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Semisynthetic Lectin–4-Dimethylaminopyridine Conjugates for Labeling and Profiling Glycoproteins on Live Cell Surfaces

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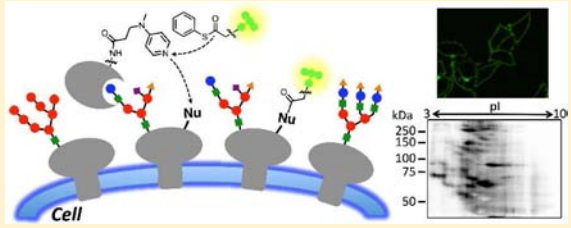
Supporting Information

ABSTRACT: Glycoproteins on cell surfaces play important roles in biological processes, including cell–cell interaction/signaling, immune response, and cell differentiation. Given the diversity of the structure of glycans, labeling and imaging of selected glycoproteins are challenging, although several promising strategies have been developed recently. Here, we design and construct semisynthetic reactive lectins (sugar-binding proteins) that are able to selectively label glycoproteins. Congerin II, an animal galectin, and wheat germ agglutinin are conjugated with 4-dimethylaminopyridine (DMAP), a well-known acyl transfer catalyst by our affinity-guided DMAP method and Cu(I)-assisted click chemistry. Selective labeling of glycoproteins is facilitated by the DMAP-tethered lectin catalysts both *in vitro* and on living cells. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis enabled us to isolate labeled glycoproteins that are uniquely exposed on distinct cell lines. Furthermore, the combination of immunoprecipitation with mass spectrometry (MS)-fingerprinting techniques allowed us to characterize 48 glycoproteins endogenously expressed on HeLa cells, and some low-abundant glycoproteins, such as epidermal growth factor receptor (EGFR) and neuropilin-1, were successfully identified. Our results demonstrate that semisynthetic DMAP-tethered lectins provide a new tool for labeling and profiling glycoproteins on living cells.



INTRODUCTION

Glycoproteins play important roles in numerous biological processes, such as cell–cell interactions/signaling, immune response, and cell differentiation. It is known that unusual expression levels of glycoproteins are strongly correlated with progression of diseases, including cancers and autoimmune diseases.[1,2] Therefore, a number of cell surface glycoproteins are therapeutic targets or important biomarkers.[3] Despite the pivotal roles of cell surface glycoproteins, chemical tools for their precise and comprehensive analysis are still limited. This is largely due to the structural heterogeneity and dynamic change of the glycans within glycoproteins and lack of conventional genetic and biochemical methods for precise control of glycans.[4]

Detection and analysis of cell surface glycoproteins are conventionally achieved by tagging strategies (e.g., biotinylation and hydrazide chemistry) coupled with advanced mass spectrometric teqniques.[5,6] Unfortunately, these methods lack selectivity between different types of glycans and are often unable to obtain crucial information about the dynamic change and spatial distribution of glycoproteins. Recently, two promising methods have been proposed that overcome these drawbacks. The first and most extensively used method is based on a combination of metabolic labeling and bioorthogonal ligation.[7a] Pioneering works by Bertozzi and co-workers exploited the metabolic uptake of unnatural monosaccharides containing reactive handles (e.g., azide, alkyne, or ketone/aldehyde groups), followed by bioorthogonal probe ligation, which enabled selective labeling of glycans on living cells and even in whole organisms.[7] As an alternative approach, Hsieh-Wilson et al. developed a chemoenzymatic method to probe glycans in living systems. They used bacterial glycosyltransferases to tether bioorthogonal reactive handles on glycans, which can then be detected by subsequent bioorthogonal ligation.[8] Although these techniques are undoubtedly powerful, they require metabolic engineering that might perturb the natural cellular conditions and can detect only a handful of mono- or disaccharides. Thus, it is highly desirable to develop new methods to selectively label endogenous glycoproteins with a variety of glycan structures.

We previously reported that 4-dimethylaminopyridine (DMAP) tethered to a small molecule ligand can be a powerful tool for selective protein labeling even under aqueous physiological conditions because of a proximity effect guided by ligand–protein interactions, which is termed affinity-guided DMAP (AGD) chemistry.[9] Using a series of ligand-tethered DMAP catalysts and thiophenylester acyl donors, we selectively labeled several sugar-binding proteins (lectins), such as congerin II (congII), and other soluble proteins in test tubes,

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as well as a G-protein-coupled receptor on the surface of living cells.[9] In comparison to other affinity-labeling methods,[10] this catalyst-based approach is unique in the way that probe-appended labeling reagent itself is less reactive but activated in the presence of ligand-tethered catalyst and transferred to nucleophilic amino acid residues near the ligand binding site of target proteins. Thus, AGD chemistry provides a way to selectively tag a protein of interest with high efficiency and site specificity.

