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Danielle H. Dube
University of California, Berkeley

Jennifer A. Prescher
University of California, Berkeley

Chi M. Quang
University of California, Berkeley

Carolyn R. Bertozzi
University of California, Berkeley

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Danielle H. Dube*†, Jennifer A. Prescher*†, Chi N. Quang‡, and Carolyn R. Bertozzi*‡§¶‖

Departments of *Chemistry and ‡Molecular and Cell Biology and §Howard Hughes Medical Institute, University of California, Berkeley, CA 94720; and ¶Molecular Foundry, Materials Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

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Changes in O-linked protein glycosylation are known to correlate with disease states but are difficult to monitor in a physiological setting because of a lack of experimental tools. Here, we report a technique for rapid profiling of O-linked glycoproteins in living animals by metabolic labeling with *N*-azidoacetylgalactosamine (GalNAz) followed by Staudinger ligation with phosphine probes. After injection of mice with a peracetylated form of GalNAz, azide-labeled glycoproteins were observed in a variety of tissues, including liver, kidney, and heart, in serum, and on isolated splenocytes. B cell glycoproteins were robustly labeled with GalNAz but T cell glycoproteins were not, suggesting fundamental differences in glycosylation machinery or metabolism. Furthermore, GalNAz-labeled B cells could be selectively targeted with a phosphine probe by Staudinger ligation within the living animal. Metabolic labeling with GalNAz followed by Staudinger ligation provides a means for proteomic analysis of this posttranslational modification and for identifying O-linked glycoprotein fingerprints associated with disease.

azide | Staudinger ligation | glycan | *N*-azidoacetylgalactosamine | metabolic labeling

Posttranslational modifications are thought to decorate nearly every eukaryotic protein (1). Their system-wide characterization has rightfully become a central challenge in the field of proteomics. Certain modifications, such as phosphorylation, sulfation, lipid modifications, and acetylation, lend themselves to identification by mass spectrometry, as they impart a predictable mass shift to the protein scaffold. By contrast, glycosylation possesses intrinsic heterogeneity in the modification itself and is therefore more difficult to profile in the context of large proteomes. Because of this complexity, fundamental questions at the intersection of proteomics and glycomics remain largely unanswered. For example, which proteins are decorated with glycans? What are the structures of these glycans? And finally, what is their functional significance? The process of answering these questions necessarily involves taking inventory of protein glycosylation at the systems level.

Because glycans are highly variable in mass, efforts to probe for their presence among large protein sets have focused on other defining features. Of the two most common forms of protein glycosylation, N-linked and O-linked, the N-linked variety has been most amenable to proteomic analysis. N-linked glycans are covalently attached to asparagine (Asn) residues within a defined consensus sequence (Asn-Xaa-Ser/Thr, where Xaa is any residue except proline), enabling prediction of the modification sites by protein sequence analysis. All N-linked glycans share a common pentasaccharide core ($GlcNAc_2Man_3$) that is recognized by lectins such as Con A and leukocytic phytohemagglutinin (2) and N-glycanase enzymes such as PNGase F (3). These reagents have been used to visualize proteins bearing N-linked glycans from cell or tissue lysates and to enrich them for mass spectrometry analysis (4, 5). For example, the lectins can be used to capture the glycoproteins from a complex mixture or detect them on Western blots. The N-glycanase can be used to release captured glycoproteins via cleavage of their N-linked glycans.

By contrast, comparable tools are lacking for the study of proteins bearing O-linked glycans. The most prevalent form of O-linked glycosylation, termed the mucin type, is characterized by an *N*-acetylgalactosamine (GalNAc) residue α-linked to the hydroxyl group of Ser or Thr (6, 7). This core GalNAc residue is installed by a family of $\approx$24 polypeptide *N*-acetyl-α-galactosaminyltransferases (6–8), then further elaborated by a series of glycosyltransferases to generate higher-order O-linked structures. Because of their complex biosynthetic origin, O-linked glycans are not installed at a defined consensus motif and their presence cannot be accurately predicted based on the protein's primary sequence (9). The one structural feature shared by all mucin-type O-linked glycans is their peptide-proximal GalNAc residue. There exists a lectin, *Helix pomatia* agglutinin (10), that binds to this residue but the GalNAc moiety must first be exposed by using a mixture of glycosidases. Additionally, there is no equivalent to N-glycanase for liberation of all O-linked glycans from the protein scaffold. The α-GalNAc/protein linkage can be cleaved under alkaline conditions (11), but the process is known to damage other posttranslational modifications and the protein backbone itself (12, 13).

Motivated by these deficiencies, we previously developed a technique for metabolic labeling of mucin-type O-linked glycans with a bioorthogonal chemical reporter, the azide (14–16). Peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz), an azido analog of GalNAc, was shown to be metabolized by cultured cells and incorporated into the core position of O-linked glycans (Fig. 1) (15). The azide is distinguished from all cellular functionality by virtue of its unique chemical reactivity with phosphine probes, a reaction termed the Staudinger ligation (17). Thus, proteins modified with GalNAz, a marker of O-linked glycans, can be selectively tagged for visualization or enrichment. More recently, azides have been used as chemical reporters for proteomic profiling of cytosolic O-GlcNAcylation (18–20) and farnesylation (21), and the potential exists for extension to numerous additional posttranslational modifications.

