Optimization of Metabolic Oligosaccharide Engineering with Ac4GalNAlk and Ac4GlcNAlk by an Engineered Pyrophosphorylase

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ABSTRACT: Metabolic oligosaccharide engineering (MOE) has fundamentally contributed to our understanding of protein glycosylation. Efficient MOE reagents are activated into nucleotide-sugars by cellular biosynthetic machineries, introduced into glycoproteins and traceable by bioorthogonal chemistry. Despite their widespread use, the metabolic fate of many MOE reagents is only beginning to be mapped. While metabolic interconnectivity can affect probe specificity, poor uptake by biosynthetic salvage pathways may impact probe sensitivity and trigger side reactions. Here, we use metabolic engineering to turn the weak alkyne-tagged MOE reagents Ac4GalNAlk and Ac4GlcNAlk into efficient chemical tools to probe protein glycosylation. We find that bypassing a metabolic bottleneck with an engineered version of the pyrophosphorylase AGX1 boosts nucleotide-sugar biosynthesis and increases bioorthogonal cell surface labeling by up to two orders of magnitude. A comparison with known azide-tagged MOE reagents reveals major differences in glycoprotein labeling, substantially expanding the toolbox of chemical glycobiology.

INTRODUCTION

Protein glycosylation is an essential modulator of biological processes. Chemical MOE reagents have developed into important alternatives to protein-based binding reagents to profile the roles of glycans in cellular processes.1−4 Monosaccharides with chemical modifications can be fed to living cells as hydrophobic caged analogues that cross the plasma membrane. Once deprotected by (thio-)esterases, these monosaccharides are metabolically activated and introduced into the glycome by the activity of glycosyltransferases (GTs).1,4−6 Modifications such as azides or alkynes can be probed by bioorthogonal ligation using Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) to allow for the visualization and characterization of glycoconjugates.2,4,7,8 While it is generally accepted that small chemical perturbations are compatible with metabolic activation, the actual fate and turnover efficiency of modified monosaccharides is only beginning to be understood. The key to being used by GTs is the biosynthesis of modified nucleotide-sugars, such as derivatives of uracil diphosphate (UDP)-activated N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) (Figure 1A). The biosynthetic salvage pathway of GalNAc derivatives features the kinase GALK2 and the pyrophosphorylases AGX1/2, while GlcNAc derivatives have to be activated by the kinase NAGK, the mutase AGM, as well as AGX1/2.5,10 In the cytosol of mammalian cells, the derivatives of UDP-GalNAc and UDP-GlcNAc can be interconverted by the UDP-GalNAc/GlcNAc 4'-epimerase GALE, which interconnects both nucleotide-sugar pools. Epimerization substantially decreases the glycan specificity while enhancing the labeling efficiency of certain MOE reagents and can be suppressed by careful choice of the chemical modification.10−12 Once biosynthesized, the derivatives of UDP-GalNAc and UDP-GlcNAc can be used as substrates by cellular GTs, including the large polypeptide GalNAc transferase (GalNAc-T) family in the secretory pathway and a myriad of GlcNAc transferases in several cellular compartments.

Recent years have seen increasing evaluation of the metabolic fate of MOE reagents. Although the enzymes of GalNAc and GlcNAc salvage pathways generally display reduced efficiency toward modifications on the acetamide side chain, the relatively small azide group is accepted as part of reliable MOE reagents.10,12−15 In contrast, bulkier modifications prevent the enzymatic activation of GalNAc and GlcNAc analogues.11,16,17 Yu et al. thus developed an...
engineered version of AGX1 (mutant F383G) to increase substrate promiscuity and biosynthesize UDP-GlcNAc analogues from the corresponding GlcNAc-1-phosphate analogues that can be delivered through caged precursors. We have used the similar F383A mutant, herein termed mut-AGX1, to biosynthesize UDP-GalNAc analogues that would not normally be made in the living cell. Somewhat surprisingly and contrary to azide-tagged analogues of similar size, Batt et al. found that, after feeding the commercial MOE reagents Ac4GalNAlk1 and Ac4GlcNAlk2, the most simple alkyne-tagged UDP-GalNAc and UDP-GlcNAc derivatives, Ac4GalNAlk3 and Ac4GlcNAlk4, are biosynthesized in varying and often low efficiency in mammalian cells (Figure 1A). Previous experience by us and Yu et al. on delivering UDP-sugar analogues with even longer side chains suggested that AGX1-mediated pyrophosphorylation may be a roadblock to the biosynthesis of UDP-GalNAc/GlcNAc derivatives. These longer derivatives could only be delivered through caged sugar-1-phosphates that are of limited stability and tedious to synthesize. We thus sought to investigate if simply enhancing pyrophosphorylation with mut-AGX1 would allow delivery from the readily available reagents Ac4GalNAlk1 and Ac4GlcNAlk2.

