**In Vivo Imaging of Caenorhabditis elegans Glycans**

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**ABSTRACT** The nematode *Caenorhabditis elegans* is an excellent model organism for studies of glycan dynamics, a goal that requires tools for imaging glycans in vivo. Here we applied the bioorthogonal chemical reporter technique for the molecular imaging of mucin-type O-glycans in live *C. elegans*. We treated worms with azido-sugar variants of *N*-acetylgalactosamine (GlcNAc), *N*-acylgalactosamine (GalNAc), and *N*-acetylmannosamine (ManNAc), resulting in the metabolic labeling of their cell-surface glycans with azides. Subsequently, the worms were reacted via copper-free click reaction with fluorphore-conjugated difluorinated cyclooctyne (DIFO) reagents. We identified prominent localization of mucins in the pharynx of all four larval stages, in the adult hermaphrodite pharynx, vulva and anus, and in the tail of the adult male. Using a multicolor, time-resolved imaging strategy, we found that the distribution and dynamics of the glycans varied anatomically and with respect to developmental stage.

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**Caenorhabditis elegans** glycans are attractive targets for molecular imaging. As a well-established model organism, *C. elegans* offers a system in which to probe the dynamics of glycans in development, disease, and normal physiological processes. Further, glycans have been documented as key players in *C. elegans* biology. For example, vulval morphogenesis and early embryo cytokinesis require the glycosaminoglycan chondroitin sulfate (1, 2). Each stage of *C. elegans* development is associated with specific N-glycan structures, and anatomical features such as the cuticle, vulva, and male tail possess a repertoire of mucin-type O-glycans (3–5). Nevertheless, the dynamics of *C. elegans* glycans have remained obscure because they have been inaccessible to analysis by molecular imaging. Here we applied the bioorthogonal chemical reporter strategy (6), which we recently described for imaging glycans in developing zebrafish (7), to the molecular imaging of glycans in live *C. elegans*. In the first step, we metabolically incorporate an azido-sugar into the worm’s glycans by using their own biosynthetic machinery (Figure 1, panel a). Per-O-acetylated azidosugars passively diffuse into the cells where their hydroxyl groups are deacetylated by promiscuous intracellular esterases (8). The free sugar is processed and integrated into newly synthesized glycans, some of which are displayed on cell-surface glycoconjugates. The azidosugars are then imaged in a second step with difluorinated cyclooctyne (DIFO)-based probes (Figure 1, panel b) via copper-free click chemistry (9).

Additionally, azide-labeled glycoconjugates can be analyzed ex vivo by reaction of tissue lysates with alkyn- or triarylphosphine-based probes (e.g., via the Staudinger ligation (10)) followed by Western blot.

Because the metabolism of azidosugars in nonvertebrate animals has not been tested, we first assayed the ability of *C. elegans* to incorporate azido analogues of ManNAc, GalNAc, and GlcNAc into glycoproteins. We seeded nematode growth medium (NGM)-agar plates containing 1 mM per-O-acetylated *N*-azidoacetylmannosamine (Ac4ManNAz), -galactosamine (Ac4GalNAz), or -glucosamine (Ac4GlcNAz) with 10 larval (L1) wild-type *C. elegans* (strain N2). We allowed the population of worms to expand until the bacterial food source was consumed. Then, we made lysates from the mixed-stage population of worms, reacted them with phosphine-Flag, and analyzed the resident glycoproteins for the presence of azides using standard Western blot techniques (Figure 1, panel c). We observed robust incorporation of GalNAz into glycoproteins, indicating that *C. elegans*’ metabolic enzymes are tolerant of this azidosugar. Samples from Ac4GlcNAz- and Ac4ManNAz-treated worms produced no observable signal in the Western blot, indicating that these azidosugars are not incorporated into *C. elegans* glycans at significant levels. This was not unexpected because, in mammalian cells, GlcNAz is incorporated into cytosolic O-GlcNAcylated proteins at low levels with no significant cell-surface glycoprotein labeling (11). ManNAz is converted by mammalian cells to the corre-
sponding azido sialic acid (10), a residue that is absent in *C. elegans* (12).