Although the chemical reporter strategy for O-linked glycoprotein profiling is now established in cultured cells, its application to probing glycosylation in living animals has not been explored. The importance of this transition is underscored by the established sensitivity of the glycome to specific cues from the tissue microenvironment (22). Thus, protein glycosylation in living tissues can differ drastically from that in cells cultured from the same tissues. Furthermore, changes in glycosylation are known to accompany cell differentiation and disease progression (23), processes that are most accurately studied *in vivo*. Profiling mucin-type glycosylation in a

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Abbreviations: GalNAz, *N*-azidoacetylgalactosamine; Ac_4GalNAz, peracetylated GalNAz; Ac_4ManNAz, peracetylated *N*-azidoacetylmannosamine; SiaNAz, *N*-azidoacetyl sialic acid; Phos-FLAG, phosphine reagent conjugated to the FLAG peptide; α-benzyl-GalNAc, α-benzyl 2-acetamido-2-deoxy-D-galactopyranoside; MFI, mean fluorescence intensity.

†D.H.D. and J.A.P. contributed equally to this work.

‖To whom correspondence should be addressed. E-mail: crb@berkeley.edu.

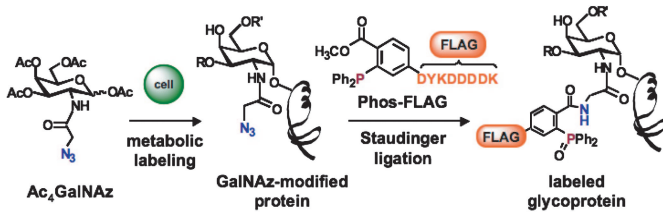

Fig. 1. Profiling mucin-type O-linked glycoproteins by metabolic labeling with an azido GalNAc analog ($Ac_4GalNAz$) followed by Staudinger ligation with a phosphine probe (Phos-FLAG). R and R′ are oligosaccharide elaborations from the core GalNAc residue.

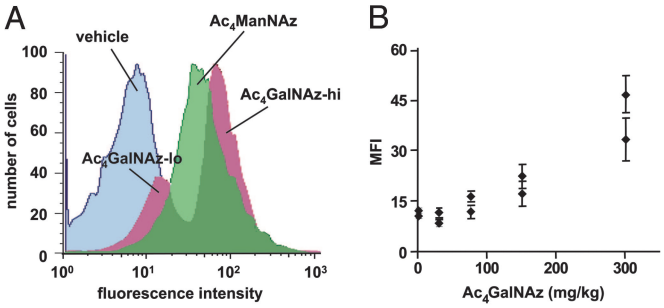

Fig. 2. $Ac_4GalNAz$ is metabolized *in vivo*. (*A*) Flow cytometry analysis of splenocytes from $Ac_4GalNAz$-treated (magenta) or $Ac_4ManNAz$-treated (green) C57BL/6 mice. (*B*) MFI of splenocytes from two B6D2F1 mice treated with various doses of $Ac_4GalNAz$. Error bars represent the SEM for three replicate Staudinger ligation reactions. Similar results were obtained in two replicate experiments.

truly physiological environment will therefore require moving beyond the cell culture dish to the living organism.

For successful *in vivo* labeling of O-linked glycoproteins with GalNAz, the azidosugar must possess suitable pharmacokinetic properties (i.e., tissue access, metabolic stability) and minimal toxicity. Classic literature on azido drug metabolism and toxicity was encouraging in this regard (24). Furthermore, Cravatt and coworkers (25, 26) administered azide-functionalized mechanism-based enzyme inhibitors to mice and achieved specific covalent labeling of target proteins without general toxicity. Most relevant to this work, we previously demonstrated that a relative of $Ac_4GalNAz$, peracetylated *N*-azidoacetylmannosamine ($Ac_4ManNAz$), is metabolized in mice and converted to the corresponding azido sialic acid (*N*-azidoacetyl sialic acid, SiaNAz) without any apparent physiological harm (27). The azide-labeled glycoproteins were chemically reacted with phosphine probes in tissue lysates or on cell surfaces within the living organism. Here, we report that mucin-type O-linked glycoproteins can be metabolically labeled with GalNAz in mice, permitting their detection and analysis by Staudinger ligation with phosphine probes. The technique provides a means for proteomic analysis of this elusive posttranslational modification and for identifying O-glycosylation signatures associated with disease.

Results and Discussion

O-Linked Glycoproteins Are Metabolically Labeled with GalNAz in Mice. Using cultured cell lines, we previously showed that $Ac_4GalNAz$ is deacetylated by cellular esterases and that the enzymes of the GalNAc salvage pathway will convert GalNAz to UDP-GalNAz *in situ* (15). Further, this azido nucleotide sugar is recognized by the polypeptide *N*-acetyl-α-galactosaminyltransferases that initiate O-glycosylation of protein substrates (28). Importantly, GalNAz was not found to be converted to any other cell surface azidosugar, such as *N*-azidoacetylglucosamine or ManNAz, in cells (15). Thus, the only products of $Ac_4GalNAz$ metabolism we observed were glycoconjugates possessing GalNAz itself. UDP-GalNAc can also be generated from UDP-GlcNAc via the action of UDP-GlcNAc/GalNAc C_4-epimerase (29). This pathway is thought to be the major source of cellular UDP-GalNAc, which competes with UDP-GalNAz generated via the salvage pathway. Thus, the maximal labeling efficiency we observed in cultured cells was ≈30% (15), achieved with cell culture doses of $Ac_4GalNAz$ in the range of 50 μM and labeling periods of several days. The direct translation of these parameters to an *in vivo* setting is not straightforward, thus we relied on previous experiments using $Ac_4ManNAz$ as a metabolic labeling substrate to guide our choice of injection doses and incubation times (27).

