Glycoprotein profiling using Tandem Mass Tag Technology and Staudinger ligation of azido sugars

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Overview

Purpose: To identify and quantify N-linked and O-linked glycoproteins.

Methods: Jurkat cells were metabolically labeled with azido sugar derivatives, detected and enriched using Staudinger phosphine probes. Glycoproteins were identified and quantified using isobaric Tandem Mass Tag (TMT) Technology analyzed by the LTQ Orbitrap XL mass spectrometer.

Results: Over 200 proteins were identified and quantified with at least two unique peptides (FDR<2%) for enriched glycoproteins encompassing sialic acid-containing cell surface proteins and O-linked GalNAc and GlcNAc intracellular proteins.

Introduction

Protein glycosylation is a diverse post-translational modification involved in numerous biological processes including protein folding, stabilization, trafficking and signaling. Azido-modified sugars, Ac_4GlcNAz, Ac_4GalNAz and Ac_4ManNAz, are bioorthogonal analogs for endogenous amino sugars that can be used in chemoselective ligations with phosphines.[1,2] Ac_4ManNAz is converted by cells to an azido sialic acid derivative that is used for *N*-linked glycosylation of cell surface proteins. Ac_4GlcNAz and Ac_4GalNAz are predominantly incorporated into *O*-linked glycoproteins which contain *O*-GlcNAc and *O*-GalNAc, respectively (Figure 1A).

Tandem Mass Tags are isobaric chemical compounds which produce up to six different reporter ions in the MS/MS spectra allowing for relative quantitation of different samples.[3] In this study, we combined the selective enrichment glycoproteins using phosphine chemistry with TMT technology. This method allowed for the simultaneous processing of different azido sugar-labeled samples in addition to providing internal controls which aided in the identification and relative quantitation of glycoproteins containing sugars from each subclass.

Methods

Sample Preparation

Jurkat cells grown in RPMI 1640 with 10% FBS were incubated three days with 40 µM Ac_4GlcNAz, Ac_4GalNAz or Ac_4ManNAz. Cells (2×10^7 each condition) were lysed with a modified RIPA buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Deoxycholate, 1 mM EDTA) containing Thermo Scientific Halt Protease Inhibitors and clarified by centrifugation. Lysates (0.1-1 mg) were incubated with 200 µM of Thermo Scientific DyLight 649-Phosphine or Biotin-PEG_3-Phosphine for 4 hours at 37°. Excess dye was removed using Thermo Scientific Dye Removal Columns before separation by SDS-PAGE (4-20% polyacrylamide gel) and detection using Typhoon™ Imager (Molecular Devices) at 630 nm. Fluorescent densitometry was performed using ImageQuant™ 5.2 software.

Biotinylated samples were desalted using Thermo Scientific ZeBa Desalting columns into wash buffer (PBS pH 7.4, 1% NP-40). Samples (2 mg each) were purified using 400 µl High Capacity streptavidin agarose overnight at 4°C. Bound proteins were washed (3 x wash buffer, 3 x PBS, 2 x water, 10 ml each) before reduced and alkylated in 6M urea/PBS and digested overnight with a proteolytic enzyme in 2M urea/PBS. Glycosylated peptides were released by incubation with PNGase F, Endo-O-Glycosidase Sialidase A, ß-N-Acetylglycosaminidase and β(1,4)-Galactosidase (Sigma) overnight at 37°C. Eluted peptides from both total proteolytic enzyme digests and endglycosidase digests were labeled in duplicate with Thermo Scientific TMT isobaric tags (TMT^0 control; TMT^6 126/129-Ac4ManNAz; TMT^6 127/130-Ac4GlcNAz; TMT^6 128/131-Ac4GalNAz), combined and desalted using Sep-Pak™ C18 columns (Waters).

LC/MS

An NanoLC-2D HPLC (Eksigent) with a Proteopep II™ C18 column 75 µm ID x 20 cm (New Objective) was used to separate peptides in using a 3-40% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at 250 nl/min over 140 min. A Thermo Scientific LTQ Orbitrap XL ETD mass spectrometer was used to detect peptides and generate MS/MS data under the following settings:, MSn Target Orbitrap = 2e5; MSn Target Ion Trap = 1e4; MS/MS = 2 µscans, 200 ms max ion time; MS = 380 -1800 *m/z*, 30,000 resolution; MS/MS = Top Four Data dependant acquisition HCD FT; Dynamic Exclusion = Repeat count 1, Duration 30 s, Exclusion duration 30 s; HCD Parameters: Collision Energy = 45%.