Here, we profile the metabolic fate of the weak MOE reagents Ac4GalNAlk1 and Ac4GlcNAlk2 in order to turn both reagents into highly efficient tools to probe cell surface glycosylation. We find that mut-AGX1 effectively biosynthesizes UDP-GalNAlk3 and UDP-GlcNAlk4 with greatly increased efficiency over WT-AGX1 from caged precursors that can thus be used to profile cell surface protein glycosylation. Bysuppressing GALE-mediated epimerization, we further find that UDP-GalNAlk3 and UDP-GlcNAlk4 are used to glycosylate non-identical glycoprotein subsets to azide-tagged analogues, potentially due to differential acceptance by GTs. We show that close monitoring of the biosynthetic fate enables the development of highly effective MOE reagents.

RESULTS AND DISCUSSION

To study the metabolic fate of UDP-GalNAlk3 and UDP-GlcNAlk4, we first assessed in vitro whether both reagents are epimerized by GALE (Figure 1B). The incubation of synthetic Ac4GalNAlk3 with either a wild type (WT) GALE-containing (ctrl sgRNA) cell lysate or a GALE-KO lysate as a control, as assessed by high-performance anion exchange chromatography (HPAEC). The reaction was also performed using purified GALE, and the retention times were compared to those of the standards (Figure S1). (C) In vitro glycosylation with purified GalNAc-Ts of synthetic peptides using UDP-GalNAlk3 or UDP-GlcNAlk as substrates. The amino acids in red are new glycosylation sites. T* denotes α-D-GalNAc-Thr. Data are individual measurements of independent duplicates and means. The reactions using UDP-GalNAlc as a substrate have been used previously.
use of UDP-GalNAlk 3 as a GalNAc-T subset-selective substrate.

We next studied the biosynthesis of UDP-GalNAlk 3 and UDP-GlcNAlk 4 in cells fed with caged, membrane-permeable precursors. Since AGX1 has been identified as a metabolic bottleneck of other modified GalNAc analogues, we synthesized caged GalNAlk-1-phosphate 5 to specifically probe the AGX1-mediated biosynthesis of UDP-GalNAlk 3.2,5,17 We tested UDP-sugar biosynthesis from 5 in K-562 cells with either normal GALE expression or a GALE-KO and stably transfected with either mut-AGX1, WT-AGX1, or an empty vector. HPAEC revealed measurable biosynthesis of both UDP-GalNAlk 3 and UDP-GlcNAlk 4 in the presence of mut-AGX1 but not WT-AGX1 (Figure 2A and Figure S2). The levels of UDP-GalNAlk 3 and UDP-GlcNAlk 4 were in the same range as the levels of native UDP-GalNac and UDP-GlcNac. Free GalNAlk-1-phosphate was detectable in all cases, as observed by comparison with a synthetic standard (Figure S2). In the absence of GALE, UDP-GlcNAlk was not detectable, indicating that UDP-GalNAlk 3 is biosynthesized by mut-AGX1 and subsequently epimerized by GALE in the cytosol.