C57BL/6 mice were injected i.p. with solutions of $Ac_4GalNAz$ or vehicle (70% DMSO) once daily for 7 days. As a positive control, a solution of $Ac_4ManNAz$ was administered to a separate group of mice (27). In all cases, no signs of toxicity were observed after the 7-day dosing regimen. On the eighth day, spleens were harvested from the mice, and isolated splenocytes were analyzed for the presence of azides in their cell-surface glycoconjugates. Briefly, the splenocytes were treated with a phosphine reagent conjugated to the FLAG peptide (Phos-FLAG) (30) followed by FITC-α-FLAG, and the resulting cellular fluorescence was quantified by flow cytometry. Splenocytes from mice treated with azidosugar ($Ac_4ManNAz$ or $Ac_4GalNAz$) showed significantly higher fluorescence than splenocytes from vehicle-treated mice (Fig. 2*A*). Furthermore, splenocytes from $Ac_4GalNAz$-treated mice exhibited dose-dependent cellular fluorescence (Fig. 2*B*), suggesting that GalNAz is metabolically incorporated into cell surface glycans.

In addition to splenocytes, we analyzed a panel of murine organs for the presence of GalNAz-labeled glycoproteins. Tissues from B6D2F1 mice treated with $Ac_4GalNAz$, $Ac_4ManNAz$, or vehicle (as above) were harvested, homogenized, and reacted with Phos-FLAG. The treated lysates were then analyzed by Western blot using horseradish peroxidase-α-FLAG. $Ac_4GalNAz$ was found to label glycoproteins from the liver, kidney, heart, and serum (Fig. 3*A*). These tissues labeled more robustly than other organs examined, such as thymus and brain, perhaps because of their extensive vascularization and abundant production of mucin-type glycoproteins (31). The lack of GalNAz labeling observed in brain glycoproteins may be caused by the privileged nature of this compartment. Consistent with these hypotheses, $Ac_4ManNAz$ was found to label glycoproteins most robustly in the same subset of murine organs (27).

$Ac_4ManNAz$ and $Ac_4GalNAz$ Label Distinct Glycoproteins in Mice. $Ac_4ManNAz$ labels sialylated glycoproteins (17), whereas $Ac_4GalNAz$ is expected to label primarily mucin-type O-linked glycoproteins (15). Although some of these targets may overlap, we expected each azidosugar to produce a unique glycoprotein labeling pattern or "fingerprint." Western blot analysis of azide-labeled glycoproteins from heart tissue confirmed that $Ac_4ManNAz$ and $Ac_4GalNAz$ are incorporated into different glycoproteins *in vivo* (Fig. 3*B*). Interestingly, the splenocytes from $Ac_4GalNAz$-treated mice exhibited two populations of labeled cells. By contrast, splenocytes from $Ac_4ManNAz$-treated mice displayed only a single population (Fig. 2*A*). Splenocytes that were harvested from untreated mice and subsequently incubated with the azidosugars *ex vivo* showed the same population distribution (data not shown). This observation further suggests that $Ac_4GalNAz$ and $Ac_4ManNAz$ label distinct glycoproteins both *in vivo* and *in vitro*.

To characterize the nature of the glycoproteins that labeled with $Ac_4GalNAz$ and $Ac_4ManNAz$, we subjected splenocytes to treatment with glycan-modifying reagents. Splenocytes from B6D2F1 mice were cultured overnight with $Ac_4GalNAz$, $Ac_4ManNAz$, or no azidosugar, and then treated with sialidase, an enzyme that cleaves sialic acid residues, including SiaNAz (27). Cell-surface azides were

