Glycoproteomics enabled by tagging sialic acid- or galactose-terminated glycans

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In this paper, we present two complementary strategies for enrichment of glycoproteins on living cells that combine the desirable attributes of "robust enrichment" afforded by covalent-labeling techniques and "specificity for glycoproteins" typically provided by lectin or antibody affinity reagents. Our strategy involves the selective introduction of aldehydes either into sialic acids by periodate oxidation (periodate oxidation and aniline-catalyzed oxime ligation (PAL)) or into terminal galactose and *N*-acetylgalactosamine residues by galactose oxidase (galactose oxidase and aniline-catalyzed oxime ligation (GAL)), followed by aniline-catalyzed oxime ligation with aminooxy-biotin to biotinylate the glycans of glycoprotein subpopulations with high efficiency and cell viability. As expected, the two methods exhibit reciprocal tagging efficiencies when applied to fully sialylated cells compared with sialic acid-deficient cells. To assess the utility of these labeling methods for glycoproteomics, we enriched the PAL- and GAL-labeled (biotinylated) glycoproteome by adsorption onto immobilized streptavidin. Glycoprotein identities (IDs) and N-glycosylation site information were then obtained by liquid chromatography-tandem mass spectrometry on total tryptic peptides and on peptides subsequently released from *N*-glycans still bound to the beads using peptide *N*-glycosidase F. A total of 175 unique N-glycosylation sites were identified, belonging to 108 nonredundant glycoproteins. Of the 108 glycoproteins, 48 were identified by both methods of labeling and the remainder was identified using PAL on sialylated cells (40) or GAL on sialic acid-deficient cells (20). Our results demonstrate that PAL and GAL can be employed as complementary methods of chemical tagging for targeted proteomics of glycoprotein subpopulations and identification of glycosylation sites of proteins on cells with an altered sialylation status.

Keywords: aniline catalysis / galactose oxidase / glycoproteomics / mass spectrometry / oxime ligation / periodate oxidation

Introduction

Glycosylation is one of the most common posttranslational modifications known, and affects >50% of proteins (Apweiler et al. 1999); yet, profiling glycosylation on a global scale has been hampered by the micro-heterogeneity of glycans arising from the nontemplate-driven nature of glycan biosynthesis and the variable occupancy of glycosylation sites. In recent years, rapid methodological advances have enabled the site-specific glycosylation analysis of proteomes using mass spectrometry (Zhang et al. 2003; Wollscheid et al. 2009; Zielinska et al. 2010; Danzer et al. 2012). The strategy followed for such analysis typically entails enrichment of glycoproteins followed by standard proteomic analysis. Release of peptides from captured glycans is often applied in conjunction with proteomic analysis, such as enzymatic digestion of *N*-linked glycoproteins with peptide *N*-glycosidase F (PNGase-F), allowing mapping of the sites of glycosylation (Zhang et al. 2003).

A key step in glycoproteomics is enrichment of glycoproteins. Lectin- and glycan-specific antibodies have been documented to selectively enrich for specific glycoprotein subpopulations (Kaji et al. 2003; Qiu and Regnier 2005; Yang et al. 2005; Durham and Regnier 2006; Schwientek et al. 2007; Xu et al. 2007). Although the specificity of lectins toward broad subpopulations of glycans limits their use as selective agents, recent efforts using mixtures of lectins to cover the glycoproteome have yielded impressive results (Ueda et al. 2010; Zielinska et al. 2010).

An alternative method of enrichment of the glycoproteome is through introduction of bioorthogonal functional groups onto glycoprotein glycans enzymatically (Khidekel et al. 2003), through glycan-specific chemical reactions (De Bank et al. 2003; Zhang et al. 2003; Wollscheid et al. 2009; Zeng et al. 2009) or by exploiting cell's own biosynthetic

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machinery (Saxon and Bertozzi 2000; Prescher and Bertozzi 2005; Baskin et al. 2007; Campbell et al. 2007; Hsu et al. 2007). These methods have the potential for selectively labeling glycoproteins on the cell surface, and subsequently enriching them. Several of these methods have been adapted to profiling proteomes (Bond and Kohler 2007; Hanson et al. 2007). In particular, introduction of aldehydes into carbohydrates by periodate oxidation has frequently been coupled with ligation of aminooxy or hydrazide tags to aid in enrichment of glycoproteins (Zhang et al. 2003; Liu et al. 2005; Lewandrowski et al. 2006; Pan et al. 2006; Ramachandran et al. 2006; Bernhard et al. 2007; Shimaoka et al. 2007; Sun et al. 2007; Tian et al. 2007; Zhou et al. 2007; Gundry et al. 2009; Nilsson et al. 2009; Wollscheid et al. 2009). These chemical tagging methods are yielding impressive coverage of the cellular glycome (Wollscheid et al. 2009; Hofmann et al. 2010; Danzer et al. 2012). However, the majority of these methods are directed towards maximal coverage of the glycoproteome. Still needed are robust methods to sample subsets of the glycoproteome that probe the chemical heterogeneity of glycosylation of cell surface glycoproteins.

We recently described a variation in the periodate oxidation strategy for labeling glycoproteins on living cells called PAL (periodate oxidation and aniline-catalyzed oxime ligation) (Zeng et al. 2009) that employs mild periodate oxidation of sialic acids (Gahmberg and Andersson 1977) and ligation with an aminooxy tag in the presence of aniline (Cordes and Jencks 1962; Dirksen et al. 2006). In principle, the high selectivity and efficiency of PAL makes it ideal to label cell surface glycoproteins bearing sialic acids for analysis of the sialo-glycoproteome. Here we describe a related but complementary method, GAL (galactose oxidase and aniline-catalyzed oxime ligation) that uses galactose oxidase (D-galactose: oxygen 6-oxidoreductase) to selectively introduce aldehydes into terminal galactose (Gal) and N-acetylgalactosamine (GalNAc) residues of glycans. Galactose oxidase oxidizes terminal Gal and GalNAc residues at the C-6 position to generate an aldehyde (Cooper et al. 1959; Avigad et al. 1962) and has been previously employed in conjunction with biotin hydrazide or radio-labeled borohydride to visualize the external Gal and GalNAc residues on cells (Gahmberg and Hakomori 1973; Heitzmann and Richards 1974; Skutelsky and Bayer 1983). By coupling galactose oxidase with aniline-catalyzed ligation with aminooxy-biotin, we demonstrate the labeling of cell surface glycans with high efficiency while retaining cell viability.