We then assessed metabolic cell surface labeling mediated by caged GalNAlk-1-phosphate 5 by flow cytometry. Clickable biotin-picolyl azide was used in noncytotoxic Cu(I)-click CuAAC conditions followed by streptavidin-DTAF to visualize labeling.8,21 The presence of mut-AGX1 led to a dose-dependent increase of cell surface labeling from Ac4GlcNAlk, indicating that UDP-GalNAlk was biosynthesized at levels that are too low to detect chromatographically. This was especially pronounced in GALE-KO cells in which no endogenous UDP-GalNac is present to compete with UDP-GalNAlk 3 as a substrate of GalNAc-Ts (Figure 2B and Figure S3B). A labeling difference of one order of magnitude was observed between cells expressing WT-AGX1 and mut-AGX1 when fed with Ac4GlcNAlk 2, indicating that mut-AGX1 also mediates UDP-GlcNAlk 4 biosynthesis (Figure S3A). Increasing the UDP-GalNac levels in GALE-KO cells by supplementing cell culture media with free GalNac led to a decrease of an UDP-GalNAlk 3-dependent labeling signal (Figure 2C and Figure S3C).17 Likewise, the labeling signal by Ac4GlcNAlk 2 was abrogated by the addition of free GlcNac (Figure 2C). In contrast, labeling by the control compound Ac4ManNAlk, a MOE reagent that enters the biosynthetic pathway of the sugar sialic acid, was unchanged irrespective of AGX1 overexpression or the addition of free GalNac or GlcNac (Figure 2C and Figure S3B). Enhancing the levels of native UDP-sugars thus competed out the incorporation of GalNac and GlcNac, but not ManNAlk, into glycoproteins. We concluded that AGX1 is likely a bottleneck in the biosynthesis of both UDP-GalNAlk 3 and UDP-GlcNAlk 4, impairing metabolic labeling, which can be enhanced by a stable overexpression of mut-AGX1 but not WT-AGX1. Our data further indicate that Ac4GalNAlk 1 exhibits low-level metabolic glycoprotein labeling without mut-AGX1 expression, in line with findings of Zaro et al.7 UDP-GalNAlk 3 formation is not measurable under these conditions, underlining the highly inefficient biosynthesis of 3 by the GalNac salvage pathway without mut-AGX1.18

As the overexpression of mut-AGX1 enabled cell surface labeling from Ac4GlcNAlk 2, GlcNAlk-1-phosphate biosynthesis from the free monosaccharide by NAGK/AGM1 was apparently not a major metabolic bottleneck. We next assessed whether UDP-GalNAlk 3 biosynthesis followed the same principles, which would, in turn, allow us to use the readily available MOE reagent Ac4GlcNAlk 1 instead of caged GalNAlk-1-phosphate 5. We found that mut-AGX1, but not WT-AGX1, efficiently biosynthesized UDP-GalNAlk 3 and UDP-GlcNAlk 4 from the peracetylated precursors Ac4GalNAlk 1 and Ac4GlcNAlk 2, respectively, in living cells (Figure S4). We note that the “upstream” precursors Ac4GalNAlk 1 and Ac4GlcNAlk 2 required longer feeding times (12–16 h instead of 6–9 h) than caged GalNAlk-1-phosphate 5 for UDP-sugar biosynthesis to be detected, in line with additional enzymatic reactions being required. At these
time points, free GalNAlk-1-phosphate is clearly detectable (Figure S4). These data indicated that WT-AGX1-mediated pyrophosphorylation is likely the rate-determining step in the biosynthesis of UDP-GalNAlk 3 and UDP-GlcNAlk 4. The expression of mut-AGX1 likely renders the upstream activation steps NAGK/AGM and GALK2 as rate-determining.

We next visualized the impact of metabolic engineering on glycoprotein labeling by Ac4GlcNAlk 1 and Ac4GlcNAlk 2. Following the feeding of AGX1-transfected K-562 cells with alkyn-containing monosaccharide precursors, cell surfaces were either treated with a neuraminidase that removes sialic acid from glycoproteins, or left untreated. The living cells were then subjected to CuAAC with the clickable near-infrared fluorophore CF680-picoyl azide, and labeled cell surface glycoproteins were analyzed by in-gel fluorescence.17 Under these conditions, the compounds Ac4GlcNAlk 1, Ac4GlcNAlk 2, and caged GalNAlk-1-phosphate 5 exhibited mut-AGX1-dependent labeling while the control reagent Ac4ManNAlk labeled glycoproteins irrespective of the AGX1 construct used (Figure 3A). Neuraminidase treatment led to an increase of signals in all cases except for Ac4ManNAlk-labeled cells, (Figure 3A). Neuraminidase treatment led to an increase of glycoprotein labeling in AGX1-expressing K-562 cells with CF680-picoyl-azide under CuAAC conditions and visualized with streptavidin-AF647. Data are representative of two independent experiments. Scale bar: 20 μm.