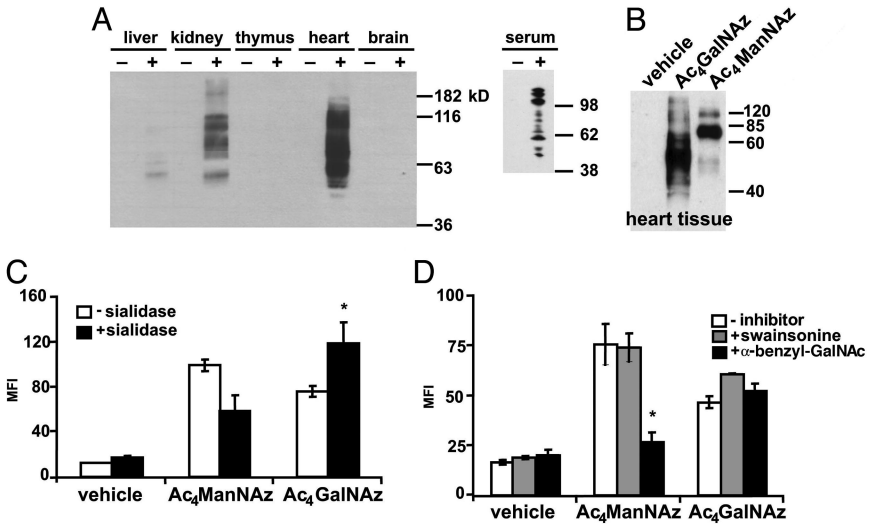

Fig. 3. Analysis of GalNAz-labeled glycoproteins on cells and in tissues. (*A*) Western blot analysis of tissue lysates from B6D2F1 mice administered $Ac_4GalNAz$ (+) or vehicle (−). An equivalent amount of protein (28 μg) was added to each lane, and similar patterns of labeling were apparent in several experiments. (*B*) Western blot analysis of heart tissue lysates (20 μg per lane) from $Ac_4ManNAz$-, $Ac_4GalNAz$-, or vehicle-treated mice. (*C*) Splenocytes from $Ac_4ManNAz$-, $Ac_4GalNAz$-, or vehicle-treated mice were incubated with (filled bars) or without (empty bars) *A. ureafaciens* sialidase. The cells were analyzed by flow cytometry; error bars represent the SEM for three replicate Staudinger ligation reactions. *, $P < 0.02$. (*D*) Splenocytes from B6D2F1 mice were treated with $Ac_4ManNAz$, $Ac_4GalNAz$, or no azidosugar in the presence of swainsonine (gray bars), α-benzyl-GalNAc (black bars), or no inhibitor (white bars). The cells were analyzed by flow cytometry, and error bars represent the SEM for three replicate Staudinger ligation reactions. *, $P < 0.002$.

quantified by reaction with Phos-FLAG followed by flow cytometry analysis as above. As expected, sialidase treatment reduced the fluorescence of $Ac_4ManNAz$-treated splenocytes (Fig. 3*C*). In contrast, sialidase treatment of $Ac_4GalNAz$-treated splenocytes resulted in a significant increase in the number of detectable azides (Fig. 3*C*). It is likely that removal of terminal sialic acid residues renders core GalNAz residues more accessible to the detection reagents (i.e., Phos-FLAG and FITC-α-FLAG).

We next used standard N- and O-linked glycosylation inhibitors to probe the nature of $Ac_4GalNAz$- and $Ac_4ManNAz$-labeled glycoproteins. The two compounds used in this study were the small molecule "primer" α-benzyl 2-acetamido-2-deoxy-D-galactopyranoside (α-benzyl-GalNAc) (32), which blocks the elaboration of core GalNAc residues on O-linked glycoproteins, and swainsonine (33), which inhibits the formation of complex N-linked glycans. Splenocytes from B6D2F1 mice were treated with $Ac_4GalNAz$, $Ac_4ManNAz$, or no azidosugar in combination with the inhibitors, and the presence of azides in their cell-surface glycoproteins was quantified by flow cytometry. Treatment with swainsonine did not substantially alter azide labeling on either $Ac_4GalNAz$-treated or $Ac_4ManNAz$-treated splenocytes. In a control experiment, swainsonine treatment increased splenocyte staining with FITC-Con A (data not shown), suggesting that the compound had disrupted complex N-glycan biosynthesis, leading to an increase in high-mannose structures (34). α-benzyl-GalNAc treatment significantly reduced the number of detectable azides on $Ac_4ManNAz$-treated splenocytes (Fig. 3*D*), suggesting that most SiaNAz residues occupy the termini of elaborated O-linked glycans. By contrast, α-benzyl-GalNAc did not affect the number of detectable azides on $Ac_4GalNAz$-treated splenocytes. This observation is consistent with a majority of GalNAz residues occupying peptide-proximal core positions, which should be unaffected by the primer.

Metabolic Labeling with GalNAz Can Distinguish Leukocyte Subpopulations. As noted above, splenocytes from $Ac_4GalNAz$-treated mice exhibited two populations, one that labeled robustly with the azidosugar ($Ac_4GalNAz$-hi) and one that was barely labeled above background ($Ac_4GalNAz$-lo) (Fig. 2*A*). We were curious as to how different leukocyte classes contributed to these populations. Splenocytes are a mixture of B cells, T cells, natural killer (NK) cells, and macrophages, among others. Combined, B and T cells comprise ≈85% of splenocytes in healthy mice, with their ratios ranging from 3:1 to 1:1. C57BL/6 mice were treated with $Ac_4GalNAz$, $Ac_4ManNAz$, or vehicle. Their splenocytes were harvested and probed by two-color flow cytometry for the presence of cell surface azides and for the B and T cell markers B220 and CD3, respectively (Fig. 4). B and T cells from $Ac_4ManNAz$-treated mice exhibited similar levels of cell surface azides (Fig. 4*A*). By contrast, B cells from $Ac_4GalNAz$-treated mice represented the $Ac_4GalNAz$-hi population, whereas T cells from the same mice represented the $Ac_4GalNAz$-lo population (Fig. 4*B*). We also performed two-color flow cytometry with antibodies specific for macrophages (anti-F4/80) and NK cells (anti-DX5). These minor leukocyte populations (<10% of total splenocytes) labeled with $Ac_4GalNAz$ at levels comparable to B cells (data not shown).

