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Bioorganic & Medicinal Chemistry Letters 17 (2007) 3851–3854
Bioorganic & Medicinal Chemistry Letters

# A high-throughput assay for *O*-GlcNAc transferase detects primary sequence preferences in peptide substrates

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Received 6 March 2007; revised 3 May 2007; accepted 4 May 2007
Available online 10 May 2007

**Abstract**—*O*-GlcNAc transferase (OGT) catalyzes the addition of *N*-acetylglucosamine (*O*-GlcNAc) onto a diverse array of intracellular proteins. Although hundreds of proteins are known to be modified by *O*-GlcNAc, a strict amino acid consensus sequence for OGT has not been identified. In this study, we describe the development of a high-throughput assay for OGT and use it to profile the specificity of the enzyme among a panel of peptide substrates.


The glycosylation of intracellular proteins with the monosaccharide β-*N*-acetylglucosamine (*O*-GlcNAc) is a posttranslational modification implicated in many vital cellular processes.[1–4] To date, hundreds of nuclear and cytosolic proteins modified by *O*-GlcNAc have been identified. These proteins vary widely in structure and function and include RNA polymerase II,[5] cell cycle regulators,[6] heat shock proteins,[7] nuclear pore complex proteins,[8] transcription factors,[9] and cytoskeletal components.[10,11] Furthermore, genetic studies have shown that the *O*-GlcNAc modification is required for survival of both embryonic stem cells and differentiated tissues, underscoring its critical biological significance.[12,13] However, the regulation of the *O*-GlcNAc modification remains poorly understood.

The *O*-GlcNAc modification is initiated by a single enzyme, *O*-GlcNAc transferase (OGT), which transfers a GlcNAc residue from UDP-GlcNAc to select serine or threonine residues of target proteins. No consensus sequence has been identified that defines the specificity of OGT for its protein substrates, and thus the mechanism of substrate selection by OGT remains a mystery. Given the physiological significance of the *O*-GlcNAc modification, there is a pressing need for an assay that allows for interrogation of OGT's peptide specificity.

Screening of peptide libraries comprising combinatorial arrays of putative peptide targets has been an effective means for elucidating the sequence preferences of kinases and proteases.[14] To perform such a study with OGT would require an assay that monitors *O*-GlcNAc transfer onto various peptide substrates in a high-throughput manner. Traditional assays for OGT activity utilize radiolabeled substrates, require product isolation, and are not easily amenable for rapid screening.[15,16] Although a high-throughput method that detects OGT binding to UDP-GlcNAc has been reported and used for small molecule screens, its adaptation to peptide specificity profiling is not straightforward.[17]

We recently reported an assay for polypeptidyl galactosaminyltransferases (ppGalNAcTs) that capitalizes on their tolerance for an azido substituent on the acetyl group of UDP-GalNAc.[18,19] The azide provides a means to detect the glycopeptide products by Staudinger ligation with phosphine probes. We termed this assay architecture the azido-ELISA.

Similar to the substrate tolerance of the ppGalNAcTs, OGT will recognize an *N*-azidoacetyl analog of UDP-GlcNAc (UDP-GlcNAz) in both biochemical assays and in cultured cells.[20] In cells, the azide-modified substrate appears to be transferred to the same spectrum of proteins as the natural sugar.[21,22] Here, we capitalize on this finding in the development of a high-throughput azido-ELISA for OGT and we employ the assay in a preliminary peptide substrate screen.

*Keywords*: Glycosylation; OGT; *O*-GlcNAc; Consensus sequence; ELISA.
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doi:10.1016/j.bmcl.2007.05.008

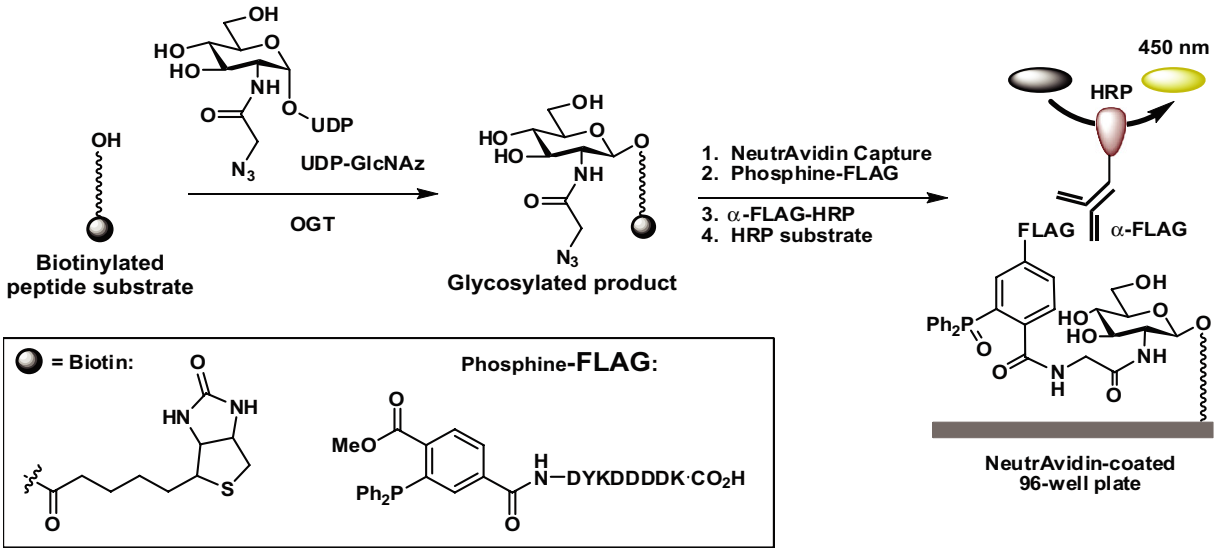


**Figure 1.** High-throughput azido-ELISA for OGT activity.

A schematic of the azido-ELISA is shown in Figure 1. Biotinylated peptide substrates are captured onto NeutrAvidin-coated 96-well plates and are subsequently covalently tagged by treatment with phosphine-FLAG.[23] The FLAG-labeled epitopes are then treated with a horseradish peroxidase (HRP)-conjugated α-FLAG antibody that allows for colorimetric readout of HRP activity.