Here, we describe a new approach to label glycoproteins in test tubes and on living cells using DMAP-tethered reactive lectins. The design principle of the reactive lectin is based on rational coupling of the ability of lectins to recognize sugars with the catalytic activity of DMAP, as illustrated in Figure 1.

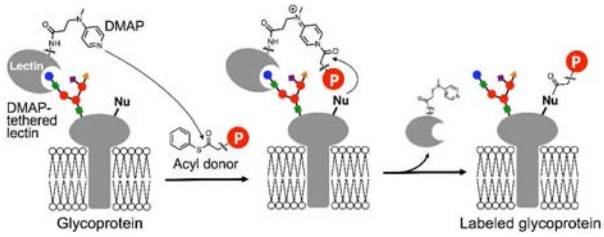

Figure 1. Schematic representation of glycoprotein labeling with a DMAP-tethered lectin.

Two reactive lectins with selectivity for specific sugars were constructed using our AGD strategy. Using the DMAP-tethered lectins and probe-bearing acyl donors, we selectively labeled fetuin and asialofetuin (ASF) in test tubes through lectin–glycan interactions. Because of its efficiency and biocompatibility, this new approach allowed for labeling and profiling of endogenous glycoproteins on living animal cells, demonstrating the potential of reactive lectins to identify selected glycoproteins with complex glycan structures in their native environment.

RESULTS AND DISCUSSION

Design, Preparation, and Characterization of DMAP-Tethered Lectins. To prove the principle of the reactive lectin–DMAP conjugate, we initially used congII,[11] a member of the galectin family, as a sugar recognition module. In the present semisynthetic lectin, the well-established acyl transfer catalyst DMAP was bioconjugated to congII and lectin–glycan interactions facilitate selective acylation of target glycoproteins (Figure 1). CongII–DMAP conjugates were prepared in two steps: introduction of an azide group, a bioorthogonal reactive handle, by AGD chemistry, followed by Cu(I)-catalyzed click chemistry to attach DMAP moieties (Figure 2A). We designed two types of DMAP tethers bearing one or four DMAP group(s) jointed via a rigid hexaproline linker (hereafter called 1DMAP and 4DMAP, respectively, **6a** and **6b** in Figure 2C). The linker was expected to keep the DMAP moieties away from congII and efficiently reach the target protein.[12]

In the first step, congII was treated with azide-appended thiophenyl ester **5** in the presence of lactose-tethered DMAP **1a** (Figure 2B), according to our protocol reported previously.[9] Progress of the reaction was monitored by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI–TOF MS), and after 5 h of incubation at room temperature, we confirmed that new peaks appeared at m/z 15 500 (+257) and 15 754 (+511), which corresponded to the calculated mass of congII modified with one (calculated m/z +256) and two (calculated m/z +512) azide handle(s), respectively (80% yield according to the MALDI–TOF MS; Figure 3A). Azide-modified congII was subsequently reacted with alkyne-appended DMAP (**6a** and **6b** in Figure 2C) using Cu(I)-assisted click chemistry to yield DMAP-tethered congII (Figure 2A). Upon incubation at room temperature for 2 h,