Because PAL and GAL differ only in the method of introducing aldehyde into sialic acid and Gal/GalNAc, respectively, they represent complementary methods for capturing subsets of the glycoproteome. To test this, we performed mass spectrometry-based glycoproteomics on BJA-B K20 cells. GAL and PAL afford highly efficient labeling of nonsialylated and sialylated cell surface glycoproteins on living cells, respectively, demonstrating their potential to profile glycoproteins with altered sialylation in physiological conditions such as inflammation, metastasis and cellular activation (Gabius and Engelhardt 1988; Sell 1990; Narayanan 1994; Taylor-Papadimitriou and Epenetos 1994; Hakomori and Zhang 1997; Amado et al. 2004; Comelli et al. 2006).

Results

Strategy for glycoproteomics using PAL and GAL

We employed a two-pronged approach for profiling the B-cell glycoproteome (Figure 1). We selectively introduced an aldehyde group into cell surface sialic acids by periodate oxidation (PAL) (Zeng et al. 2009) or terminal Gal/GalNAc using galactose oxidase (GAL) and then reacted the cells with aminooxy-biotin in an aniline-catalyzed ligation to biotinylate the corresponding glycoprotein glycans. We then affinity captured the biotinylated glycoproteins on streptavidin-coated beads, performed on-bead trypsin digest to elute peptides and infer glycoprotein IDs, and then subsequently performed on-bead PNGase-F digest to specifically release and identify the peptides linked to N-linked glycans to map the glycosylation sites (Zhang et al. 2003).

Galactose oxidase with aniline-catalyzed oxime ligation (GAL)

To label glycoproteins with glycans lacking sialic acids, we employed galactose oxidase to introduce an aldehyde group into Gal/GalNAc. To optimize GAL, we employed the cell line BJA-B K20 that cannot synthesize its own sialic acids because of a deficiency in a key enzyme, uridine diphosphate (UDP)-GlcNAc 2-epimerase, but will readily incorporate sialic acids added to the culture medium (Keppler et al. 1999; Oetke et al. 2001). Thus, if BJA-B K20 cells are grown in serum-containing media, glycoproteins are largely sialylated (Sialo-K20), while those grown in serum-free medium (SFM) express glycoproteins predominately with terminal Gal residues (Asialo-K20). Alternatively, Asialo-K20 cells could be produced by the treatment of BJA-B K20 cells with *Arthrobacter ureafaciens* sialidase (AUS).

Asialo-K20 cells were incubated with 50 U/mL galactose oxidase and 250 μM aminooxy-biotin, and biotinylated glycans were then visualized by staining with dichlorotriazinyl amino fluorescein (DTAF)–streptavidin and flow cytometry. Biotinylation was dramatically enhanced upon addition of 10 mM aniline, yielding ∼7-fold greater biotinylation over that without aniline and ∼250-fold greater biotinylation than cells without galactose oxidase in 30 min (Figure 2A). There was no background labeling of the cells in reactions when galactose oxidase was omitted (Figure 2A). Over 90% viability was retained following the labeling procedure as determined by Trypan Blue exclusion (Supplementary data, Figure S1). We varied the concentration of galactose oxidase and time of reaction and found 50 U/mL galactose oxidase for 30 min to be optimal for labeling Asialo-K20 cells by GAL with high efficiency (Supplementary data, Figure S2). This step uses a very large excess of enzyme such that there was no significant change in labeling when the galactose oxidase reaction was conducted at 4°C instead of at 25 or 37°C (Supplementary data, Figure S2). Labeling of discrete glycoprotein bands was detected by western analysis of cell lysates (Figure 2B).

Next, we determined whether GAL was specific for the Gal/GalNAc residues by employing ldlD-Chinese hamster ovary (CHO) cells that are deficient in UDP-Gal/UDP-GalNAc 4-epimerase, an enzyme required for the synthesis of UDP-Gal and UDP-GalNAc (Kingsley et al. 1986).
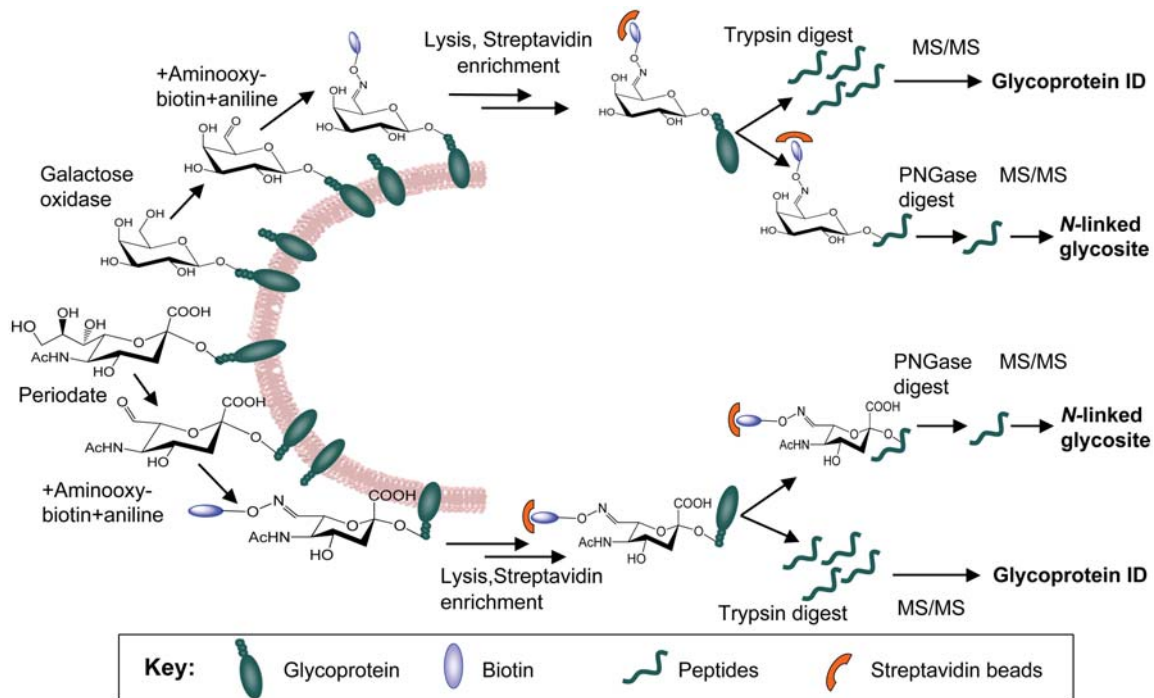
Fig. 1. Strategy for profiling the glycoproteome. Aldehydes are introduced selectively into either sialic acids by mild periodate oxidation or terminal galactose and GalNAc residues by galactose oxidase treatment. Aldehydes are then reacted with aminooxy-biotin in the presence of aniline to biotinylate the glycans. Biotinylated glycoproteins are enriched by streptavidin, and subjected to on-bead trypsin digestion and PNGase-F digestion. Glycoprotein IDs and *N*-glycosylation site information are obtained by LC-MS/MS of the tryptic and PNGase-F-eluted peptides.

