

Novel Rabbit Monoclonal Antibodies for Profiling of Ser/Thr O-GlcNAc Modified Proteins.

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INTRODUCTION

A distinct form of protein glycosylation, beta-linked N-acetyl-glucosamine (GlcNAc) moieties can be added to serine or threonine residues of proteins (1,2). This differs from other forms of glycosylation, as it typically is a single moiety rather than the complex branched sugars that are more commonly studied. It is thought that these modifications happen in a dynamic cycle reminiscent of phosphorylation (3). GlcNAc modified proteins are found in the cytoplasm and nucleus and are modulated by means of specific O-GlcNAc transferases (OGT) as well as GlcNAcase activity (OGA). O-GlcNAc modification of proteins plays an important role in many cellular processes, including metabolism, growth, morphogenesis, apoptosis, transcription, and cancer (4).

METHODS

Polyclonal antibodies were produced by modifying serine and threonine residues with O-linked GlcNAc randomized peptide libraries coupled to KLH. Rabbits were selected for monoclonal antibody development based on reactivity in ELISA and western blot assays. Rabbit monoclonal clones were then produced, tested, and selected for scale-up and additional testing. ELISA and western blot assays were performed as described (5). GlcNAcase activity was inhibited using Thiamet-G (TMG) on COLO205 and HeLa cells at 10 μ M for 6-8 hr at 37°C. PTMScan™ analysis was performed using immunoaffinity enrichment with a mixture of O-GlcNAc positive clones on trypsin digested peptides from HeLa cells treated with TMG.

RESULTS

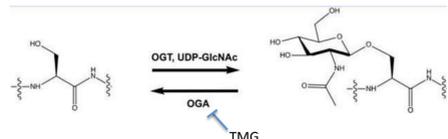
Four final clones were acquired and tested further by western blot analysis and on PTMScan™ analysis. Whole cell extracts from COLO205 and HeLa cells untreated or treated with TMG for 6 hours were analyzed by western blot and it was found that two of the four clones gave strong signal that shows induction with TMG treatment. These clones have also shown specificity with blocking of the signal using free free N-acetyl-D-glucosamine, or N-acetyl-D-galactosamine. Similarly these two clones were also found to enrich for more GlcNAc modified peptides using our PTMScan™ protocols followed by LC-MS/MS analysis. Analysis of hundreds of non-redundant peptides shows different GlcNAc motifs found for each clone. A mixture of these two clones has been used for further testing and analysis for use in both western blotting and for PTMScan™ analysis.

CONCLUSIONS

- New CST mixed IgG monoclonal #82332 appears stronger than mouse IgM CTE110.6 (1)
- Expected induction of signal with TMG treatment observed by Western blotting
- Specificity for GlcNAc over GalNAc demonstrated through blocking studies
- New rabbit monoclonal antibody clones show promise in PTMScan protocols for specific enrichment of O-GlcNAcylated peptides upstream of LCMS analysis

REFERENCES

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2. Slawson, C. et al. (2011) *Nat Rev Cancer* 11, 678-84.
3. Hart GW and Akimoto Y. (2009) *Essentials of Glycobiology*
4. Capotosti, F. et al. (2011) *Cell* 144, 376-88.
5. Halim, A. et al. (2014) *Journal of Proteome Research* p. 6024-32.



O-GlcNAcylation Implicated Roles

- Cancer
- Neurodegeneration
- Metabolism
- Growth
- Morphogenesis
- Apoptosis
- Transcription
- Immune Avoidance

Antibody Development

Polyclonal Antibody Screening

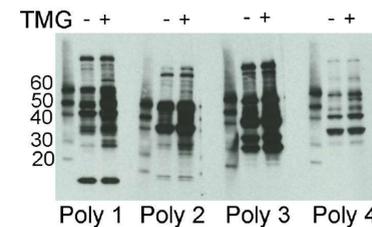


Figure 1A

Polyclonal antibodies were screened and selected for reactivity by Western blot on HeLa cells treated with Thiamet G (TMG) for 6-8 hours at a final concentration of 10 μ M. Rabbit polyclonal 3 was selected to move into monoclonal development