Due to the GALE-mediated interconversion of UDP-GalNAlk 3 and UDP-GlcNAlk 4, the glycoprotein profiles labeled by both MOE reagents Ac4GlcNAlk 1 and Ac4GlcNAlk 2 were identical (Figure 3A). To assess the contribution of each UDP-sugar to the signal, we profiled the glycoprotein patterns in GALE-KO cells that functionally separate UDP-GalNAlk 3 and UDP-GlcNAlk 4 (Figure 4A). Cells were grown in GalNAc-containing media to maintain the native levels of metabolites such as UDP-GalNAc, allowing for comparison with GALE-expressing control cells when all cell lines were transfected with mut-AGX1. DMSO feeding did not lead to discernible labeling (Figure 4A, lanes 1 and 2).

While GALE-expressing control cells displayed identical labeling patterns when fed with either Ac4GalNAlk 1 or Ac4GlcNAlk 2 (Figure 4A, lanes 3 and 4), GALE-KO had a striking effect on labeling patterns. In GALE-KO cells, Ac4ManNAlk 1 contributed highly intense glycoprotein bands at approximately 100 and 40 kDa (Figure 4A, lane 5), while Ac4GlcNAlk 2 contributed a diffuse pattern of lower overall intensity (Figure 4A, lane 6). These results suggested that separating the UDP-GalNAlk 3 and UDP-GlcNAlk 4 pools led to labeling of different subsets of glycoproteins. In contrast, feeding Ac4ManNAlk led to similar band patterns in both GALE-expressing and GALE-KO cell lines (Figure 4A, lanes 7 and 8), indicating that sialylation is not affected by GALE-KO. We speculated that the intense bands labeled by UDP-GalNAlk 3 (Figure 4A, lanes 3–5) are highly GalNAc-glycosylated mucin-domain-containing glycoproteins. To test this notion, we treated cagedGalNAlk-1-phosphate 5-fed, mut-AGX1-expressing K-562 cells with CF680-picoyl-azide under CuAAC conditions to fluorescently tag the GalNAlk-
containing glycoproteome. We then subjected the living cells to different concentrations of the mucin protease StcE or the more promiscuous O-glycoprotease OpeRATOR (Figure S6). Treatment with both proteases led to a decrease of cell surface glycoprotein signal in a dose-dependent manner, while a signal was recovered as fluorescently-labeled broad bands of lower molecular weight in the supernatant. Several glycoprotein bands were digested by OpeRATOR, but not StcE, indicating that labeling of nonmucins containing O-GalNAc glycans was observed. These data confirm that GalNAc enters mucin-domain-containing proteins and other O-GalNAc-glycosylated proteins.

We next compared the Ac4GalNAk and Ac4GlcNAk labeling band patterns in GALE-KO or control cells with previously characterized, azide-containing MOE reagents Ac4GalNAz and Ac4GlcNAz (Figure 4B). Both reagents are converted to azide-tagged UDP-GlcNAc/GalNAc analogues that are interconvertible by GALE.10–12 We further used the O-GalNAc-specific reagent Ac3GalNAzMe-1-P-(SATE)2, a precursor to an epimerization-resistant, azide-tagged UDP-GalNAc analogue that is not a substrate for GALE in the living cell.11 To ensure that band patterns are comparable between azide- and alkyne-tagged monosaccharides, we used the same NIR-fluorophore CF680 with either alkyne or picolyl azide groups for CuAAC. Compound 8 showed a band pattern attributable to O-GalNAc glycosylation in GALE-containing and GALE-KO cells (Figure 4B, lanes 1 and 6). Ac4GalNAz/6/Ac4GlcNAz 7 (Figure 4B, lanes 2 and 3) labeled the same band pattern in GALE-containing cells, consistent with the interconversion of azide-tagged UDP-sugar pools.10,11 This labeling pattern was somewhat different from the pattern observed after feeding GALE-containing cells Ac4GalNAk 1/Ac4GlcNAk 2 (Figure 4B, lanes 4 and 5), with more bands being visible with azide-tagged monosaccharide analogues. These findings can be explained by UDP-GalNAz being a better substrate for the commonly expressed glycosyltransferases GaINAc-T1 and T2 than UDP-GalNAk 3 (Figure 1C).11,19 Upon GALE-KO, Ac4GalNAz 6 and Ac4GlcNAz 7 led to different band patterns, as reported before (Figure 4B, lanes 7 and 8).11 In comparison, the Ac4GalNAk-labeled band pattern in GALE-KO cells resembled only a subset of the pattern seen from Ac4GalNAz 6 or compound 8 feeding (Figure 4B, lane 9), indicating that UDP-GalNAk 3 labels a subset of O-GalNAc glycoproteins. Finally, Ac4GlcNAk 2 exhibited a diffuse labeling pattern in GALE-KO cells (Figure 4B, lane 10) similar to that of the azide-tagged counterpart Ac4GalNAz 7. Taken together, these data suggest that UDP-GalNAk 3 and UDP-GlcNAk 4 label different sets of glycoproteins but are interconnected by GALE in the living cell. Structurally simple azide- and alkyne-tagged GaINAc/GlcNAc derivatives label particular glycoprotein subsets and should thus serve as orthogonal but potentially complementary MOE reagents in the presence of mut-AGX1.