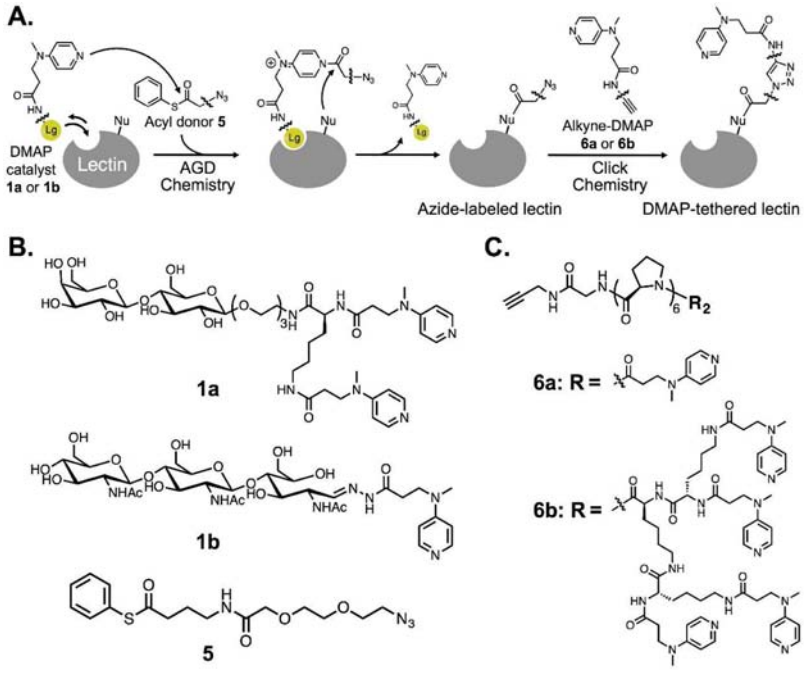

Figure 2. (A) Two-step conjugation of DMAP catalysts to lectins and chemical structures of (B) lactose-tethered and [*N*-acetylglucosamine (GlcNAc)]$_3$-tethered DMAP catalysts **1a** and **1b**, respectively, and azide-type acyl donor **5** and (C) alkyne-appended DMAP catalysts with one and four DMAP group(s) (**6a** and **6b**, respectively).

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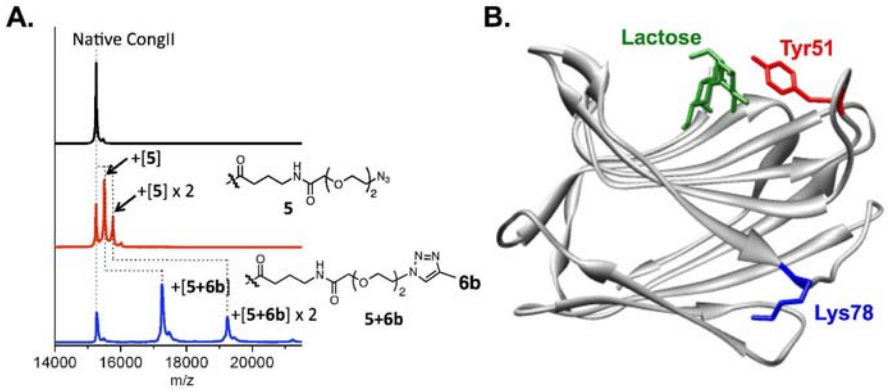

Figure 3. (A) MALDI−TOF MS spectra of native congII (top), congII−**5** (middle), and congII−**5**+**6b** (bottom) and (B) crystal structure of lactose-bound congII. The red and blue residues are Tyr51 and Lys78 labeled by AGD chemistry, and the green molecule is lactose (PDB ID 1IS4).

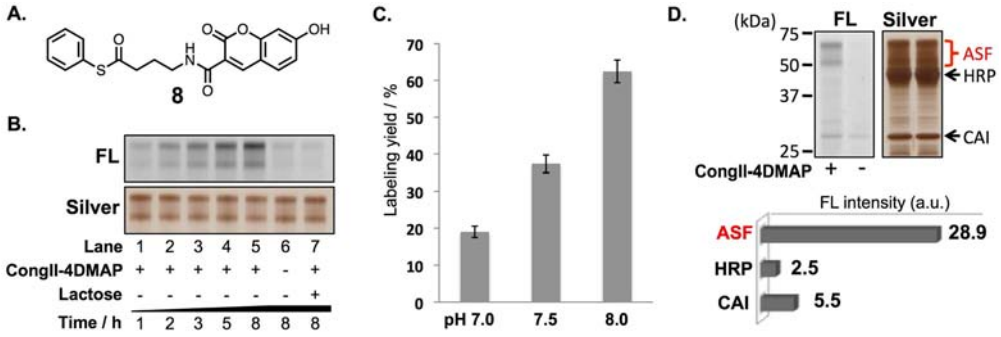

Figure 4. (A) Chemical structure of HC-type acyl donor **8**. (B) Fluorescence and silver-stained gel images of ASF labeled by congII−4DMAP. (C) pH dependence of the labeling reaction with an incubation time of 5 h. (D) Selective labeling of ASF by congII−4DMAP in the presence of ASF, HRP, and CAI.

quantitative conversion of azide-modified congII to congII−DMAP was demonstrated by the appearance of new peaks at m/z 17 239 (+1974) and 19 223 (+3946), along with the disappearance of the peaks from the azide-modified congII species (Figure 3A and see Figure S2 of the Supporting Information). The modification sites of congII were determined by conventional peptide mapping, revealing that mainly lysine 78, which is located at the entrance of the sugar-binding crevice, was modified (63%), along with tyrosine 51 (23%), which is positioned close to the sugar-binding pocket (Figure 3B and see Figures S3 and S4 of the Supporting Information).[9a] Such distribution of the labeling sites may be reasonably ascribed to the flexible linker of DMAP catalyst **1a**. The total isolation yields of these two reactions were typically 50%, and the purity of DMAP-tethered congII was 81% (native, 19%; 4DMAP×1, 54%; and 4DMAP×2, 27%).