ldlD-CHO cells cultured in SFM lacked Gal and GalNAc residues as detected by staining with fluorescein isothiocyanate (FITC)-*Erythrina cristagalli* lectin (ECL) that recognizes sequences containing terminal Gal/GalNAc (Figure 2C) and were not labeled by GAL (Figure 2D). Addition of Gal/GalNAc to the culture medium of ldlD-CHO cells resulted in increased staining by FITC-ECL and significant GAL labeling (Figure 2D), consistent with labeling specific to Gal/GalNAc residues.

It is well known that galactose oxidase will not oxidize galactose capped with sialic acid in α2–6 linkage since the C-6 position is required for activity. To check whether galactose capped with α2–3 sialic acid is susceptible to oxidation by galactose oxidase, we performed GAL with aminooxy-AF488 on desialylated and native CHO cells that have α2–3 but not α2–6 sialic acids, and subjected them to flow cytometry. The removal of α2–3 sialic acids dramatically increased GAL labeling, indicating that α2–3 sialic acids also interfere with galactose oxidase activity (Supplementary data, Figure S3), and that GAL only targets Gal/GalNAc-terminated glycans uncapped by sialic acids.

PAL and GAL are complementary probes of glycosylation status

GAL was developed as a method complimentary to PAL (periodate oxidation coupled with aniline-catalyzed oxime ligation) that we previously described for selective labeling of cell surface glycans containing terminal sialic acids (Zeng et al. 2009). Since oxidation by periodate and galactose oxidase target terminal sialic acids and terminal Gal/GalNAc residues uncapped by sialic acids, respectively, they can be employed to label glycoprotein subsets that differ in the sialylation state of their glycans. As a proof of principle, we employed the cell line, BJA-B K20 that cannot synthesize its own sialic acids, but incorporate sialic acids added to the culture medium (Keppler et al. 1999; Oetke et al. 2001). Sialo and Asialo-K20 cells were obtained by culturing cells in medium containing serum or in SFM, respectively. Alternatively, Sialo-K20 cells could be enzymatically converted to Asialo cells by treatment with AUS. Similarly, Asialo-K20 cells could be resialylated by enzymatic engineering with cytidine monophosphate (CMP)–neuraminic acid (NeuAc) and the sialyltransferase, ST6Gal I (Sadler et al. 1979) resulting in *Sambuccus nigra* agglutinin (SNA) staining equivalent to Sialo-K20 cells (Figure 3A). Note that the difference in sialic acid content of Asialo and Sialo-K20 cells is only 5–10-fold, as determined by staining with FITC-SNA, a lectin that recognizes the abundant NeuAcα2-6Gal sequence on B-cells (Figure 3A).