Data Processing

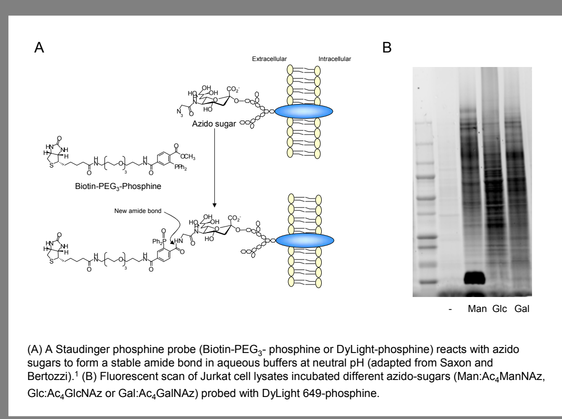
Thermo Scientific BioWorks 3.3.1 with SEQUEST™ was used for protein ID based on protein probability 1e-002 and peptide mass accuracy 10 ppm. Quantitation based on TMT reporter ions was accomplished by Thermo Scientific PepQuan software within BioWorks™ 3.3.1. Thermo Scientific Proteome Discoverer 1.1 software was also used for protein ID based on precursor mass accuracy of 10 ppm and <2% FDR. At least two peptides were required for assignment of protein IDs.

Human IPI database was used for both search engines. All samples were searched with mod of +57 Da for carbamidomethylation of cysteine residues by iodoacetimide. Glycopeptides were also searched using the diffmod of +1 Da for Asn to Asp conversion by PNGase F.

Results

There are several classes of glycoproteins grouped by the type of carbohydrate and amino acid linkage site. *N*-linked glycosylation is a modification of asparagine amines, whereas *O*-linked glycosylation occurs through the hydroxyl of serine and threonine residues. To demonstrate the utility of azido-sugars for differentiating these subclasses, Jurkat cells were incubated with each sugar derivative for 3 days. Whole cell lysates were probed with DyLight™ 649-phosphine to detect the presence of azido-labeled glycoproteins (Figure 1). Using this reagent revealed differential glycoprotein staining patterns among the three azido sugars with high specific labeling, more than ten fold over background.

FIGURE 1. Detection and purification of azido-labeled glycoproteins



(A) A Staudinger phosphine probe (Biotin-PEG_3- phosphine or DyLight-phosphine) reacts with azido sugars to form a stable amide bond in aqueous buffers at neutral pH (adapted from Saxon and Bertozzi).[1] (B) Fluorescent scan of Jurkat cell lysates incubated different azido-sugars (Man:Ac_4ManNAz, Glc:Ac_4GlcNAz or Gal:Ac_4GalNAz) probed with DyLight 649-phosphine.

Glycoprotein profiling with azido-labeled sugars

Using the Biotin-PEG_3-phosphine, azido-labeled glycoproteins from Jurkat cell lysates were also enriched using streptavidin agarose for mass spectrometry identification (Figure 2). TMTsixplex isobaric tags were used to label proteins from each sample in order to aid in the relative quantitation of each glycoprotein subclass as well as provide an internal duplicate control. Affinity captured proteins were denatured, reduced and aklyated on beads before enzymatic digest (total). Glycopeptides were subsequently released using a mixture of endoglycosidases which are specific for *N*-linked (PNGase F) or *O*-linked (Endo-O-Glycosidase) sugars (glyco). Peptides were analyzed using an LTQ Orbitrap XL™ mass spectrometer to identify glycoproteins contained in each starting sample and quantify the relative abundance of each glycoprotein.