Following the same scheme, we also modified wheat germ agglutinin (WGA), another lectin showing affinity for GlcNAc and sialic acid, with azide using $GlcNAc_3$-tethered DMAP **1b**.[13] This conjugate was then converted to DMAP-tethered WGA by the click reaction with alkyne-appended DMAP **6b** (see Figure S5A of the Supporting Information). The labeling sites were identified as lysine 33 (50%) and lysine 149 (27%) (see Figures S5B and S6 of the Supporting Information). Similar to the case of congII, both sites are proximal to the sugar-binding pocket of WGA (see Figure S5B of the Supporting Information).[13] The total yields were approximately 60%, and the purity of WGA−4DMAP was 71% (native, 29%; 4DMAP×1, 70%; and 4DMAP×2, 1%) (see Figure S5A of the Supporting Information).

Glycoprotein Labeling by DMAP-Tethered Lectin in Test Tubes. With the DMAP−lectin conjugates in hand, we subsequently examined their ability to label glycoproteins, such as ASF, a glycoprotein displaying terminal galactose moieties as major glycoforms (see Figure S7A of the Supporting Information). In the presence of congII−4DMAP and hydroxycoumarin (HC)-appended acyl donor **8** (Figure 4A), a new fluorescent band corresponding to HC-modified ASF appeared in the sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) fluorescence image of the reaction mixture, and the intensity of this band increased for 8 h (Figure 4B and see Figure S7B of the Supporting Information). We also noticed that additional fluorescent bands from congII−4DMAP resulted from self-labeling during this reaction (see Figure S7C of the Supporting Information). It should be noted that a fluorescent band from HC-modified ASF was not observed for the reaction in the absence of DMAP-tethered congII or in the presence of excess lactose, a competitive ligand for congII with K_a of 2.3×10^4 M^{-1} (lanes 6 and 7 in Figure 4B),[11c] demonstrating that the labeling reaction was effectively driven by the sugar−lectin interaction.[14] The congII−1DMAP catalyst did not readily label ASF (see Figure S7D of the Supporting Information). Therefore, the multivalent DMAP catalyst is required for efficient labeling, which is in good agreement with our previous report.[9c] The labeling reaction proceeded around physiological pH (7−8), with an increased rate at higher pH, suggesting that deprotonation of certain nucleophilic amino acid side chains accelerated the acyl transfer reaction (Figure 4C and see Figure S8 of the Supporting Information). Furthermore, we were able to

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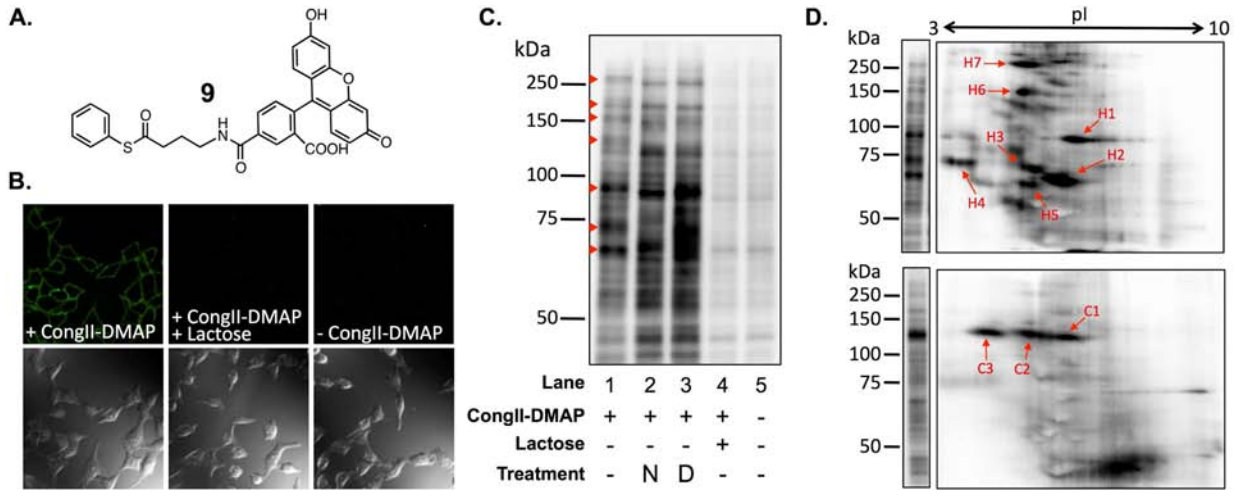