Sialo, Asialo and enzymatically engineered (resialylated) K20 cells were subjected to PAL or GAL with aminooxy-biotin, stained with DTAF-streptavidin, and subjected to flow cytometry (Figure 3B and C). Significantly, in accordance with the ∼5-fold lower SNA staining, Asialo-K20 cells displayed ∼5-fold lower PAL labeling and ∼5-fold greater GAL labeling when compared with Sialo-K20 cells (Figure 3B and C). Also, Sialo-K20 cells displayed ∼5-fold higher PAL labeling than GAL labeling, and Asialo-K20 cells displayed ∼5-fold higher GAL labeling than PAL labeling. Resialylation
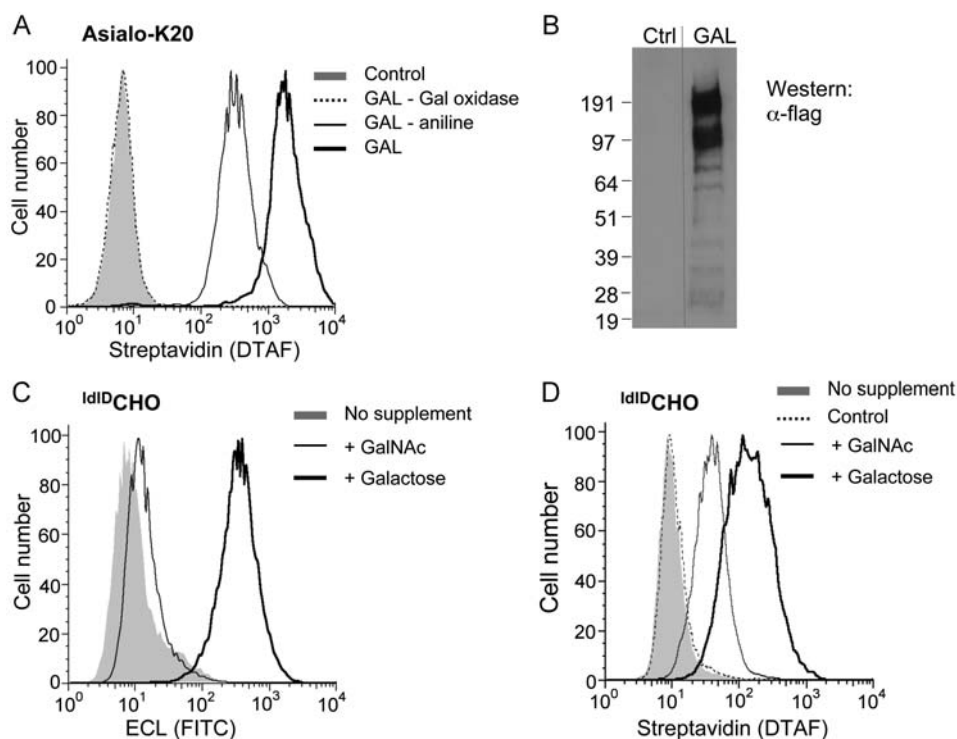
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Fig. 2. GAL efficiently labels Gal and GalNAc residues on cell surface glycoproteins. (**A**) Asialo-K20 cells were incubated in PBS with 5 mg/mL BSA, pH 6.7, containing 250 μM aminooxy-biotin in the presence or absence of 10 mM aniline and 50 U/mL galactose oxidase (Gal oxidase) for 30 min at 37°C. Cells were then washed with PBS, stained with DTAF–streptavidin and subjected to flow cytometry. "Control" refers to untreated Asialo-K20 cells stained with DTAF-streptavidin. "GAL – Gal oxidase" refers to an additional control sample where galactose oxidase was omitted from the reaction mixture. (**B**). Asialo-K20 cells subjected to GAL at pH 6.7 with 250 μM aminooxy-flag peptide, 10 mM aniline and 50 U/mL galactose oxidase for 30 min at 37°C, or control (untreated) K20 cells were lysed. Proteins in the cell lysates were resolved by gel electrophoresis and subjected to western blot analysis with anti-flag antibody. (**C**). [ldID]CHO cells cultured in SFM with or without 20 μM galactose or 200 μM GalNAc were stained with FITC-labeled-(ECL) and subjected to flow cytometry. (**D**). [ldID]CHO cells were cultured in SFM with or without 20 μM galactose or 200 μM GalNAc and then subjected to GAL at pH 6.7 with 250 μM aminooxy-biotin, 10 mM aniline and 50 U/mL galactose oxidase for 30 min at 37°C. The cells were stained with DTAF–streptavidin, and subjected to flow cytometry. "Control" refers to untreated [ldID]CHO cells stained with DTAF–streptavidin.
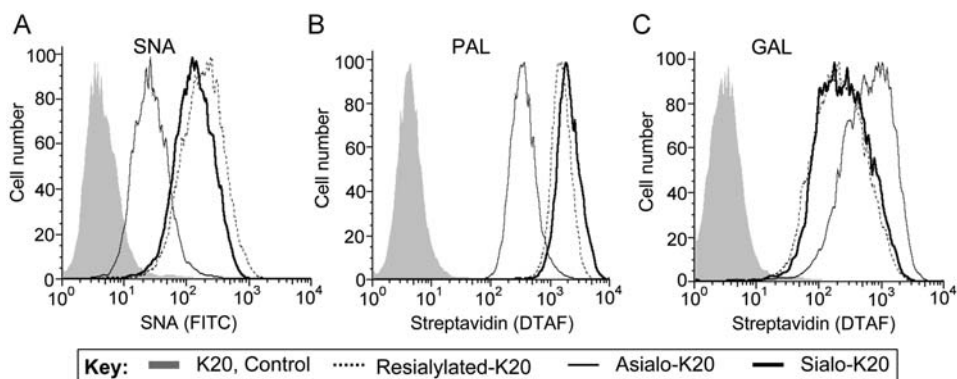

Fig. 3. PAL and GAL are complementary probes for visualizing cell surface glycans on B-cells. (**A**). Sialo, Asialo or resialylated K20 B-cells were stained with FITC-labeled SNA and subjected to flow cytometry. "K20, Control" refers to cells not stained with FITC–SNA. Sialo and Asialo-K20 cells were obtained by culturing cells in medium with serum (a source of sialic acids) or in SFM, respectively. Re-sialylated K20 cells were obtained by enzymatically engineering Asialo-K20 with CMP-NeuAc and the sialyltransferase, ST6Gal I. (**B**) Sialo, Asialo or resialylated K20 cells were subjected to PAL at pH 6.7 with 1 mM sodium periodate, 250 μM aminooxy-biotin and 10 mM aniline for 30 min at 4°C. Cells were stained with DTAF–streptavidin, and subjected to flow cytometry. K20, Control refers to untreated cells stained with DTAF–streptavidin. (**C**) Sialo, Asialo or resialylated K20 cells were subjected to GAL at pH 6.7 with 250 μM aminooxy-biotin, 10 mM aniline and 50 U/mL galactose oxidase for 30 min at 37°C. Cells were stained with DTAF–streptavidin, and subjected to flow cytometry. K20, Control refers to untreated cells stained with DTAF-streptavidin.

of Asialo-K20 cells by enzymatic engineering reversed GAL and PAL profiles (Figure 3B and C), confirming that terminal sialic acids abrogate GAL labeling and enable PAL labeling.

B-cell glycoproteomics enabled by PAL and GAL labeling

We employed the strategy outlined in Figure 1 to profile the glycoproteome of Sialo- and Asialo-K20 cells by PAL and GAL labeling. Cell surface glycoproteins of K20 cells were biotinylated by PAL or GAL labeling, enriched with streptavidin agarose and subjected to trypsin digestion. Following mass spectrometry of tryptic peptides, the protein entries identified were sorted by their abundance in the PAL and GAL samples relative to control samples (cells not subjected to labeling). To do this, two measures, "score" and "ratio" (see Materials and methods), were employed. By adopting a score of ≥4 and ratio of ≥15, 384 and 224 protein IDs were identified as enriched from PAL-labeled Sialo-K20 to Asialo-K20 cells, respectively and 147 and 194 protein IDs were identified as enriched from GAL-labeled Sialo-K20 to Asialo-K20 cells, respectively. Of the proteins that could be annotated by GO analysis with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics tool, 72, 73, 86 and 89%, respectively, comprised membrane, extracellular and secreted proteins (Supplementary data, Figure S4 and Tables S1 and S2a). In the GAL-labeled asialo and Sialo-K20 datasets, a higher number of total protein IDs with enrichment of membrane and secreted proteins comparable with the PAL datasets could be achieved by adopting a lower cutoff score and ratio (Supplementary data, Figure S4 and Table S2b). Given the relatively limited abundance of membrane proteins (Ishihama et al. 2008) and considering that the GO ID, GO:0016020 (corresponding to "membrane") is attributed to only ∼40% (7441 of 18,581 gene products, GO database release 11–15 November 2009, AmiGO version 1.7) (Carbon et al. 2009) of all human annotated proteins, we achieved a significant enrichment of proteins annotated as secreted or localized on the cell surface (∼80%) with just a single streptavidin affinity step without any additional isolation of the membrane fraction. We attribute this to the specific and robust labeling afforded by PAL and GAL.

