The Development and Characterization of Antibodies to Site-specific O-GlcNAc modified Histones for Epigenetic Research

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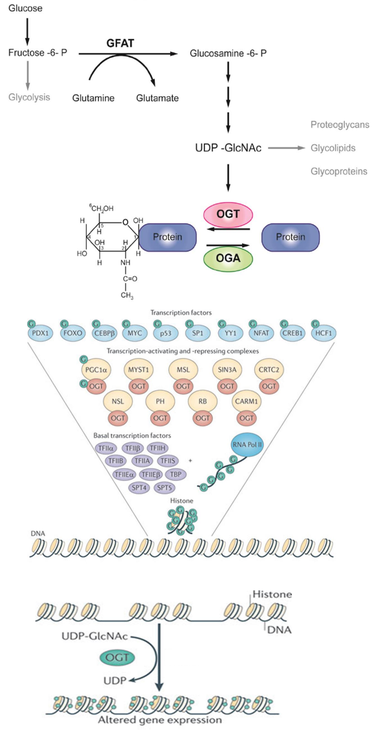
Overview

There is a growing body of evidence linking O-GlcNAcylation to the Histone Code and epigenetics, and it has been suggested that this modification may play a central role in the mechanism of diabetes and obesity risk inheritance. Our goal is to create a repertoire of well-characterized site-specific antibodies to enable research into the role that O-GlcNAc plays in epigenetics and in disease inheritance.

Introduction

O-glycosylation of nuclear and cytoplasmic proteins by a single β-N-acetyl-D-glucosamine moiety (O-GlcNAc) is a common post-translational modification that is highly dynamic and fluctuates in response to cellular stimuli. This type of glycosylation has been found on approximately a thousand human proteins to date, and is thought to be nearly as wide-spread and abundant as protein phosphorylation. In fact, O-GlcNAc often competes with protein phosphorylation, and these two modifications have extensive crosstalk in the regulation of signaling, transcription, and the functions of oncogenes and tumor suppressors.

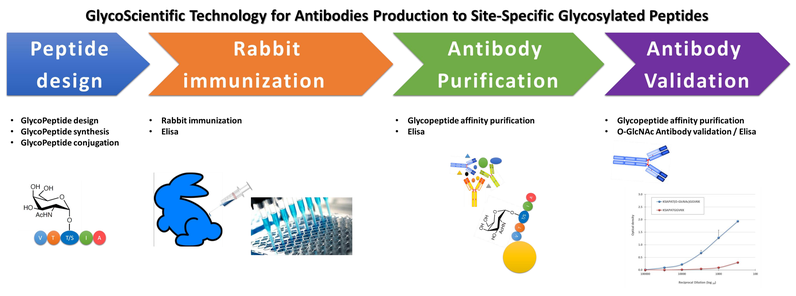
O-GlcNAc plays a role in nutrient sensing since the level of this modification is closely related to the cellular concentration of UDP-GlcNAc. Intracellular glucose is predominetly utilized as energy and catabolized by glycolysis. Approximately 3% of glucose is diverted into the hexosamine biosynthetic pathway (shown to the right). The final product of this pathway is being UDP-GlcNAc, which is the substrate for O-GlcNAc transferase (OGT) the enzyme responsible to adding O-GlcNAc to the seriene and threonine residues of various proteins. The modification is removed by O-GlcNAcase (OGA).



Many of the first proteins identified carrying this modification were transcription factors, and it has become clear in the last several years that O-GlcNAc plays a major role in chromatin remodeling and gene expression. All 4 core histones have been shown to be O-GlcNAc modified, and these sites reside at or near sites of other PTMs including phosphorylation, methylation and ubiquitylation. OGT's activity and specificity appears to be mediated by interaction with a variety of other proteins via formation of holoenzyme complexes, the make-up of which varies by cell type, developmental stage and cell cycle phase. Many of these co-factors are players in the epigenetic code and include CARM1, PGC-1a, FOX1, TET2 and TET3.

Consequently, it is believed that the O-GlcNAc modification may play a central role in the mechanism of diabetes and obesity risk inheritance.