We used a peptide from human α-A crystallin ($NH_2$-AIPVSREEK(biotin)-COOH), a bona fide physiological OGT substrate,[24] as the basis for the substrate library. We confirmed the activity of the peptide by treatment with UDP-GlcNAz and purified OGT[25] and analysis of the product by mass spectrometry (data not shown). For the azido-ELISA, enzymatic reactions were carried out under saturating conditions and were initiated with the addition of purified OGT (0.4 nM, 4 μL) to a solution containing the α-A crystallin peptide (125 μM), UDP-GlcNAz (62.5 μM), $MgCl_2$ (12.5 μM), and β-mercaptoethanol (1 mM) in a total volume of 40 μL. Reaction mixtures were incubated at 37 °C for 4 h and reactions were terminated with the addition of sodium acetate (0.4 M, 40 μL, pH 4.5). The reaction solutions were then transferred to a NeutrAvidin-coated 96-well plate (Pierce) and incubated at 25 °C for 1 h. The NeutrAvidin plate was then subsequently washed (3 × 100 μL) with phosphate-buffered saline (PBS) and treated with a solution of phosphine-FLAG (0.2 mM, 200 μL in PBS) for 2 h at 37 °C. Each well was then washed (3 × 100 μL) with a BSA blocking buffer (PBS containing 0.1% BSA and 0.05% Tween, pH 7.2) followed by the addition of a solution of α-FLAG HRP (1 μg/5 mL, 100 μL, in blocking buffer) and the plate was incubated for 1 h at 25 °C. After 3 washes with PBS (100 μL), the α-FLAG-HRP activity was then quantified by the addition of a tetramethyl benzidine (TMB) peroxide solution (100 μL, TMB Substrate Kit reagent from Pierce) for 5 min at 25 °C. Peroxidase activity was terminated upon treatment with $H_2SO_4$ (2N, 50 μL) and absorbance at 450 nm was monitored on a Molecular Devices SpectraMAX 190 microplate reader. The assay allowed for the calculation of kinetic parameters for UDP-GlcNAz ($K_m$ = 22 ± 4 μM, $V_{max}$ = 3.6 ± 0.3 μM/min) and the α-A crystallin peptide

**Table 1.** Library of α-A crystallin-derived peptides

| Peptide designation | Peptide sequence[a] | Relative activity[b] |
|---|---|---|
| **α-A crystallin** | AIPVSREEK | 1.0 ± 0.1 |
| **−4I** | **I**IPVSREEK | 1.6 ± 0.2 |
| **−4E** | **E**IPVSREEK | 1.0 ± 0.2 |
| **−4R** | **R**IPVSREEK | 2.0 ± 0.2 |
| **−4P** | **P**IPVSREEK | 1.8 ± 0.04 |
| **−3A** | AA**P**VSREEK | 0.4 ± 0.1 |
| **−3E** | A**E**PVSREEK | 0.5 ± 0.1 |
| **−3R** | A**R**PVSREEK | 0.0 ± 0.01 |
| **−3P** | A**P**PVSREEK | 0.1 ± 0.01 |
| **−2A** | AI**A**VSREEK | 0.1 ± 0.01 |
| **−2E** | AI**E**VSREEK | 0.0 ± 0.01 |
| **−2R** | AI**R**VSREEK | 0.2 ± 0.04 |
| **−1I** | AIPI**S**REEK | 0.3 ± 0.1 |
| **−1A** | AIPA**S**REEK | 0.1 ± 0.1 |
| **−1E** | AIPE**S**REEK | 0.2 ± 0.02 |
| **−1R** | AIPR**S**REEK | 0.0 ± 0.02 |
| **−1P** | AIPP**S**REEK | 0.1 ± 0.01 |
| **+1I** | AIPVS**I**EEK | 0.1 ± 0.1 |
| **+1A** | AIPVS**A**EEK | 0.0 ± 0.07 |
| **+1E** | AIPVS**E**EEK | 0.0 ± 0.05 |
| **+1P** | AIPVS**P**EEK | 0.0 ± 0.04 |
| **+2A** | AIPVSR**A**EK | 5.0 ± 0.06 |
| **+2R** | AIPVSR**R**EK | 0.6 ± 0.02 |
| **+2P** | AIPVSR**P**EK | 4.7 ± 0.7 |
| **+3I** | AIPVSRE**I**K | 0.1 ± 0.05 |
| **+3A** | AIPVSRE**A**K | 0.6 ± 0.03 |
| **+3R** | AIPVSRE**R**K | 0.2 ± 0.01 |
| **+3P** | AIPVSRE**P**K | 0.3 ± 0.1 |

Amino acid substitutions in the sequence of the parent peptide are shown in bold. Activities of the modified peptides are shown relative to the parent α-A-crystallin peptide.
Each independent experiment was performed in triplicate.
[a] Lysine residue of each peptide is biotinylated.
[b] Values shown are representative data from 3 separate experiments.