Monoclonal Antibody Screening

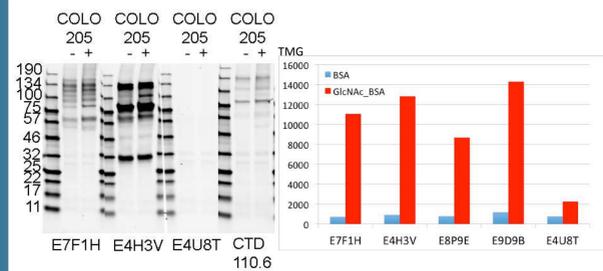


Figure 1B

Recombinant monoclonal antibodies were created using the CST XMT process. Final clones were screened and selected by Western blot and ELISA assays. For Western blots, COLO205 cells were treated with TMG for 6-8 hours at a final concentration of 10 μ M (left) and screened by ELISA assay using BSA alone or BSA conjugated to GlcNAc antigen peptide (right). A previously published mouse monoclonal antibody against O-GlcNAc CTD110.6 was used as a control (1).

Antibody Characterization

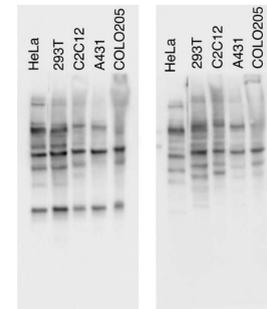
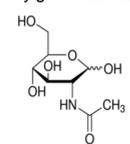


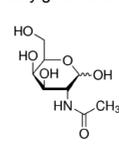
Figure 2A

Western blot analysis of various cell lines using O-GlcNAc MultiMab™ Rabbit mAb mix #82332 (left) and CTD110.6 mouse monoclonal (right).

N-Acetylglucosamine



N-Acetylgalactosamine



Sugar Specificity Blocking

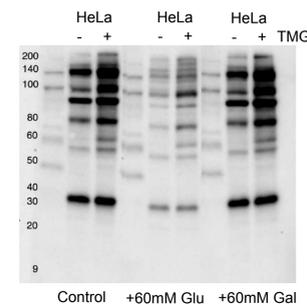
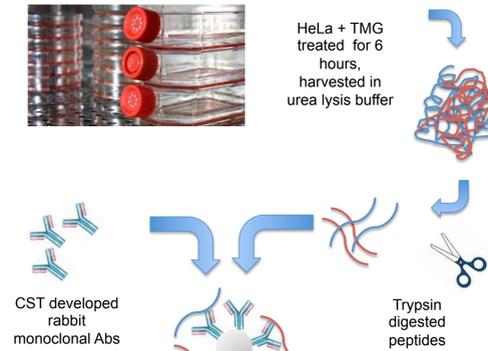


Figure 2B

Blocking experiment with highly related free sugars showed appropriate blocking by glucosamine and no cross-reactivity with galactosamine. Western blot analysis of HeLa cells untreated (-) or treated (+) with 10 μ M Thiamet G (TMG) for 6 hours, using O-GlcNAc MultiMab™ Rabbit mAb mix #82332 with no blocking (left), blocking with free N-acetyl-D-glucosamine (center), or N-acetyl-D-galactosamine (right).

O-GlcNAc Peptide Preparation



Immunoprecipitated peptides with GlcNAc monoclonal antibodies, desalted and purified

LC - MS/MS



Figure 3A

HeLa cells were grown to 75% confluence and then treated with TMG 10 μ M final concentration for 6 hours and then harvested in urea lysis buffer according to PTMScan® protocols. Proteins were then alkylated, reduced, and trypsin digested to yield peptides that were purified and lyophilized. These peptides were next immunoprecipitated using our newly developed monoclonal antibodies and further purified and desalted before being analyzed by LC-MS/MS.