Mapping N-glycosylation sites

To reliably identify N-glycoproteins and map N-glycosylation sites, we employed mass spectrometry following on-bead PNGase-F digestion to release previously N-glycosylated and biotinylated peptides from streptavidin beads. The change in mass of +1 Da corresponding to the asparagine (Asn) to aspartic acid (Asp) conversion during PNGase-F digestion was used to map N-linked glycosylation sites in Sialo-K20 cells subjected to PAL and Asialo-K20 cells subjected to GAL. Although spontaneous deamidation of Asn residues could in principle lead to false-positive assignments of N-glycosylation sites (Palmisano et al. 2012), it is not a major concern for this type of experiment. Because peptides are being released from beads, we expect the majority of the peptides to contain at least one N-linked glycosylation site. Moreover, in datasets from control beads exposed to samples not treated with PAL or GAL (e.g. with peptides nonspecifically bound to beads), we noted only three peptides with potential Asn deamidation, none of which had a consensus glycosylation motif.

PNGase-F digestion was conducted on PAL-labeled Sialo-K20 cells and GAL-labeled Asialo-K20 cells. These two samples were chosen as they would be most likely to generate high-quality mass spectrometry data based on the flow cytometry results. In total, 125 unique N-glycosylation sites belonging to 88 nonredundant proteins were identified in Sialo-K20 cells labeled by PAL (Figure 4A, Supplementary data, Table S3). Our data verified 51 potential glycosylation sites (predicted in the UniProtKB database but not experimentally verified) (2009) and identified six new glycosylation sites (not found in UniProtKB database) (Figure 4A). Interestingly, while most of the glycosylation sites comprised the commonly found N*XT or N*XS sequence, three of the glycosylation sites identified comprised the rare glycosylation site, N*XC (Figure 4B). Information about one glycosylation site was used to identify 72% of the glycoprotein IDs; however, two glycosylation sites identified 22% of the glycoprotein IDs and three–six glycosylation sites identified the remaining 6% (Figure 4C). In accordance with the subcellular distribution of N-glycoproteins, 97% of the glycoproteins were membrane proteins, 2% were extracellular by GO cellular component annotation and the remaining unannotated (Figure 4D). GO biological process and molecular function annotations of the identified N-glycoproteins are represented in Supplementary data, Figure S5.

Further, we identified 112 unique glycosylation sites belonging to 68 nonredundant proteins in Asialo-K20 cells labeled by GAL (Figure 4A, Supplementary data, Table S4). These glycosylation sites included 4 new sites (sites not annotated as glycosylation sites in UniprotKB) and 42 potential sites (sites predicted in the UniprotKB but not experimentally verified) in addition to 66 previously verified sites (Figure 4A). Again, though the majority of sites comprised the sequence, N*XS or N*XT, a single glycosylation site of the sequence N*XC was also identified (Figure 4B). Two-thirds (67%) of all glycoproteins were identified by information from a single glycopeptide, and the rest were identified by two or more glycopeptides (Figure 4C). Of these, 67 of the 68 proteins were annotated as membrane proteins, with 57 belonging to the plasma membrane by GO cellular component analysis (Figure 4E). GO molecular function and biological process annotations are summarized in Supplementary data, Figure S5.

Of the 108 glycoproteins identified, 48 were identified by both methods of labeling, and the remainder was identified using PAL on Sialo-K20 cells (40) or GAL on Asialo-K20 cells (20) (Figure 4F, Supplementary data, Tables S5 and S6). Altogether, 175 unique N-glycosylation sites (95 verified, 71 potential and 9 new) were identified that belonged to 108 nonredundant proteins (Figure 4A and F, Supplementary data, Table S5). Of these, 97 sites were identified by higher spectral counts in Sialo-K20 PAL samples when compared with the Asialo-K20 GAL samples, 6 were identified by equal spectral counts in the PAL and GAL samples, and 72 were identified by higher spectral counts in Asialo-K20 GAL samples. Interestingly, while 62 glycosylation sites were identified by both PAL and GAL, 63 glycosylation sites were identified
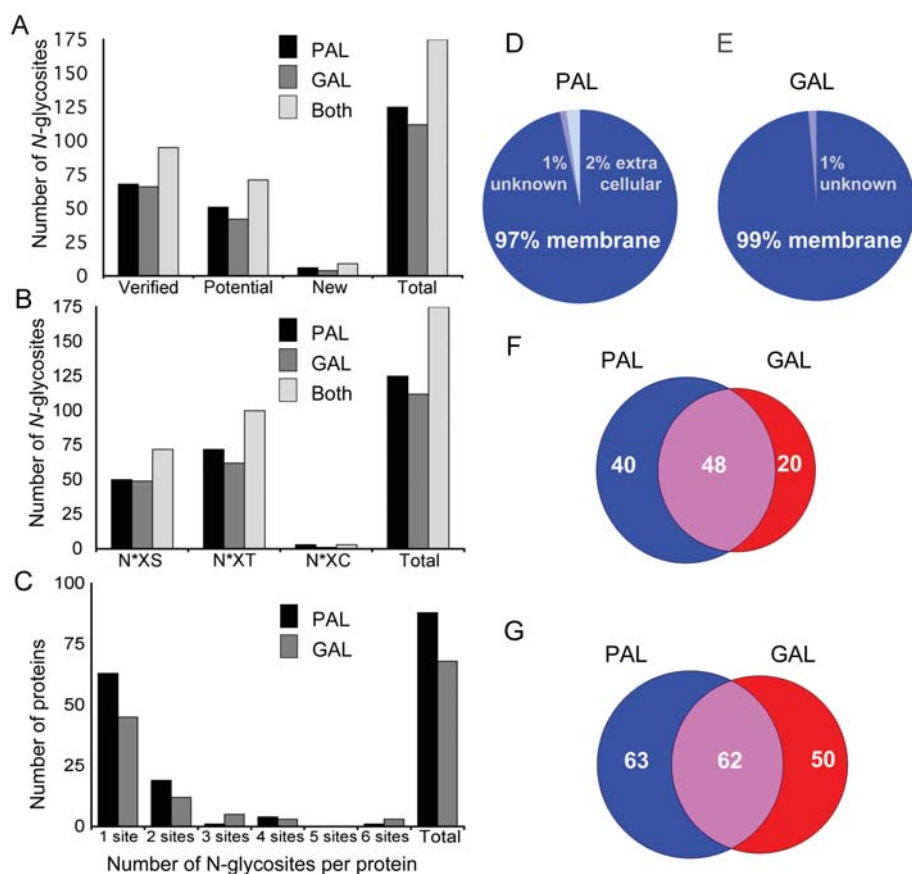
Fig. 4. B-cell *N*-linked glycosylation sites identified. (**A**) Number of verified, potential, new and total *N*-linked glycosylation sites identified upon mass spectrometry of PNGase-F eluted peptides from glycoproteins on K20 B-cells by PAL or GAL or both. (**B**) Number of N-glycosylation sites with the sequence, N*XS, N*XT or N*XC identified upon mass spectrometry of PNGase-F-eluted peptides from glycoproteins on K20 B-cells by PAL or GAL or both. (**C**) Number of N-linked glycosylation sites identified per glycoprotein identified on K20 B-cells after PAL or GAL by mass spectrometry of PNGase-F-eluted peptides. (**D**). Subcellular localization of glycoproteins identified on K20 B-cells after PAL by mass spectrometry of PNGase-F-eluted peptides. (**E**). Subcellular localization of glycoproteins identified on K20 B-cells after GAL by mass spectrometry of PNGase-F eluted peptides. (**F**) The number of *N*-glycoproteins identified by PAL and GAL. (**G**) The number of *N*-glycosylation sites identified by PAL and GAL.