We further characterized the fingerprint of GalNAz-labeled glycoproteins from purified B cells. Splenocytes were harvested from C57BL/6 mice and cultured with $Ac_4GalNAz$ *ex vivo*. The B and T cells were separated by using magnetic beads. The purified B and T cell populations were lysed, reacted with Phos-FLAG, and analyzed by Western blot using horseradish peroxidase-α-FLAG. Analogous to flow cytometry data, glycoproteins from B cells, but not T cells, were intensely labeled with GalNAz (Fig. 4*D*).

The observed difference in global metabolic labeling of B cells and T cells with $Ac_4GalNAz$ could be attributed to a number of factors. A simple interpretation is that B cells harbor more mucin-type O-linked glycoproteins than T cells, with GalNAz acting as a reporter of this phenomenon. However, T cells are known to be rich in mucins (e.g., CD43) (35), and flow cytometry analysis using the Jacalin lectin (36) revealed higher levels of target O-linked glycan epitopes on T cells than on B cells (*Supporting Text* and Fig. 6, which are published as supporting information on the PNAS web site). It is also possible that the observed labeling patterns reflect differences in the abilities of these lymphocyte subpopulations to metabolize GalNAz. For example, it is possible that the polypeptide *N*-acetyl-α-galactosaminyltransferases expressed in T cells are less tolerant of the azide-modified substrate than those expressed in B cells. However, splenocytes cultured with ^{14}C-labeled $Ac_4GalNAc$

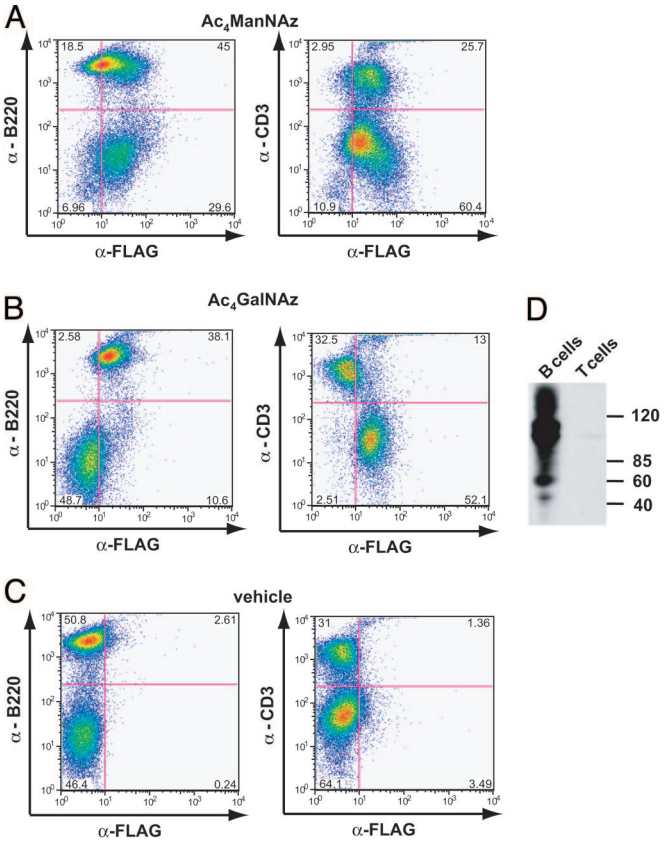

Fig. 4. $Ac_4GalNAz$ metabolism can distinguish leukocyte subpopulations. (*A–C*) Flow cytometry analysis of splenocytes from C57BL/6 mice treated with $Ac_4ManNAz$, $Ac_4GalNAz$, or vehicle. Plots are shown with B cell (α-B220) and T cell (α-CD3) staining on the *y* axis and azide probing (α-FLAG) on the *x* axis. (*D*) Western blot analysis of B and T cell lysate (30 μg per lane) from C57BL/6 murine splenocytes cultured in media supplemented with $Ac_4GalNAz$. The same patterns of labeling were apparent in several experiments.

revealed a similar disparity in glycoprotein labeling between T cells and B cells (*Supporting Text* and Fig. 7, which are published as supporting information on the PNAS web site). No differences in sugar uptake were observed.

These results suggest that B cells may be intrinsically more active than T cells in the *de novo* production of O-linked glycoproteins. The observation that T cells metabolize $Ac_4ManNAz$ and label glycoproteins with SiaNAz at high levels may seem contradictory to this hypothesis. However, it should be noted that GalNAz is a core residue and should therefore preferentially label O-glycans of newly biosynthesized glycoproteins. In contrast, SiaNAz is a terminal glycan modification that can be added to both newly synthesized proteins and proteins that are recycled from the cell surface (37). In this scenario, the two azidosugars access distinct pools of glycoproteins, which could account for their differential labeling of B and T cells.