Western blot analysis with an antibody against AGX1 indicated that our expression constructs lead to an approximately 2-fold overexpression, suggesting that metabolic engineering does not require abundant expression levels of mut-AGX1. While our approach relies on cell transfection, orders of magnitude, substantially expanding the toolbox for glycobiology. Our work relied on cell transfection, the plasmids we used are based on transposase-mediated stable integration, which is compatible even with hard-to-transfect cell lines and more complex model systems such as organoids.11 Our work focused on improving metabolic labeling efficiency with Ac4GalNAk 1/Ac4GlcNAk 2. While we showed that the corresponding activated sugars UDP-GalNAk 3/UDP-GlcNAk 4 can be incorporated in GaINAc- and GlcNAc-containing glycoconjugates, we did not assess their fine specificity for certain subtypes of glycans. We and others have previously focused on assessing such specificity for similar MOE reagents,11,20 and further studies extending this work are underway.
Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00034.

Primary data and plasmids are available upon request. Compounds are available upon request as long as stocks last.

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Author Contributions

A.C. and G.B.-T. contributed equally. A.C., M.F.D., J.T.B., C.R.B., and B.S. conceived the project and planned experiments. A.C., G.B.-T., A.J.A. H.L.D., and B.S. performed experiments. J.C., T.M.W., W.M.B., C.R., and S.K. made and contributed key reagents. A.C., G. B.-T., A.J.A., M.F.D., and B.S. analyzed data. A.C., G.B.-T., and B.S. wrote the paper with input from all authors.

Funding

The authors are thankful for generous funding by Stanford University, Stanford Chemistry, Engineering and Medicine for Human Health (ChEM-H), and Howard Hughes Medical Institute. This work was supported by NIH Grant RO1 CA200423 (to C.R.B.) and by the Francis Crick Institute (A.C., G.B.-T., and B.S.), which receives its core funding from Cancer Research UK Grant FC001749, UK Medical Research Council Grant FC001749, and Wellcome Trust Grant FC001749. M.F.D. was supported by a Dutch Research Council (NWO) Rubicon Postdoctoral Fellowship. W.M.B. was supported by a PhD studentship funded by Engineering and Physical Sciences Research Council (EPSRC) Centre for Doctoral Training in Chemical Biology – Innovation for the Life Sciences Grant EP/S023518/1 and GlaxoSmithKline. H.L.D. acknowledges funds from Wellcome Trust New Investigator Award 104785/B/14/Z. A.J.A. was supported by a Stanford ChEM-H undergraduate scholarship. This research was funded in whole, or in part, by the Wellcome Trust [FC001749 and 104785/B/14/Z]. For the purpose of Open Access, the author has applied a CC BY public copyright license to any Author Accepted Manuscript version arising from this submission.

Notes

The authors declare no competing financial interest.

Acknowledgments

The authors would like to thank D. Fox for help with HPAEC experiments, K. Pedram for providing StcE, M. Pratt for helpful discussions, P. Walker for advice on vector choice, and A. Garza-Garcia for helpful discussions on HPLC. The authors would like to thank R. D’Antuono of the Crick Advanced Light Microscopy STP for support and assistance in this work. The authors are grateful for support by the Francis Crick Institute Cell Services and Peptide Chemistry Science Technology Platforms.

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