We sought to determine the types of glycoproteins being labeled by Ac₄GalNAz. The naturally occurring counterpart of GalNAz, GalNAc, is found in mucin-type O-glycans (13), chondroitin sulfate (14), and rarely, N-glycans (15). In order to probe for GalNAz residues within chondroitin sulfate and N-glycans, we reacted lysates from Ac₄GalNAz-treated *C. elegans* with chondroitinase ABC or peptide N-glycosidase F (PNGase F), enzymes that digest the respective glycans. Then, we reacted the enzyme-treated lysates with phosphine-Flag and probed the glycoproteins by Western blotting with an α-Flag antibody. Neither enzyme-treated sample showed a significant decrease in chemiluminescence signal compared to undigested lysate (Supplementary Figure 1a,b), indicating that a majority of GalNAz residues are situated in other types of glycans.

We recently found that mammalian cells epimerize the nucleotide sugar derived from GalNAz, uridine diphosphatidyl (UDP)-GalNAz, to UDP-GlcNAz and that the enzyme O-GlcNAc transferase (OGT) further installs the azidosugar on nuclear and cytoplasmic proteins that would normally be O-GlcNAcylated (16). In order to determine if this phenomenon occurs in worms, we treated an OGT-deficient *C. elegans* strain, RB653 (17), with Ac₄GalNAz. Western blots of phosphine-Flag-labeled lysates from Ac₄GalNAz-treated RB653 worms were indistinguishable from similarly treated wild-type samples (Supplementary Figure 1c). This observation suggests that GalNAz does not metabolically label O-GlcNAcylated proteins appreciably in this organism.

Finally, we probed the binding of the azide-labeled glycoproteins to lectins specific for N- or O-glycans. We observed no detectable binding of azide-labeled glycoproteins to the N-glycan specific lectin concanavalin A (ConA), consistent with the hypothesis that a majority of labeled glycans are O-linked. Moreover, a small fraction of GalNAz-labeled species were retained on peanut agglutinin (PNA) conjugated agarose beads, and judging by the faint chemiluminescence signal of the relevant phosphine-Flag-modified sample, some of them were selectively eluted with galactose (Supplementary Figure 2). These results indicate that only a subset of GalNAz-labeled glycoproteins possess the unmodified form of the core 1 O-glycan, galactoseβ1–3GalNAc, which is recognized by PNA (18). That only a fraction of GalNAz-labeled glycoproteins bound to PNA-agarose was not unexpected because *C. elegans* O-glycans possess extended peripheral structures that would mask the PNA epitope (19).

Collectively, our *in vitro* characterization experiments suggest that GalNAz is incorporated into mucin-type O-glycans in *C. elegans*. We should note, however, that Western blot analysis of lysates does not probe glycolipids, which may possess GalNAc residues (20), nor does the technique analyze those species that are not soluble in the detergent lysis buffer. Thus, we cannot rule out the possibility that other glycans, such as insoluble cuticle-associated chondroitin sulfate proteoglycans (21), are metabolically labeled with GalNAz.

We next sought to image the azide-labeled glycans in live *C. elegans* in order to reveal their anatomical distribution at different stages of development. To accomplish this, we employed copper-free click chemistry using an Alexa Fluor 488-conjugated difluorinated cyclooctyne (DIFO) reagents (green) enables imaging of glycans by fluorescence microscopy. b) The DIFO-488 and -568 probes used in this study. c) Lysates from mixed-stage *C. elegans* that had been incubated with Ac₄GlcNAz, Ac₄GalNAz, or Ac₄ManNAz were reacted with phosphine-Flag and subsequently analyzed by Western blot, probing with HRP-conjugated α-Flag clone M2 (top panel) or mouse α-actin (bottom panel).
in the production of 40% male worms (compared to the wild-type occurrence of 0.1%) and thus allows the visualization of male-specific structures (22).