only by PAL and 50 glycosylation sites were identified only by GAL and not by PAL (Figure 4G, Supplementary data, Tables S5 and S7). It is possible that the latter glycosylation sites are not sialylated even in Sialo or native cells, and therefore not identifiable by mild periodate oxidation coupled with oxime ligation. Thus our results validate our hypothesis that GAL and PAL may be used as complementary methods to chemically tag the glycoproteome on living cells.

Discussion

We have developed two complementary chemical labeling methods to selectively and efficiently label the glycoproteome under mild conditions that are compatible with labeling living cells. The two methods introduce aldehydes into either terminal sialic acid (PAL) or Gal (GAL) of native glycoprotein glycans using mild periodate oxidation or galactose oxidase, respectively, followed by a common step of aniline-catalyzed ligation with an aminooxy tag (e.g. biotin). Because PAL and GAL employ membrane-impermeable reagents, they can be

used for the selective tagging of cell surface proteins (Wollscheid et al. 2009). It is also noteworthy that the metabolic activity of the cell is not required for labeling. While methods that employ metabolic labeling with monosaccharides containing chemical tags do accomplish selective and efficient labeling of glycoprotein populations (Prescher and Bertozzi 2005; Campbell et al. 2007), they are not applicable to certain proteomes such as plasma or tissue lysates and body fluids, or to glycoproteomes of metabolically inert cells. GAL and PAL do not suffer from these limitations. Although the number of glycoproteins we identify in BJA-B K20 cells is lower than the relative size of glycoproteomes recently reported for other cells (Hofmann et al. 2010; Zielinska et al. 2010; Danzer et al. 2012), we attribute this to the increased sensitivity of the downstream processing of the samples in these studies. This is not a reflection on the efficiency of the labeling method, since we have shown previously that PAL labels ∼50% of all cell-surface sialic acids (Zeng et al. 2009).

Further on-bead analysis should be possible with glycoproteomics enabled by PAL and GAL. While we have only employed PNGase-F in this study to selectively elute formerly

N-glycosylated peptides, it should be possible to specifically elute peptides with *O*-glycans by beta elimination. Additionally, the use of the aminooxy tag provides further possibilities in the simultaneous analysis of both glycan and peptide moieties through the use of transoximization (Shimaoka et al. 2007) or mild acid hydrolysis of sialic acids (Nilsson et al. 2009).

Little is currently known concerning the degree to which individual glycoproteins on a cell will express different glycosylation from other glycoproteins on the same cell. Based on the preferential labeling of Asialo and Sialo cells by GAL and PAL, respectively, we suggest that these labeling methods might be used to probe subsets of a glycoproteome that are differentially glycosylated. Indeed, the fact that PAL and GAL each identified overlapping and distinct subsets of glycoproteins is in concordance with the fact that Asialo- and Sialo-K20 were only ~5-fold different in sialic acid content and suggests that they may be differentially sialylated. It should be emphasized, however, that many if not most glycoproteins would carry both sialylated and nonsialylated termini on their antennae, and would likely be detected with GAL and PAL, and that a mere detection of one glycoprotein with PAL and another with GAL is not a sufficient criterion for establishing them as having different glycosylation.

It is also notable that we could enzymatically convert Asialo-K20 cells to Sialo-K20 cells with high efficiency using the sialyltransferase ST6Gal I that adds sialic acids only to glycans with the terminal Galβ1-4GlcNAc sequence, effectively adding a chemical "tag" to glycoproteins containing this sequence. In principle, this approach could be used to explore other subsets of the glycoproteome. Several reports have demonstrated the utility of this basic approach, using an exogenous galactosyltransferase (Boeggeman et al. 2007; Rexach et al. 2008), fucosyltransferase (Zheng et al. 2011) or blood group A GalNac transferase (Chaubard et al. 2012) to probe the glycan structures of cell surface glycoproteins by transferring monosaccharides with ketone or azide functionality, followed by chemically ligating biotin or fluorescent tags. These approaches can be readily adapted for use in parallel with GAL and PAL to probe the glycoproteome for differential glycosylation.

In summary, GAL and PAL are powerful and robust, enabling technologies for analyzing distinct subpopulations of glycoproteins on a proteomic scale. We have demonstrated their efficacy in this report by exploring the glycoproteome of a B-cell line. Future experiments will not only serve to establish additional glycosylation sites and provide evidence for occupancy by specific glycans, but may also assist in identifying altered states of sialylation in glycoproteins especially under different states of health and disease.