FIGURE 2. Schematic of sample prep workflow using TMT labeling

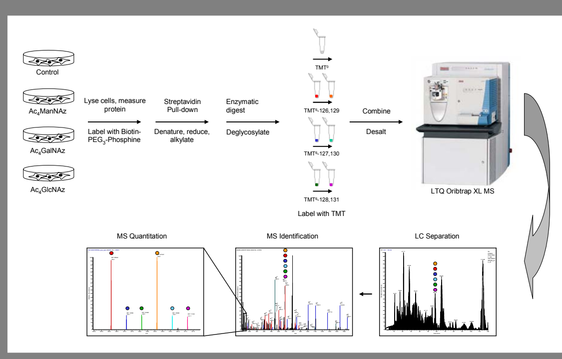


Figure 3 shows examples of MS quantitation results for four TMT-labeled peptides from four different glycoproteins. Using the TMT tags as internal standards for replicate samples, specific glycoproteins from each azido sugar treatment could easily be assigned. Likewise, abundant, non-specific proteins were found to be equally represented in all conditions (TMT ratio 1:1:1:1:1:1). Most of the proteins identified were known glycoproteins or glycoprotein-associated proteins as annotated by SwissProt. However, some of glycopeptides identified also included novel sites of glycosylation which have not been previously annotated. Finally, we also this annotation to address the subcellular distribution of identified glycoproteins. Consistent with known distribution of sugar linkage subtypes, a majority of *N*-linked identified glycoproteins from Ac_4ManNAz-labeled lysate were membrane-associated proteins where as those from Ac_4GlcNAz-labeled were primarily known intracelluar *O*-linked glycoproteins.

FIGURE 3. MS/MS spectra of TMT-labeled glycoprotein peptides

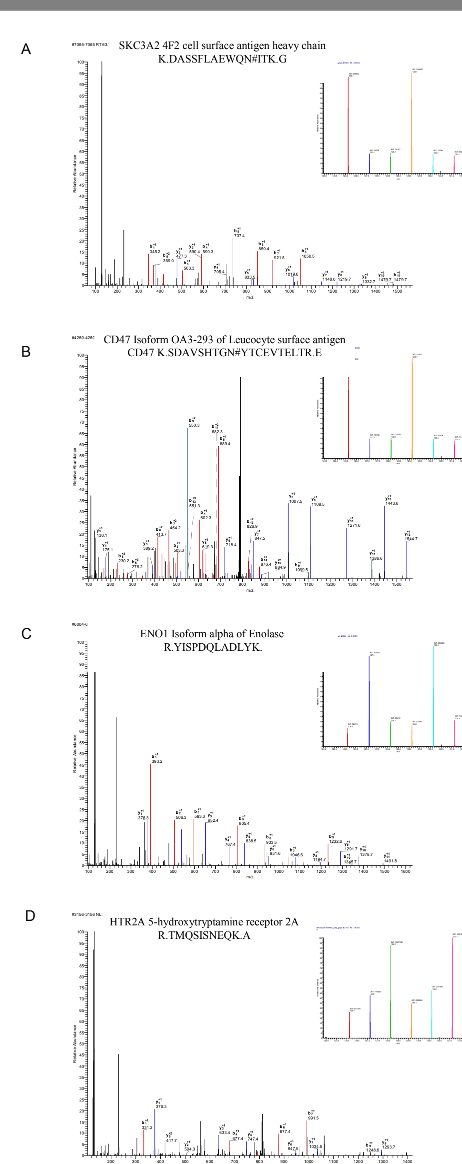


Figure 3: MS/MS spectra of four representative peptides of azido labeled glyoproteinss, (A) SKC3A2, (B) CD47, (C) ENO1 and (D) HRT2A. Asn to Asp conversion by PNGase F is marked by N#. TMT reporter ion spectra are shown as an insert for each peptide.

Conclusion

- Staudinger phosphine probes are excellent tools which can detect and capture azido sugar-containing glycoproteins with high specificity.
- TMT tags can be used to provide absolute internal standardization of multiple samples in addition to assessing relative protein abundance.
- Over 200 glycoproteins encompassing sialic acid-containing cell surface proteins and O-linked GalNAc and GlcNAc intracellular proteins were identified and quantified.

References

1. Saxon, E. and Bertozzi, C. (2000). Cell surface engineering by a modified Staudinger reaction. *Science*, 287:2007-10.
2. Agard, N. *et al.* (2006). A comparative study of bioorthogonal reactions with azides. *ACS Chemical Biology*, 1(10):644-48.
3. Thompson, A., *et al.* (2003). Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.* **75(8)**, 1895-1904.

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