Mixed-stage populations of Ac4GalNAz-treated or untreated (“no sugar”) C. elegans were reacted with DIFO-488 for 1 h, washed briefly, and then anesthetized prior to imaging (Figure 2). In the adult hermaphrodite, we observed GalNAz-specific labeling in the pharynx, vulva, and anus (Figure 2, panels a–d). The labeled glycans were concentrated in the pharynx of all larval and adult worms (Figure 2, panels a, b, e), though higher exposure times did reveal some staining of the worms’ outer coat, the cuticle (Figure 2, panel d). Like the adult hermaphrodites, the adult males stained intensely in the pharynx region and minimally in the anus and cuticle. However, the male tail also showed striking labeling of the fan, spicule, and rays (Figure 2, panel f).

Figure 2. In vivo imaging of glycan distribution in larval and adult C. elegans. a) C. elegans adult hermaphrodites that had been incubated with Ac4GalNAz (+) or no sugar (−) were reacted with DIFO-488 and imaged alive. Fluorescence was observed in the pharynx (b), vulva (c), and anus (d) regions. e) C. elegans hermaphrodites at larval stage L1–L4 that had been incubated with Ac4GalNAz (+) or no sugar (−) were reacted with DIFO-488. Fluorescence was observed in the pharynx region of Ac4GalNAz-treated worms. f) C. elegans strain CA151 adult males metabolically labeled with Ac4GalNAz (+) or no sugar (−) were reacted with DIFO-488. Fluorescence was observed in the tail region of Ac4GalNAz-treated worms. DIC denotes differential interference contrast. 488 denotes DIFO-488 fluorescence. Scale bars: (a) 100 μm; (b–f) 25 μm.

The one-color labeling protocol enabled the visualization of the GalNAz-containing glycans that were produced throughout C. elegans larval development and into adulthood; however, this strategy does not highlight nascent glycans, as would be necessary to study their dynamics. To identify “hot spots” of O-glycan biosynthesis, we employed a two-color labeling strategy (Figure 3, panel a). We metabolically labeled mixed-stage strains N2 and CA151 with Ac4GalNAz and then reacted the worms with DIFO-488 as described above. We then transferred the specimens back to plates containing Ac4GalNAz (or to control plates) for an additional 12 h. During this second incubation, only the newly synthesized glycans were metabolically labeled with GalNAz. We reacted these worms with Alexa Fluor 568-conjugated DIFO (DIFO-568, Figure 1, panel b). Thus, at the end of the two labeling steps, the older population of glycans should be labeled with DIFO-488 (false-colored green) and the newer glycans with DIFO-568 (false-colored red). The anatomical distributions of the two dyes at various developmental stages were then analyzed by fluorescence imaging of live anesthetized worms.

Figure 3, panel b shows two-color fluorescence images of worms at three different developmental stages: L1, L2, and adult. Worms in the first larval stage (L1) when the image was acquired would have been eggs at the time of the initial labeling reaction. Accordingly, these worms show no labeling with DIFO-488 (green) but intense labeling with DIFO-568 (red), which was administered 12 h later (Figure 3, panel b, top row). By contrast, worms in the second larval stage (L2) when the image was acquired were in stage L1 during the DIFO-488 labeling reaction. These worms display roughly equal and overlapping green and red fluorescence, indicating that O-glycans were produced in the same anatomical regions during the first two larval stages. One region of the L2 larvae labeled more intensely with DIFO-568 (red) than with DIFO-488: the posterior pharynx (also called the “grinder,” marked in Figure 3, panel b by arrowheads). This local increase in the production of O-glycans was even more pro-
nounced in adult worms (Figure 3, panel b, marked by arrowheads).