Figure 5. (A) Chemical structure of FL-type acyl donor **9**, (B) CLSM images, and (C) western blots of Hela cells labeled by congII–4DMAP and acyl donor **9**. Red arrowheads indicate glycoproteins labeled by congII–4DMAP. N and D denote treatment of cells with neuraminidase and a deglycosylation mixture of PNGase, *O*-glycosidase, neuraminidase, β1–4 galactosidase, and β-*N*-acetylglucosaminidase, respectively, prior to the labeling reaction. (D) 2D-PAGE western blotting analysis of HeLa (top panel) and COS7 (bottom panel) cells treated with congII–4DMAP and acyl donor **9**.

determine the labeled amino acid residues of ASF by in-gel digestion and deglycosylation followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. HC-labeling occurred at four Lys residues (Lys57, Lys67, Lys232, and Lys238) and one Ser residue (Ser65) of ASF (see Figure S9 and S10 of the Supporting Information). These results demonstrated that DMAP-tethered congII allows for covalent labeling of glycoproteins without any structural information (i.e., three-dimentional structure).

The glycan selectivity of the labeling reaction catalyzed by congII–4DMAP was subsequently evaluated using a custom-made protein mixture containing ASF, another glycoprotein-bearing mannose-terminated sugar group, horseradish peroxidase (HRP), and non-glycoprotein carbonic anhydrase I (CAI). Incubation of the protein mixture with congII–4DMAP and acyl donor **8** resulted in a fluorescent band corresponding to ASF to appear, revealing the glycan-based selectivity of the congII–4DMAP catalyst (Figure 4D).[15] In contrast, substantial labeling of ASF did not occur in the absence of congII–4DMAP (Figure 4D).

Using WGA–4DMAP, we confirmed that the fluorescent labeling of fetuin, a glycoprotein with sialic acid moieties at its terminals, occurred in the presence of acyl donor **8**. A new fluorescent band corresponding to HC-modified fetuin appeared in the SDS–PAGE fluorescence image of the reaction mixture, and the intensity of this band increased for 8 h (see panels A and B of Figure S11 of the Supporting Information). This band was not observed in the absence of DMAP-tethered WGA or in the presence of excess $GlcNAc_3$, a competitive ligand for WGA with K_a of 1.1×10^4 M^{-1} (see lanes 6 and 7 in Figure S11A of the Supporting Information).[13c] These behaviors are quite similar to those observed for congII–4DMAP. We then compared the glycan selectivity of the two DMAP-tethered lectins. A mixture of fetuin and ASF was incubated in the presence of each lectin–DMAP (i.e., congII–4DMAP or WGA–4DMAP) and acyl donor **8**. Distinct band patterns were obtained with congII–4DMAP and WGA–4DMAP; that is, both fetuin and ASF were labeled by WGA–4DMAP, whereas only ASF was labeled by congII–4DMAP. This result is consistent with the fact that WGA recognizes both GlcNAc and sialic acids, whereas congII only binds to galactose/lactose moieties. Taken together, these observations demonstrate that the design of our DMAP-tethered lectins is valid and the selectivity of glycoprotein labeling can be controlled by the lectin scaffold in the DMAP-tethered lectin.

On-Cell Labeling of Glycoproteins by Lectin–DMAP. Exploiting the reaction driven by sugar recognition, we finally used the lectin–DMAP conjugates to selectively label glycoproteins endogenously expressed on living cells. Cultured HeLa and COS7 cells were treated with fluorescein (FL)-appended acyl donor **9** (Figure 5A) in the presence of congII–4DMAP for 90 min, during which time the cell morphology did not changed. As shown in Figure 5B and Figure S12A of the Supporting Information, strong fluorescence was predominantly observed from the cell membrane of both HeLa and COS7 and this fluorescence remained even after extensive washing with buffer solution containing lactose. In contrast, no or minimal fluorescence was detected in the absence of congII–4DMAP or the presence of excess lactose (Figure 5B). These results indicate that covalent labeling with **9** was greatly facilitated by sugar–lectin interactions even on the live cell surface, similar to the test tube experiments.

To further analyze these FL-labeled proteins, we performed western blotting analysis of the cells after treatment with congII–4DMAP and acyl donor **9**. Several bands were clearly stained by an anti-FL antibody at 65, 75, 100, and 130–250 kDa for HeLa (red arrowheads next to lane 1 in Figure 5C) and at 130 kDa for COS7 (see Figure S12B of the Supporting Information). Consistent with the confocal laser scanning microscope (CLSM) images, no appreciable bands were observed in the absence of congII–4DMAP or presence of excess lactose (lanes 4 and 5 in Figure 5C and see lanes 4 and 5 in Figure S12B of the Supporting Information). Interestingly, we found that pretreatment of these cells with neuraminidase (sialidase) or glycosidase cocktail resulted in the loss or downshift of most of these bands, strongly suggesting that these FL-labeled bands originate from glycosylated proteins (lanes 2 and 3 in Figure 5C and see lanes 2 and 3 in Figure S12B of the Supporting Information).