To address the hypothesis that GalNAz is preferentially incorporated into newly synthesized glycoproteins, we used cycloheximide (CHX), an inhibitor of *de novo* protein biosynthesis (38). Partial inhibition of this cellular process had a more pronounced effect on $Ac_4GalNAz$ labeling than on $Ac_4ManNAz$ labeling of both B and T cells (*Supporting Text* and Fig. 8, which are published as supporting information on the PNAS web site). Presumably, B and T cells recycle membrane glycoproteins at comparable levels and therefore label robustly with $Ac_4ManNAz$ in the presence or absence of CHX.

There are numerous alternative explanations for the differential metabolism of $Ac_4GalNAz$ by B and T cells. For example, B and T cells may possess different mechanisms for sugar uptake/transport that result in differential labeling with GalNAz but not SiaNAz. Finally, endogenous UDP-GalNAc levels may differ between the lymphocyte populations, perhaps because of varying activities of their UDP-GlcNAc/GalNAc C_4-epimerase enzymes. Unfortunately, cultured cell lines are unlikely to be instructive in defining the origins of the metabolic difference observed with B and T cells. We screened a panel of B cell (Namalwa, Daudi, Raji) and T cell (Jurkat, Loucy, HuT78) lines for cell-surface labeling with $Ac_4GalNAz$, and unlike primary splenocytes, the cultured cells labeled at comparable but low levels (*Supporting Text* and Fig. 9, which are published as supporting information on the PNAS web site). Thus, further studies will require analysis of primary splenocytes *in vivo* or in cell culture.

Selective Targeting of B Cells in Mice by GalNAz Metabolism Followed by Staudinger Ligation. Regardless of the mechanism underlying the preferential metabolic labeling of B cells with GalNAz, the phenomenon can be exploited for *in vivo* targeting of B cells and other GalNAz-labeled tissues with phosphine probes. As demonstrated previously with SiaNAz as a target, the Staudinger ligation can be performed in living animals for delivery of phosphine probes to the most richly labeled sites (27). Analogously, metabolism of $Ac_4GalNAz$ followed by *in vivo* Staudinger ligation with detectable probes may prove useful for tracking changes in O-linked glycosylation in living animals. To investigate the reaction of phosphine probes with GalNAz-labeled glycoproteins *in vivo*, B6D2F1 mice were treated with $Ac_4GalNAz$ once daily for 7 days, followed by one bolus of Phos-FLAG on the eighth day. In control experiments, mice were treated with $Ac_4ManNAz$ or vehicle followed by Phos-FLAG by using the same protocol. Ninety minutes postinjection, splenocytes were harvested and probed for the presence of FLAG epitopes (the reaction product of the Staudinger ligation) by flow cytometry with FITC-α-FLAG. Splenocytes from mice treated with azidosugar (either $Ac_4GalNAz$ or $Ac_4ManNAz$) and Phos-FLAG displayed significantly higher fluorescence than splenocytes from mice treated with azidosugar, Phos-FLAG, or vehicle alone (Fig. 5*A*). This result confirmed the successful Staudinger ligation of Phos-FLAG with GalNAz *in vivo*. To estimate the yield of the reaction, we quantified the remaining azides on splenocytes by treating these cells with an additional bolus of Phos-FLAG *ex vivo*. No fluorescence increase was observed on splenocytes from mice that had been injected with Phos-FLAG, suggesting that the *in vivo* Staudinger ligation was quantitative with respect to chemically accessible azides.

To assess the selectivity of the *in vivo* Staudinger ligation for those cells most richly labeled with GalNAz, we analyzed splenocyte subpopulations by two-color flow cytometry. As shown in Fig. 5*B*, B cells were strongly labeled with Phos-FLAG *in vivo*, whereas T cells were not, mirroring the GalNAz distribution observed with harvested splenocytes (Fig. 4). Similarly, the Staudinger ligation product was detected on glycoproteins from kidney lysates (*Supporting Text* and Fig. 10, which are published as supporting information on the PNAS web site). The correlation between azidosugar labeling and chemical tagging suggests that an abundance of cell surface azides can be exploited to target a particular cell population with a bioorthogonal probe.

Conclusion

Altered posttranslational modification of proteins is a hallmark of many disease states (39–41). Analysis of these changes at the proteome scale has already revealed clinical biomarkers for diagnosis and therapeutic monitoring. Changes in glycoconjugate expression levels, or in the structures of their pendant glycans, are characteristic of many cancers and have been exploited in a clinical setting. For example, the membrane-bound mucin glycoprotein MUC1 is highly overexpressed on