Experimental



Acknowledgement

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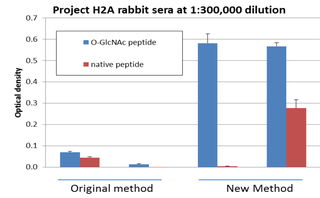
Optimized Immunization

Typical approaches for antibody generation have difficulty producing an immune response to the small neutral O-linked GlcNAc moiety attached to the peptide backbone, and thus typically produce antibodies that bind equally well to glycosylated and un-modified targets; we refer to these as agnostic and are not the desired product.

We have developed an immunization protocol that reliably produce high titers of site-specific antibodies that have >100x preference for the glycan-modified target over the non-modified form. We call these glycophilic as they are glycan "loving".

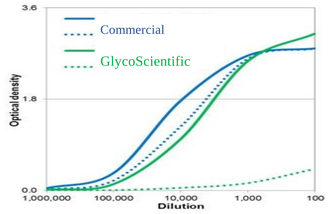


Comparison of antibody titer obtained with the original and new immunogen methods. Shown are 2 rabbits from each method. The sera is tested as is (e.g. not enriched by affinity chromatography.)

Antibody Preference to O-GlcNAcylated Peptide

The difficulty encountered while generating antibodies that recognize both the O-GlcNac and the AA sequence flanking site, necessitates evaluating the antibody's preference to the O-GlcNAcylated peptide over the unmodified species. These tests employ ELISA, as we feel that this is more indicative of solution phase interactions. to determine the

Results from the GlycoScientific antibody to O-GlcNAc on S112 of human histone H2B show that this antibody has a clear preference for the O-GlcNAc species (solid green line) over the unmodified form (dashed green line) and demonstrates that this antibodies exceeds the release specification of >50x preference for the modified form. For comparison, similar experiments were performed on a commercially available polyclonal antibody marketed as being specific for O-GlcNAc to this same site, i.e., S112 of human histone H2B. The similarity in the results obtained for the glycosylated and non-glycosylated forms of this peptide (blue traces) implies that this antibody has nearly an equal preference for the O-GlcNAcylated and native peptide (less than 3-fold preference).

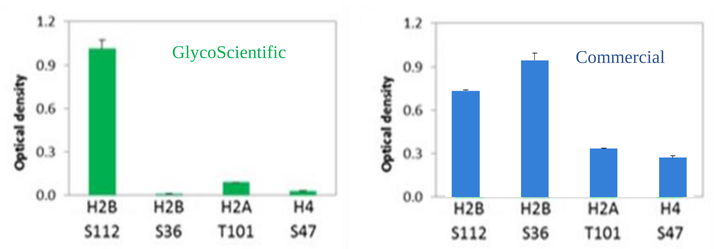


Comparing the preference of a Glycoscientific's GoPHab (Green) to a commercial antibody (Blue), both developed to detect GlcNAcylated serine 112 on human histone H2B. Binding was assessed to GlcNAcylated (solid curves) and native (dotted curves) H2B peptides via ELISA.

Antibody Specificity to Peptide Sequence

Tests are performed to evaluate the antibody's amino acid specificity, by evaluating the cross-reactivity of the antibody to other peptide sequences modified with O-GlcNAc. This experiment measures the amount of antibody remaining bound, after washing, to microtiter plate wells coated with BSA conjugated to either the target glycopeptide or O-GlcNAcylated peptides having amino acid sequences that differ from the target. Results from this type of study are shown for GlycoScientific's GlcNAc-S112 H2B. In this experiment, the binding of this antibody to the target glycopeptide (left most bar) was compared to several O-GlcNAc peptides whose sequences corresponded to other known modification sites on human histones (S36 on H2B, T101 H2A, and S47 on H4.) As displayed by the differences in optical densities, this antibody has approximately a 20x preference for the glycopeptide with the correct sequence.