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Table 1. Cell Surface Glycoproteins on HeLa Cells Labeled by CongII−4DMAP and WGA−4DMAP

protein[a]	MW (kDa)	glycosylation	membrane localization	protein function
EGFR	134	+	single-pass type I	proliferation
integrin α-5	116	+	single-pass type I	cell adhesion
NP-1	103	+	single-pass type I	coreceptor
TfR	85	+	single-pass type II	iron uptake
CD44	82	+	single-pass type I	cell adhesion
intercellular adhesion molecule 1 (ICAM1)	59	+	single-pass type I	cell adhesion
PLAP	58	+	single-pass type II	dephosphorylation

[a]Only proteins that are confirmed by immunoblots are listed.

We next labeled the surface of HeLa cells with WGA−4DMAP and acyl donor **9** and compared the data from western blotting analysis to that obtained using congII−4DMAP. Similar to the case of congII−4DMAP, labeled protein bands were detected at molecular weights ranging from 65 to 250 kDa and no significant labeling occurred in the absence of WGA−4DMAP or presence of excess inhibitor $GlcNAc_3$ (see Figure S13 of the Supporting Information). It should be noted that the overall intensity of the bands from the reaction with WGA−4DMAP were higher and the number of clear bands was greater compared to those from the congII−4DMAP experiment. This suggests that the catalytic activity of WGA−4DMAP is higher than that of congII−4DMAP and/or that sialylated glycoproteins are expressed on tumor cell surfaces more than galactosylated glycoproteins.

To further analyze the FL-labeled glycoproteins, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) combined with western blotting was subsequently carried out using anti-FL antibody. Figure 5D shows representative blotting images for the whole cell lysates of HeLa and COS7 cells treated with congII−4DMAP and acyl donor **9**. Well-resolved blotting patterns were observed on the 2D-PAGE blots. To our surprise, the band pattern was explicitly different for HeLa and COS7 cells. That is, H1−H7 were selectively labeled in HeLa cells, whereas C1−C3 were exclusively labeled in COS7 cells (Figure 5D), indicating the presence of different glycoproteins and/or glycoforms on these two cell lines. This is presumably due to the distinct glycosylation states or different expression levels of corresponding glycoproteins in these two types of cells. The blotting image of HeLa cells labeled by WGA−4DMAP showed a slightly different pattern from that using congII−4DMAP; that is, several new bands at ∼65, 75, 110, and 130 kDa were observed (see Figure S14 of the Supporting Information). This suggests that the two lectin−DMAP species have distinct catalytic activity and/or that sialylated glycoproteins are more abundant than galactosylated glycoproteins on HeLa cells.

We then sought to identify the proteins labeled by the present method. The labeled proteins were isolated by either immunoprecipitation followed by SDS−PAGE or 2D-PAGE experiments (as mentioned above). The isolated protein bands were then subjected to in-gel tryptic digestion followed by LC−MS/MS analysis. We found that 26 glycoproteins were labeled by both lectin−DMAP catalysts, and 8 and 14 glycoproteins were uniquely labeled by congII−4DMAP and WGA−4DMAP, respectively (see Figure S15 of the Supporting Information).[16] We confirmed some of the labeled proteins by a reciprocal immunoblot using an antibody for each protein (see Figure S16 of the Supporting Information), which is summarized in Table 1. Among the identified proteins, epidermal growth factor receptor (EGFR), a receptor tyrosin kinase, plays crucial roles in normal cell proliferation as well as tumor growth and progression, and its glycosylation is regarded as important for its function (i.e., dimerization).[17] Neuropilin-1 (NP-1), a membrane-bound coreceptor for receptor tyrosin kinases, was recently shown to activate vascular endothelial growth factor receptors (VEGFRs) via interaction with galectin-1 to regulate the neuron regeneration, angiogenesis, and tumor metastasis.[18] Transferrin receptor (TfR) was recently reported to mediate infection of arenaviruses, causative agents of hemorrhagic fever.[19] Placental-type alkaline phosphatase (PLAP) has long been known as a tumor marker for seminoma and ovarian cancer.[20] Notably, some low-abundant glycorpoteins, such as EGFR and NP-1, were labeled and characterized by our methods,[21] and most of these proteins (EGFR, TfR, PLAP, and integrin α-5) were also identified on prostate cancer cells using metabolic labeling.[22] These results strongly validate the potential utility of our lectin−DMAP for glycoproteomic analysis of live cells.