Materials and methods
Cell culture
The B-JA-B K20 B-cell line was routinely maintained in RPMI supplemented with 10% fetal bovine serum (FBS) which is a source of sialic acids. These cells are referred to as native or "sialo" K20 cells in the text. Asialo-K20 cells were obtained by weaning cells to SFM, Hyq-SFM (Clontech, Thermo Fisher Scientific) and culturing cells for at least 4

days in Hyq-SFM prior to use in experiments. Alternatively, K20 cells cultured in RPMI/10% FBS were subjected to treatment with 200 mU/mL AUS for 30 min at 37°C. Resialylation of Asialo cells by enzymatic engineering was conducted as described previously (Zeng et al. 2009). The degree of sialylation of Asialo cells obtained by culturing in SFM and Asialo cells produced by treatment with sialidase was similar as observed by staining with FITC-SNA and flow cytometry (data not shown). Asialo cells obtained by culturing in SFM were used for most of the flow cytometry experiments reported in the manuscript. Sialidase-treated Asialo cells were used for experiments that were conducted to obtain proteomics data after PAL and GAL labeling.

CHO-FlpIn (Invitrogen) and ldlD-CHO cells were routinely maintained in F12-Dulbecco's modified Eagle's medium (DMEM)/10% FBS. ldlD-CHO cells without Gal and GalNAc residues in glycoprotein glycans were obtained by culturing cells in F12-DMEM supplemented with 1X ITS (containing 0.625 mg/mL each of insulin, transferrin and selenium; BD Biosciences) for 3 days. ldlD-CHO cells with terminal Gal or GalNAc residues on the glycans were obtained by culturing cells in F12-DMEM supplemented with ITS and 20 μM Gal (Sigma) or 200 μM GalNAc (Sigma), respectively.

Periodate oxidation and aniline-catalyzed oxime ligation (PAL)
PAL was performed essentially as described previously (Zeng et al. 2009). BJA-B K20 cells were washed in Dulbecco's phosphate-buffered saline (PBS), pH 7.4 (PBS; Invitrogen Corporation) and suspended to 1×10^7 cells/mL in PBS containing 1 mM sodium periodate (Sigma–Aldrich). Periodate oxidation was performed for 30 min on ice and quenched with 1 mM glycerol, then cells were thoroughly washed with PBS. For the oxime ligation step, cells were suspended to 1×10^7 cells/mL in PBS/5% BSA, pH 6.7 containing 250 μM aminooxy-biotin, aminooxy-AF488 (Invitrogen Corporation) or aminooxy-FLAG peptide and 10 mM aniline (Sigma–Aldrich) for 90 min at 4°C with end-over-end mixing.

Galactose oxidase treatment and aniline-catalyzed oxime ligation (GAL)
GAL was performed as follows. Cells were suspended to 1×10^7 cells/mL in PBS/5% BSA, pH 6.7, containing 50 U/mL *Dactylium dendroides* galactose oxidase (Sigma), 250 μM aminooxy-biotin aminooxy-AF488 or aminooxy-FLAG peptide and 10 mM aniline for 30 min at 37°C with end-over-end mixing.

Lectin and streptavidin staining and flow cytometry
BJA-B K20 or ldlD-CHO cells (1×10^6 cells in 100 μL PBS/ 5% BSA) were stained with 2 μg FITC-SNA (Vector labs), 2 μg FITC-ECL (Vector labs), 2 μg FITC-Peanut agglutinin (PNA, Vector labs) or 3.2 μg (DTAF)-labeled streptavidin (Jackson Immunoresearch Laboratories) for 1 h on ice. Cells were washed with PBS/5% BSA, resuspended in PBS/5% BSA and subjected to analysis by flow cytometry.

Affinity capture of biotinylated proteins

Cells subjected to PAL or GAL were lysed at 1×10^7 cells/mL in lysis buffer (50 mM Tris, pH 8.0; 5 mM EDTA; 150 mM NaCl; 1% Triton X-100; Protease inhibitor cocktail (Merck Biosciences-Calbiochem)), and lysates cleared of debris by centrifugation at 15,000 rpm for 15 min at 4°C. Lysates were subjected to pull down with streptavidin agarose (200 µL for 1×10^8 cells; Pierce Net) at 4°C for 2 h, and washed four times with lysis buffer, thrice with PBS containing urea and thrice with PBS.

Western analysis

Cells subjected to GAL with 250 µM aminooxy-flag peptide were lysed as above. Cell lysates were resolved on NuPAGE gels (Invitrogen Corporation), transferred to nitrocellulose and blocked for 1 h with 5% milk in Tris-buffered saline with 0.1% Tween-20. Blots were incubated with mouse monoclonal anti-Flag antibody (1:5000, Sigma) and horse radish peroxidase-anti-mouse antibody (1:20,000, Jackson Immunoresearch) and visualized with chemiluminescence.

On-bead trypsin digestion

Reduction, alkylation of cysteines and protein digestion with trypsin were performed on-bead essentially as described previously (Hanson et al. 2007). All steps were performed at 37°C with end-over-end mixing. Streptavidin beads were suspended in freshly prepared 6 M urea in PBS containing 10 mM dithiothreitol. Iodoacetamide was then added to a final concentration of 20 mM and alkylation allowed to proceed for 30 min. Beads were centrifuged, supernatant discarded and beads washed with 2 M urea in PBS. Trypsin digestion was initiated by suspending the beads in 2 M urea in PBS containing 1 mM $CaCl_2$ and 10 µg/mL sequence grade modified trypsin (Promega). Trypsin digestion was performed overnight, and the tryptic peptides recovered by centrifugation of the beads. The beads were additionally washed twice with water, and the washes pooled with the tryptic digest. Formic acid was added to a final concentration of 5% and the digest stored at −20°C.

On-bead PNGase-F digestion

PNGase-F digestion was employed to elute the remaining affinity captured, biotinylated glycopeptides. Following trypsin digestion and elution, streptavidin beads were washed thrice with PBS and thrice with water. The beads were then suspended in G7 buffer, and glycerol-free PNGase-F added to a concentration of 2500 U/mL. The digestion was performed overnight at 37°C, and PNGase-F-eluted peptides recovered by centrifugation. The beads were washed twice with water, and the washes pooled with the PNGase-F eluate. Formic acid was added to 5% final volume and the PNGase-F eluate stored at −20°C.