Assignment of Saccharide IDs by LC-MS/MS Analysis

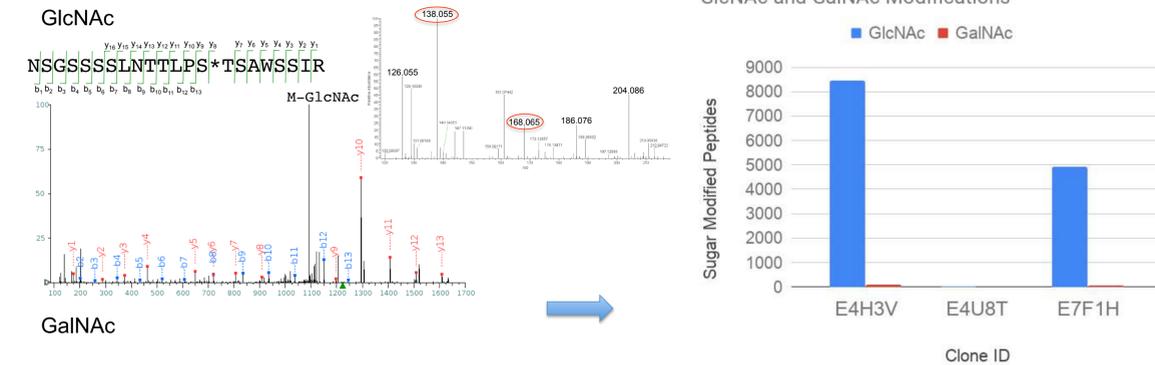


Figure 5A

LC-MS/MS analysis was performed using a 90 minute gradient on a Fusion Lumos Orbitrap to classify saccharide modified peptides after immunoaffinity-enrichment using three different recombinant monoclonal antibodies. Ion ratios (Figure 4A, B) were used to assign the saccharide modifications. Two of the clones show specificity for GlcNAc enrichment.

Figures 4A and 4B

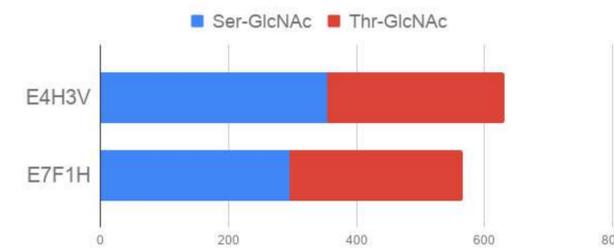
Red circles denote m/z values to assign O-GlcNAc or O-GalNAc sugar modifications. Seven key ion fragments (Table 1) were monitored to assign the different sugar modifications on peptides based on previously published studies (5).

- GlcNAc m/z peaks at 138 and 168 are higher intensity
- GalNAc m/z peaks at 126 and 144 are higher intensity
- N-Glycan core sugar structure m/z peak at 366 is absent

Table 1

Mass (m/z)	Structure	Structure	Assignment
126.055	[C ₆ H ₈ NO ₂] ⁺	[HexNAc - C ₂ H ₅ NO ₃] ⁺	High for GalNAc
138.055	[C ₇ H ₈ NO ₂] ⁺	[HexNAc - CH ₃ NO ₂] ⁺	High for GlcNAc
144.065	[C ₆ H ₁₀ NO ₂] ⁺	[HexNAc - C ₂ H ₄ O ₂] ⁺	High for GalNAc
168.066	[C ₆ H ₁₀ NO ₂] ⁺	[HexNAc - 2H ₂ O] ⁺	High for GlcNAc
186.076	[C ₆ H ₁₂ NO ₂] ⁺	[HexNAc - H ₂ O] ⁺	Found in both
204.087	[C ₆ H ₁₄ NO ₂] ⁺	[HexNAc] ⁺	Found in both
366.140	[C ₁₄ H ₂₄ NO ₁₀] ⁺	[HexHexNAc] ⁺	Branched Glycan

Ser-GlcNAc and Thr-GlcNAc



Identified O-GlcNAc Peptides

Figure 5B

LC-MS/MS Identification of peptides containing saccharide modifications on a Fusion Lumos Orbitrap with a 90 minute gradient post immunoaffinity-enrichment with two monoclonal antibodies. Fragmentation without ETD is more difficult due to neutral loss of the GlcNAc modification and leads to an overall low annotation rate.