■ CONCLUSION

We have developed semisynthetic lectin− conjugates as a new chemical tool for efficient labeling and analysis of glycoproteins. The rational coupling of the glycan recognition ability of lectins with the reactivity of organocatalyst DMAP accelerated the acyl transfer reaction driven by sugar−lectin recognition, which allowed for not only covalent labeling of glycoproteins in test tubes but also glycoprotein profiling of live mammalian cells. The present example establishes a powerful design strategy for unique semisynthetic enzymes that is applicable under live cell conditions.[23] Given that we transferred the weak glycan binding ability of lectins to stable covalent labeling, this method may also be used to identify lectins with unknown partners (glycoproteins). Future work includes construction of a DMAP−lectin library with varied glycan selectivity to facilitate detailed analysis of dynamic changes in glycoprotein expression during cell differentiation and/or tumor progression. We envision that such effort will contribute to discovery of new glycoproteins as potential biomarkers/therapeutic targets and provide new functional insight into biological processes.

■ EXPERIMENTAL SECTION

Materials and Methods. All chemical and biochemical reagents were purchased from commercial sources (Wako Pure Chemical, TCI Chemical, Sigma-Aldrich, and Watanbe Chemical) and were used without further purification. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminum sheets (Merck) and visualized by fluorescence quenching or ninhydrin staining. Chromatographic purification was performed using flash column chromatography on silica gel 60 N (neutral, 40−50 μm, Kanto Chemical).

Preparation of DMAP-Tethered CongII and WGA. The expression and purification of recombinant congII was carried out as

described previously.[11b] The congII concentration was calculated on the basis of the molar extinction coefficient at 280 nm of 11 500 M^{-1} cm^{-1}. Purified protein solution (50 μM) was incubated with DMAP catalyst **1a** (100 μM) and acyl donor **5** (250 μM) in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 8.0) at room temperature for 5 h. The conjugation reaction was monitored by MALDI−TOF MS to confirm the conversion to azide-labeled congII. The protein solution was then subjected to gel filtration on Toyopearl HW-40 (Toso, Tokyo, Japan) using an ÄKTA purifier system (GE Healthcare) equilibrated with 50 mM HEPES at pH 7.2 to remove the remaining labeling reagents (see Figure S1 of the Supporting Information). The protein fractions were monitored at 280 nm and bicinchoninic acid (BCA) assay (Pierce, Rockford, IL), collected using Frac-920 (GE Healthcare) (see Figure S1 of the Supporting Information), and if necessary, concentrated by Amicon Ultra-4 centrifugal filter device (Millipore Corporation, Billerica, MA). The purified azide-labeled congII was then incubated with alkyne-appended DMAP **6a** and **6b** in the presence of 0.5 mM Cu(II)-[tris-(ethylestertriazolylmethyl)amine] (TEtOTA)[24] and 1 mM ascorbate for 2 h at room temperature. The conversion to DMAP-tethered congII was monitored by MALDI−TOF MS. The protein solution was then subjected to gel filtration, and the protein fractions were collected as described above (see Figure S1B of the Supporting Information). The protein concentration and modification yield were determined by BCA assay and ultraviolet−visible (UV−vis) absorption with a molar extinction coefficient at 280 nm of 18 000 M^{-1} cm^{-1} per DMAP group.

WGA, a mixture of WGA1 and WGA3 isomers, was purchased from Funakoshi and used without further purification. The WGA concentration was calculated on the basis of the molar extinction coefficient at 280 nm of 25 500 M^{-1} cm^{-1}. The azide-labeling, following Click reaction, and gel filtration purification were performed by the same procedure as congII (see Figure S1 of the Supporting Information).

Glycoprotein Labeling by Lectin−DMAP in Test Tubes. ASF from fetal calf serum (Sigma-Aldrich, St. Louis, MO) (1 μM) was incubated with congII−DMAPs (6 μM) and HC-type acyl donor **8** (8 μM) with or without 15 mM lactose inhibitor in 50 mM HEPES (pH 8.0) at 17 °C. For mixed protein experiments, ASF, HRP, and CAI from human erythrocytes (Sigma-Aldrich, St. Louis, MO) at final concentrations of 100 μg/mL for ASF and 200 μg/mL for HRP and CAI were incubated with congII−4DMAP (6 μM) and acyl donor **8** (8 μM) under the same conditions as above. At indicated time points, each sample was mixed with an equal volume of 2× SDS−PAGE loading buffer [125 mM Tris-HCl, 4% SDS, 20% glycerol, and 0.01% bromophenol blue (BPB) at pH 6.8] with or without 100 mM dithiothreitol (DTT) and boiled in a heatblock for 3 min. The samples were subjected to SDS−PAGE, and HC-labeled proteins were detected by an in-gel fluorescence image. The fluorescence band intensity was quantified using HC-modified bovine serum albumin (BSA) as a standard. After fluorescence imaging, the gels were stained by silver stain (Wako, Osaka, Japan).