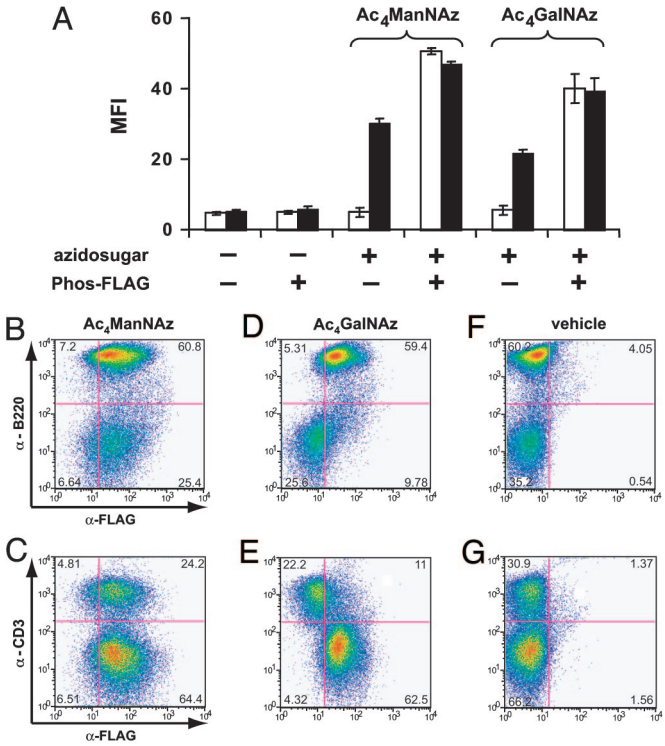

Fig. 5. Selective targeting of B cells in mice by GalNAz labeling followed by Staudinger ligation. Mice were administered Ac_4ManNAz, Ac_4GalNAz, or vehicle once daily for 7 days. On the eighth day, the mice were administered Phos-FLAG (18 μmol in $\approx$150 μl of water) or an equal volume of vehicle. (*A*) After 1.5 h, splenocytes were harvested and treated with FITC-α-FLAG, then analyzed by flow cytometry (empty bars). A portion of the isolated splenocytes was further reacted with Phos-FLAG and analyzed as in Fig. 2 (filled bars). Error bars represent the SEM for three replicate FITC-α-FLAG labeling reactions or Staudinger ligation reactions. (*B–G*) Splenocytes from mice treated with Ac_4ManNAz (*B* and *C*), Ac_4GalNAz (*D* and *E*), or vehicle (*F* and *G*) and Phos-FLAG, as in *A*, and subsequently treated with FITC-α-FLAG and α-B220 or α-CD3. Flow cytometry plots are shown with B cell (α-B220) and T cell (α-CD3) staining on the *y* axis and azide probing (α-azide) on the *x* axis.

breast cancers and displays O-linked glycans that are truncated compared with MUC1 on healthy breast tissue (42, 43). The mucin glycoprotein CA125 is found at elevated levels in the serum of ovarian cancer patients and also displays aberrant glycosylation (44, 45). The prostate-specific antigen, a widely used serum marker of prostate cancer, possesses altered glycans that may more accurately reflect the presence of the tumor than the protein's abundance alone (46, 47). Given that most epithelial cancers show some form of altered mucin-type glycosylation (48), the ability to profile this posttranslational modification could generate a multitude of novel clinical biomarkers.

Metabolic labeling with Ac_4GalNAz introduces a bioorthogonal chemical reporter into mucin-type O-linked glycoproteins that can be tagged with phosphine probes for detection or enrichment. The technique is amenable to rapid profiling of O-linked glycoproteins from cultured cells or tissues from living animals and can potentially be applied to profiling glycolipids and other biopolymers. Importantly, serum glycoproteins were found labeled with GalNAz, suggesting applications to the identification of additional serum diagnostic markers. An interesting future direction will involve comparison of the GalNAz-labeled glycoprotein fingerprint from sera of normal and tumor-bearing mice. Furthermore, the ability to tag GalNAz residues with phosphine probes *in vivo* may permit noninvasive imaging of changes in O-linked glycosylation.

The metabolic labeling technique selects for glycoconjugates that are biosynthesized *de novo* and cells that are highly metabolically active. Thus, the fingerprint generated by metabolic labeling with Ac_4GalNAz should reflect a combination of the glycan structure, the activity of the glycosylation machinery, and the overall metabolic state of the cell. Perturbations to any of these parameters could alter the labeling fingerprint. In this regard, the technique is distinct from and complementary to other high-throughput profiling methods that probe the steady-state levels of glycoproteins from tissue samples. For example, lectin microarrays have been used to define glycan fingerprints of complex mixtures (49, 50), and lectin blotting and histology have identified glycosylation patterns associated with disease (40, 41). Based on our observations with B and T cell labeling, we expect that fingerprints generated by Ac_4GalNAz metabolism will be distinct from those observed with lectins. The metabolic labeling technique should therefore permit visualization of glycosylation from a different vantage point.

Materials and Methods

Mice. B6D2F1/J mice were purchased from The Jackson Laboratory, and C57BL/6 mice were purchased from Charles River Laboratories. Animals were handled in accordance with Animal Use Protocol R234-0505B (approved by the Animal Care and Use Committee at the University of California, Berkeley).

Compound Administration. Ac_4ManNAz (51), Ac_4GalNAz (15), and Phos-FLAG (30) were synthesized according to previously published procedures. For metabolic labeling experiments, wild-type mice (B6D2F1 or C57BL/6) were administered daily doses of Ac_4ManNAz or Ac_4GalNAz (0–300 mg/kg in $\approx$200 μl of 70% aqueous DMSO) i.p. for 7 days. Organs were harvested 24 h after the final azidosugar injection. For experiments in which the Staudinger ligation was performed *in vivo* B6D2F1 mice were administered Ac_4ManNAz or Ac_4GalNAz (300 mg/kg in 70% aqueous DMSO) or vehicle (70% aqueous DMSO) i.p. once daily for 7 days. Twenty-four hours after the final azidosugar bolus, mice were injected with Phos-FLAG i.p. (18 μmol in $\approx$150 μl of water) or vehicle (water). Organs were harvested 90 min after the Phos-FLAG injection.

