of [³H]-galactose into neuronal CREB (upper panel). Ovalbumin, a protein containing *N*-linked GlcNAc moieties, is shown as a positive control for PNGase F (lower panel). (B) CREB expressed in Sf9 cells shows increased incorporation of [³H]-galactose in the presence of OGT (upper panel). Coomassie-staining shows a ~2:1 ratio of protein in lane 1 as compared to lane 2 (lower panel).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to investigate whether CREB was modified with a single GlcNAc sugar or a more complex oligosaccharide. As *O*-GlcNAc is a regulatory modification present only in low cellular abundance,¹⁰ we overexpressed CREB in Sf9 cells¹¹ to generate sufficient amounts of glycoprotein for analysis. For comparison, we also coexpressed CREB with OGT, reasoning that coexpression might enhance the extent of glycosylation. Radiolabeling with GalT and [³H]UDP-galactose indicated that both forms of purified CREB were *O*-GlcNAc glycosylated, with a 5.8-fold increase in glycosylation resulting upon coexpression with OGT (Figure 1B). MALDI-TOF MS analysis of the tryptic digests of CREB revealed three major fragment ions corresponding to amino acid residues 256–284 of CREB (2821.44 *m/z*), residues 256–284 plus a single GlcNAc saccharide (3024.54 *m/z*), and residues 256–284 plus two GlcNAc saccharides (3227.62 *m/z*; Figure 2A). To confirm these assignments, CREB was reacted with UDP-galactose and GalT. As expected, the peaks at 3024.54 and 3227.62 *m/z* shifted by +161.89 and +323.73 *m/z*, indicating the covalent attachment of one and two galactose moieties, respectively.

We established that two residues within 256–261 of CREB were *O*-GlcNAc glycosylated using liquid chromatography and tandem mass spectrometry (LC-MS/MS). Four distinct peptides encompassing region 253–268 were resolved and sequenced: the unglycosylated peptide, two monoglycosylated forms, and a diglycosylated form (Supporting Information). Figure 2B summarizes the *b* and *y* ion fragments observed by LC-MS/MS for the two monoglycosylated forms. A [*b*₁₀ + GlcNAc]⁺ ion (1272.4 *m/z* obsd, 1272.7 *m/z* calcd) and [*b*₁₁ + GlcNAc]⁺ ion (1343.4 *m/z* obsd, 1343.7 *m/z*) were detected in each case, indicating two distinct sites of glycosylation within the TAPST region. Importantly, these sites are completely conserved in CREB across various species. Moreover, the same glycosylation sites were observed from tryptic digests of CREB purified from rat brain (Supporting Information). Together, these observations suggest a physiologically important role for the *O*-GlcNAc modification.

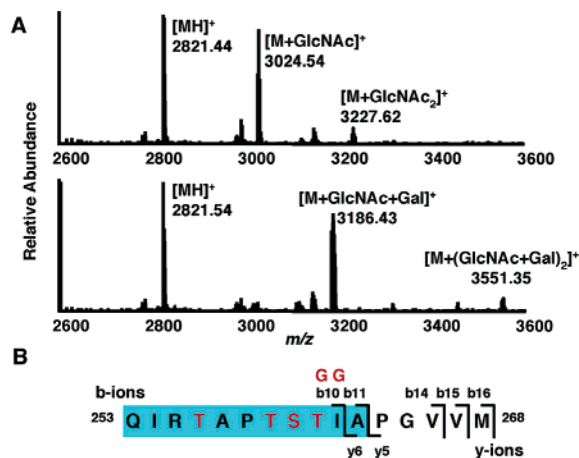


Figure 2. (A) MALDI-MS analysis of the tryptic digest of CREB coexpressed with OGT before (upper panel) and after (lower panel) reaction with GalT. (B) Correlation of all ions observed by LC-MS/MS for the two monoglycosylated species following chymotryptic digestion of CREB. Potential glycosylation sites are indicated in red. See Supporting Information for conditions and MS characterization.

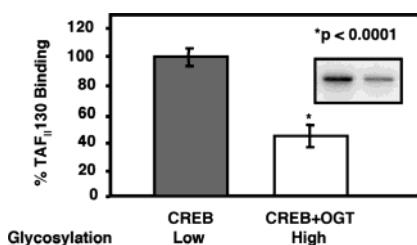


Figure 3. Ability of CREB containing low or high levels of *O*-GlcNAc glycosylation to bind to [³⁵S]-TAF_{II}130. Results are the means of 14 experiments. See Supporting Information for conditions and stoichiometry measurements.

Residues 256–261 are located in the Q2 domain of CREB, a region that recruits the TFIID transcriptional complex via an interaction with TAF_{II}130. As the recognition interface between CREB and TAF_{II}130 is known to be hydrophobic,¹² we postulated that introduction of two polar GlcNAc groups would destabilize the interaction. To test this hypothesis, we analyzed the relative ability of two forms of CREB to interact with [³⁵S]-labeled TAF_{II}130. One form contained low levels of glycosylation (9.6%, CREB expressed alone in Sf9 cells), while the other form contained higher levels of glycosylation (55.4%, CREB coexpressed with OGT in Sf9 cells). *O*-GlcNAc glycosylation significantly impaired the ability of CREB to bind to TAF_{II}130 by $57.3 \pm 14.9\%$ (Figure 3). Moreover, the reduction in binding affinity correlated with the difference in glycosylation stoichiometry between the two forms.

As loss of TAF_{II}130 binding would be expected to repress the transcriptional activity of CREB, we performed *in vitro* transcription assays using HeLa cell nuclear extracts and the two glycoforms of CREB. Transcriptional activity was analyzed by autoradiography of the RNA products and was quantified by densitometry. Consistent with the TAF_{II}130 binding studies, *O*-GlcNAc glycosylation inhibited CREB-mediated transcription by $41.5 \pm 10.7\%$ (Figure 4).

In conclusion, we provide the first demonstration that the transcription factor CREB is covalently modified by *O*-linked β -*N*-

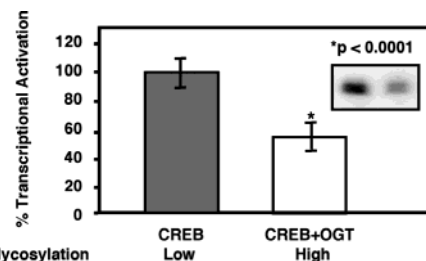


Figure 4. Transcriptional activity of CREB containing low or high levels of *O*-GlcNAc glycosylation. Results are the means of 12–14 experiments. See Supporting Information for conditions.

acetylglucosamine. *O*-GlcNAc glycosylation disrupts the binding interaction between CREB and TAF_{II}130, thereby repressing transcriptional activity *in vitro*. Our results provide the first link between *O*-GlcNAc and information storage processes in the brain. Moreover, these studies lend support to the emerging theme that *O*-GlcNAc modifications to proteins play an important role in orchestrating the dynamic and specific expression of genes.

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Supporting Information Available: Complete experimental procedures, LC-MS/MS analysis, and stoichiometry measurements (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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