Glycoprotein Labeling by CongII−4DMAP on Live HeLa and COS7 Cells. HeLa and COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotic−antimycotic (Gibco, Carlsbad, CA) and incubated in a 5% CO_2 humidified chamber at 37 °C. Prior to labeling experiments, HeLa and COS7 cells (3×10^4 cells) were incubated on 35 mm dishes treated with polystyrene (BD Falcon, Franklin Lakes, NJ) at 37 °C. After 24 h, the media was removed, and the cells were washed twice with phosphate-buffered saline (PBS). To the cells were added labeling mixtures containing congII−4DMAP and acyl donor **9** in HBS (20 mM HEPES, 107 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, and 1.2 mM $MgSO_4$ at pH 8.0) and incubated at 4 °C for 90 min. For glycosidase-treated samples, the cells were washed once with DMEM and incubated with 100 units/mL neuraminidase or deglycosylation mix (New England Biolabs) in DMEM at 37 °C for 2 h prior to the labeling reactions.

For imaging experiments, the cells were washed further with ice-cold HBS buffer containing 15 mM lactose 3 times. Cell imaging was performed with a CLSM (FV1000 IX81, Olympus) equipped with a 60×, numerical aperture (NA) = 1.40 oil objective. Fluorescence images were acquired using the 488 nm excitation derived from an Ar laser. To minimize internalization of membrane proteins, samples were maintained at 10 °C with Peltier-type stage cooling unit (Japanhightech, Fukuoka, Japan) during image acquisition.

For western blotting analysis, cells were washed twice with ice-cold PBS, lysed with radio immunoprecipitation assay (RIPA) buffer, and mixed with an equal volume of 2× SDS−PAGE loading buffer containing 100 mM DTT. The samples were applied to SDS−PAGE and electrotransferred onto immune-blot polyvinylidene fluoride (PVDF) membranes (Biorad, Hercules, CA), followed by blocking with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.05% Tween (Sigma-Aldrich, St. Louis, MO). The membranes were stained with rabbit anti-fluorescein antibody (Abcam, ab19491), followed by a HRP-conjugated goat anti-rabbit immunoglobulin G (IgG) (Santa Cruz, SC-2004). The membranes were developed with Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan).

2D-PAGE Analysis of HeLa and COS7 Cells. Protein extracts of HeLa and COS7 in sample buffer containing 40 mM Tris base, 7 M urea, 2 M thiourea, 1% (w/v) ABS-14, 0.001% BPB, 2 mM tributylphoshine, and 0.2% (w/v) Bio-Lyte 3/10 ampholyte were applied to a 7 cm immobilized pH gradient (IPG) strip at pH 3−10 or 5−8. Rehydration was carried out for 12 h at 50 V using a PEOTEAN i12 IEF system, followed by isoelectric focusing (IEF). All IEF equipment and reagents were purchased from Biorad. Immediately after IEF, IPG strips were equilibrated with buffer solutions I and II containing 375 mM Tris-HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, and 130 mM DTT for buffer solution I or 137 mM iodoacetamide for buffer solution II. The equilibrated IPG strips were placed on the top of acrylamide SDS−PAGE minigels, sealed with low-melt agarose. After second-dimension separation, proteins were detected by silver staining or western blotting, as described above.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, including chemical synthesis of DMAP catalysts and acyl donors, modification site determinations, and protein identifications. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(14) We examined the individual roles of congII−4DMAP×1 and congII−4DMAP×2 in the ASF labeling using the congII−4DMAP×1 sample containing a little congII−4DMAP×2 (native, 43%; 4DMAP×1, 51%; and 4DMAP×2, 6%), which showed that the contribution from congII−4DMAP×2 was minimal in both labeling yield and kinetics. On the other hand, the attempt to assign the role of two 4DMAP×1 species [i.e., 4DMAP×1(Lys78) and 4DMAP×1(-Tyr51)] in acyl transfer chemistry was unsuccessful, because we were unable to separate the two 4DMAP×1 species. However, the facts that the lectin−glycoprotein interaction is dynamic and that the linker of the DMAP catalyst possesses some structural flexibility may allow for compensation of such differences in the labeling site.

(15) A weak fluorescence band corresponding to HC-labeled CAI was observed even in the absence of congII−4DMAP, suggesting some non-catalytic reaction of CAI with the acyl donor. Such a reaction is unavoidable in the test tube experiments, where the concentrations of proteins are relatively high (i.e., a few micromolars).

(16) It should be noted that the current approach is not designed to detect all glycoproteins, because it requires nucleophilic amino acid residues, such as Lys, Tyr, and Ser, adjacent to glycans recognized by the lectin−DMAPs.

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