In the adult vulva, the products from both labeling reactions were apparent, though the DIFO-568 fluorescence signal was less intense. The limited fluorescence associated with new glycans indicates that O-glycan biosynthesis occurred at relatively low levels in the vulva of adult hermaphrodites (Figure 3, panel c). In the male sex organs, we observed intense DIFO-488 labeling of the fan but minimal DIFO-568 labeling. Rather, the DIFO-568 highlighted the spicule and cloaca, indicating that new glycans are continually produced in these areas in the adult worm (Figure 3, panel d). These components of the male sex organs are known to have motive capabilities (24), a property shared with the pharynx (25). It is possible that mucin-type O-glycan production is required to supply lubricating mucin glycoproteins for these processes (26). Also, the organs most readily labeled with DIFO probes are those that are accessible to solution (i.e., pharynx, vulva, and male tail). This observation most likely reflects the Alexa Fluor probes’ limited penetrance into the organism. Thus, an important area of future optimization involves the development of fluorescent dyes capable of greater dissemination among the tissues of live worms.

In conclusion, we demonstrated that O-glycans can be imaged in live C. elegans, enabling studies of their distribution and dynamics from larvae to adulthood in both hermaphrodites and males. The technique may also have utility in other studies of C. elegans glycomics that would benefit from spatial and temporal resolution, such as pathogenesis or aging.

**METHODS**

**Materials and General Methods.** All chemicals were of analytical grade, obtained from commercial suppliers, and used without further purification. Phosphine-Flag, DIFO-488, DIFO-568, Ac₄GalNAz, Ac₄ManNAz, and Ac₄GlcNAz were synthesized as previously described (9, 16, 27). C. elegans strains N2, CA151, and RB653 were obtained from the C. elegans Genetics Center. α-Actin clone C4 was obtained from AbCRep. Phosphate-buffered saline solution without calcium and magnesium (PBS) and SeeBlue Plus2 prestained protein molecular weight markers were obtained from Invitrogen Life Technologies, Inc. Nematode growth media (NGM) was obtained from United States Biological. Complete mini protease inhibitor tablets with ethylene diamine tetraacetic acid (EDTA) were obtained from Roche Applied Biosciences. Bis-Tris polyacrylamide gels and the detergent-compatible (DC) protein assay kit were obtained from Bio-Rad. HRP-conjugated α-mouse IgG was obtained from Jackson ImmunoResearch Laboratories. Peptide N-glycosidase F (PNGase F) was obtained from New England Biolabs. Restore Western blot stripping buffer and SuperSignal West Pico chemiluminescent substrate were obtained from Pierce Biotechnology. 

Figure 3. Two-color imaging of nascent glycans in live C. elegans. a) C. elegans embryos that were previously labeled with Ac₄GalNAz and reacted with DIFO-488 (green) were incubated again with Ac₄GalNAz to metabolically label a second, newer population of glycans with azides (blue). These newly labeled glycans were then reacted with DIFO-568 (red) to distinguish the old from the new populations. b) L1 (top panels), L2 (middle panels), or adult (bottom panels) C. elegans metabolically labeled with Ac₄GalNAz were reacted with DIFO-488 and DIFO-568 as described in panel a. Arrowheads denote the “grinder” in the anterior pharynx region. c, d) Strain N2 adult hermaphrodite (c) or strain CA151 adult male (d) C. elegans that were metabolically labeled with Ac₄GalNAz were reacted with DIFO-488 and DIFO-568 as described in panel a. DIC denotes differential interference contrast, 488 denotes DIFO-488 fluorescence, and 568 denotes DIFO-568 fluorescence. Scale bars: (b, d) 25 μm; (c) 10 μm.
Mixed-stage strain N2 or CA151 procedures, probing with a 1:5000 dilution of HRP-quently assayed using standard Western blot pro-
were anesthetized with 10 mM NaN3 and imaged by sonication on ice with 30-s,
mini protease inhibitor tablet with EDTA for every idet P 40 Substitute, and one Roche complete
added (150 mM NaCl, 20 mM Tris pH 7.4, 1% Non-
verted, and 200
centrifugation (16,000
radim plating density at 600 nm (OD600) of 50, and these
worms were transferred from the final PBS wash to
scope. For the two-color imaging experiments, the
strain and the population was grown at 21 °C for
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