Mass spectrometry analysis of tryptic peptides

Liquid chromatography–mass spectrometry (LC–MS) analysis was performed on an LTQ ion trap mass spectrometer (ThermoFisher) coupled to an Agilent 1100 series high-pressure (or high-performance) liquid chromatography (HPLC). Tryptic digests were pressure loaded onto a 250 µm fused silica desalting column packed with 4 cm of Aqua C18 reverse phase resin (Phenomenex). The peptides were then eluted onto a biphasic column (100 µm fused silica with a 5 µm tip, packed with 10 cm C18 and 3 cm Partisphere strong cation exchange resin (SCX, Whatman) using a gradient 5–100% Buffer B in Buffer A (Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid; Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid). The peptides were then eluted from the SCX onto the C18 resin and into the mass spectrometer using four salt steps as outlined in Speers et al. (2005) and Weerapana et al. (2007). The flow rate through the column was set to ∼0.25 µL/min and the spray voltage was set to 2.75 kV. One full MS scan (400–1800 MW) was followed by seven data-dependent scans of the *n*th most intense ions with dynamic exclusion enabled (repeat count = 1; exclusion list size = 300, exclusion duration = 60).

Mass spectrometry analysis of PNGase-F-eluted glycopeptides

Peptides from PNGase-F digests were pressure loaded onto a fused silica desalting column coupled to a biphasic column as described above. The PNGase-F samples were analyzed on an LTQ ion trap mass spectrometer (ThermoFisher) coupled to an Agilent 1100 series HPLC. The peptides were eluted from the SCX onto the C18 resin and into the mass spectrometer using four salt steps as outlined in Speers et al. (2005) and Weerapana et al. (2007). The flow rate through the column was set to ∼0.25 µL/min and the spray voltage was set to 2.75 kV. One full MS scan (400–1800 MW) was followed by 18 data-dependent scans of the *n*th most intense ions with dynamic exclusion disabled.

Database searches of MS^2 spectra

The tandem MS data were searched using the SEQUEST algorithm using a concatenated target/decoy variant of the human IPI database (version 3.33, released on September 13, 2007). No enzyme specificity was stated, and a static modification of +57 Da was specified on cysteine to account for iodoacetamide alkylation. Mass tolerance for precursor ions and fragment ions was set to 1.00 Da. SEQUEST output files were filtered using DTASelect 2.0 (Tabb et al. 2002; Cociorva et al. 2007). Reported peptides were required to be fully tryptic and discriminant analyses were performed to achieve a peptide false-positive rate below 5%. The *actual* false-positive rate was assessed at this stage according to established guidelines (Elias and Gygi 2007) and found to be <1%. When combining peptides into "proteins identified," all isoforms/individual members of a protein family supported by the data were considered, and the entries with the most annotation are reported here.

For the PNGase-F-eluted peptides, SEQUEST analysis was carried out as above, with a differential modification of +1 Da specified on Asn to account for the Asn to Asp modification resulting from PNGase-F cleavage of N-linked glycopeptides. SEQUEST output files were filtered using DTASelect 2.0 and reported peptides were required to be fully tryptic and contain the desired differential modification and

discriminant analyses were performed to achieve a peptide false-positive rate below 5%.

Analysis of tryptic digest data

Protein IDs were sorted by their average score (sum of spectral counts in PAL or GAL samples minus sum of spectral counts in control samples) and average ratio (sum of spectral counts in PAL or GAL samples divided by sum of spectral counts in control samples). Protein IDs with score <2 or ratio >2.5 were discarded. Functional analysis of proteins was performed using the UniProtKB database (2009) and the DAVID knowledgebase online tool (Dennis et al. 2003) in June 2011.

Analysis of PNGase-F digest data

Single peptide identifications were also considered since the number of peptides obtained for any protein would be dependent on the number of N-linked glycosylation sites as well as if the glycosylation site were present within a suitably sized and charged tryptic peptide. Frequently, the same peptide sequence was observed in more than one replicate sample, and in a given sample, more than once (as multiple observations of the same or different charge state) or a single peptide was observed two or more times, increasing the confidence of the identification. MS/MS spectra are included as Supplementary data for peptides that were identified by <2 spectra. Data were filtered to retain only peptide IDs with a modified Asn within the established N-glycosylation consensus sequence N-X-T/S/C. Typically >75% peptide IDs were retained (overall 89% in PAL samples and 79% in GAL samples), and negative control glycoproteomes showed negligible IDs after PNGase-F treatment, demonstrating selective enrichment of labeled glycopeptides. Functional analysis of glycoproteins was performed using the UniProtKB database (2009) and the DAVID knowledgebase online tool (Dennis et al. 2003) in May 2011.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Conflict of interest

None declared.

Authors' contribution

T.N.C.R. designed and performed the experiments, analyzed data and wrote the initial draft of the manuscript. E.W. performed the mass spectrometry and analyzed data. B.F.C. provided use of mass spectrometry equipment and reagents. J.C.P. provided reagents and scientific input during all stages of the work. All authors participated in editing the manuscript.

Abbreviations

Asn, asparagine; Asp, aspartic acid; AUS, *Arthrobacter ureafaciens* sialidase; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CMP, cytidine monophosphate; DAVID, Database for Annotation, Visualization, and Integrated Discovery; DMEM, Dulbecco's modified Eagle's medium; DTAF, 5-(4,6-dichlorotriazinyl) aminofluorescein; DTAF, dichlorotriazinyl amino fluorescein; ECL, *Erythrina cristagalli*; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; Gal, galactose; GAL, galactose oxidation and aniline-catalyzed oxime ligation; GalNAc, *N*-acetyl galactosamine; PNGase F, peptide *N*-glycosidase F; HPLC, high-pressure (or high-performance) liquid chromatography; ID, identity; LC-MC, liquid chromatography-mass spectrometry; NeuAc, neuraminic acid; PAL, periodate oxidation and aniline-catalyzed oxime ligation; PBS, phosphate-buffered saline; PNA, peanut agglutinin; SCX, strong cation exchange; SFM, serum-free medium; SNA, *Sambucus nigra* agglutinin; UDP, uridine diphosphate.

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