Glycoprotein Labeling on Isolated Splenocytes. Splenocytes were isolated from vehicle-, Ac_4ManNAz-, and Ac_4GalNAz-treated mice by using a standard protocol. The presence of azides in splenocyte cell-surface glycoproteins was determined by reaction with a phosphine probe as reported (51). Briefly, splenocytes were incubated with Phos-FLAG [0–250 μM in PBS + 1% FBS (FACS buffer)] for 1 h at room temperature, then treated with FITC-α-FLAG (Sigma, 1:900 dilution in FACS buffer) or FITC-conjugated mouse IgG_1 isotype control (BD Pharmingen, 1:900 dilution in FACS buffer) for 30 min on ice and analyzed by flow cytometry on a FACSCalibur instrument (BD Biosciences, Franklin Lakes, NJ). For single-color analysis, 10,000 live cells were analyzed for each replicate experiment. The Phos-FLAG labeling reaction was performed in triplicate, and data are reported as the mean fluorescence intensity (MFI) of a population of cells from replicate experiments.

GalNAz-labeled glycoproteins on B and T cells were detected by using the assay described above. Concurrent with FITC-α-FLAG treatment, the splenocytes were treated with phycoerythrin (PE)-Cy5-α-mouse CD45R/B220 (BD 1:20 dilution in FACS buffer) to label B cells, PE-Cy5-α-mouse CD3 (BD Pharmingen, 1:20 dilution in FACS buffer) to label T cells, or PE-Cy5 Rat IgG_{2a} isotype control (BD Pharmingen, 1:20 dilution in FACS buffer) to quantify nonspecific antibody binding. Multicolor flow cytometry experiments were performed on a FACSCalibur instrument, gating on 50,000 live cells per data point. Data were analyzed by using FLOJO software (Tree Star, Ashland, OR).

Preparation of Organ Lysates and Western Blot Analysis of Labeled Glycoproteins. Isolated murine organs were rinsed with PBS (pH 7.4) and homogenized in 2 ml of lysis buffer (20 mM Tris·HCl, pH

7.4/1% Igepal/150 mM NaCl/1 mM EDTA/0.5 mM PMSF/Roche protease inhibitor mixture) with a Dounce homogenizer. To probe for the presence of azides, aliquots of the tissue lysates were diluted 1:1 with 500 μM Phos-FLAG and incubated at room temperature for 12 h. The samples were analyzed by Western blot with horseradish peroxidase-α-FLAG as described (20).

Sialidase Treatment of Splenocytes. Splenocytes from Ac_4ManNAz-treated, Ac_4GalNAz-treated (300 mg/kg i.p. once daily for 7 days), or vehicle-treated B6D2F1 mice were incubated with *Arthrobacter ureafaciens* sialidase as reported (27).

Metabolic Labeling of Splenocyte Glycoproteins *ex Vivo*. After isolation from B6D2F1/J or C57BL/6 mice, splenocytes were cultured in RPMI media 1640 (GIBCO) supplemented with 10% FBS, penicillin/streptomycin, and Ac_4ManNAz or Ac_4GalNAz (10–50 mM). Cells were plated at a density of $\approx 1 \times 10^6$ cells/ml and incubated 1–2 days in a 5% CO_2, water-saturated incubator at 37°C.

Treatment of Cultured Murine Splenocytes with Glycosylation Inhibitors. Splenocytes from B6D2F1 mice were cultured in media supplemented with 50 μM Ac_4ManNAz or Ac_4GalNAz (as described above) and with swainsonine (Calbiochem; 10 μM) or α-benzyl-GalNAc (Sigma; 0.65 mM). Control experiments were performed in the absence of azidosugar or glycosylation inhibitor. After 2 days, splenocytes were rinsed and probed for the presence of azide-labeled glycoproteins by using Phos-FLAG (250 μM, 1 h) and FITC-α-FLAG (1:900 dilution, 30 min on ice) as described above.

Purification and Lysis of B and T Cells. Splenocytes from three C57BL/6 mice were isolated and cultured in media supplemented with 10 μM Ac_4GalNAz. After overnight incubation, the splenocytes were pelleted, rinsed, and resuspended in FACS buffer to a density of 14×10^7 cells/ml. The B cells and T cells were separated and isolated by using Pan B Dynabeads (Dynal, Great Neck, NY, 114.01) and Pan T Dynabeads (Dynal, 114.03) according to the manufacturer's instructions. The captured beads were rinsed with FACS buffer, resuspended in 100 μl of lysis buffer, and subjected to 10 freeze/thaw cycles. The cell lysates were separated from the beads, and the lysate protein concentration was determined by using a Bio-Rad DC Protein Assay kit. B and T cell lysates were standardized to a concentration of 1.6 mg/ml, diluted 1:1 with 500 μM Phos-FLAG, and reacted overnight at room temperature. The samples were analyzed by Western blot probing with horseradish peroxidase-α-FLAG as described above.

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