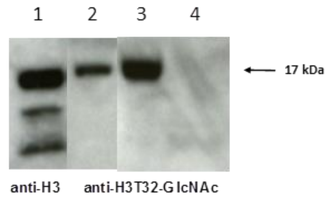
This type of specificity test was performed on a commercial antibody to O-GlcNAc on S112 H2B. Here, the antibody was found to have a higher affinity to a non-target glycopeptide, an O-GlcNAc a different site of H2B than to the targeted site of O-GlcNAc. Amazingly there is <20% amino acid sequence homology between these two glycopeptides.



Comparing the specificity of a Glycoscientific GoPHab (Green) to a commercial antibody (Blue), both developed to detect GlcNAcylated serine 112 on human histone H2B. Specificity was assessed for binding to its target glycopeptide vs glycopeptides corresponding to known O-GlcNAc sites on histones H2B, H2A and H4.

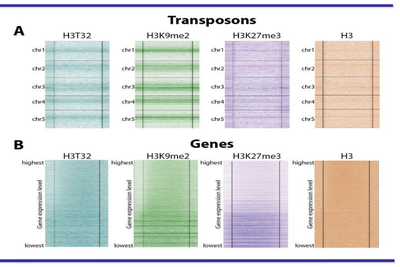
Antibody Specificity to Peptide Sequence

Tests are performed to evaluate the antibodies ability to recognize the full-length target protein, often this is performed by immunoprecipitation. Results from immunoprecipitation using Glycoscientific histone 3 with the site of O-GlcNAc attachment to threonine 32 (H3-T32) is shown. Briefly, proteins were obtained from Arabidopsis seedlings. The protein extract was incubated overnight with either anti-H3 (lane 1, Abcam ab1791) or our H3-T32-G coupled magnetic protein A beads, and washed four times before eluting with SDS loading buffer. Western detection was performed with anti-H3 (ab1791). These experiments demonstrate that H3-T32-G does indeed capture the target histone. It is interesting to note that there are multiple species caught by anti-H3, which are not present with the O-GlcNAc antibody. Presumably, these other bands are H3 fragments that are not glycosylated, supporting the selectivity of the H3-T32-G towards the O-GlcNAcylated species. To further evaluate the specificity of our H3-T32-G antibody, the glycopeptide target or the non-glycosylated peptide target were used to wash the beads before elution with SDS. The peptide had no effect on the amount of H3 retained on the beads (lane 3), while a wash with the glycopeptide (lane 4) resulted in the loss of all H3 signal. These results strongly suggest that our H3-T32-G is selective for the presence of the O-GlcNAc on the intact protein.



Antibody Specificity to Peptide Sequence

Having exceeded the ENCODE criterian, we evaluated several of our site-specific O-GlcNAc histone antibodies for their potential as ChIP-Seq reagents. Results from these experiments on *Arabidopsis thaliana* using H3-T32-G, H3K9me2, H3K27me3 and H3 (control) are shown, with (A) being transposons and (B) being genes. The H3K9me2 (antibody to histone H3 that is dimethylated at Lys9) and the H3K27me3 (antibody to histone H3 that is trimethylated at Lys 27) are standard Chip-Seq antibodies and used as positive controls. The intensity of the color indicates enrichment of the histone modification. Generally, H3T32-G enriched in transposons and genes that are lowly expressed, which are regions typically silenced. The pattern observed with H3T32-G is similar to K9me2 suggesting that H3T32-G is associated with heterochromatin formation. These results demonstrate the ability to perform ChIP-Seq experiments with the antibodies we have generated.



Conclusions:

Combined the results described above demonstrate that we can generate site-specific O-GlcNAc antibodies that have a strong preference towards the modified species over the non-modified form and have high specificity to the correct amino acid sequence, and that these are superior to the commercial antibodies evaluated. Furthermore, our antibodies are capable of capturing the correct target via immunoprecipitation and can be used with CHIP-sequencing. We have created such antibodies to GlcNAc T101 on H2A, GlcNAc S112 on H2B, GlcNac T32 H3, GlcNAc S47 on H4, and are in the process of developing more site-specific antibodies of interest to epigenetic researchers. You can see all of our site-specific O-GlcNAc antibodies and other reagents at **https://www.glycoscientific.com/**