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Probing Glycosyltransferase Activities with the Staudinger Ligation

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A fundamental challenge in the field of glycobiology is to identify the substrates and functions of the glycosyltransferases that build glycans within the secretory compartments.[1] Numbering about 250 in humans, these enzymes assemble complex glycans by glycosyl transfer from nucleotide sugar donors.[2] Although the specificities of glycosyltransferases for their glycosyl donors are usually well-defined, the profile of acceptors they can modify is not known for many enzymes. The development of microarray formats for peptide[3] and carbohydrate[4,5] display offers a new platform for rapid screening of glycosyltransferase acceptor specificities. However, a general nonradioactive method to detect glycosyltransferase products in array format has not been reported. The enzymes transfer various monosaccharides, defined by the enzyme subfamily (i.e., fucose for fucosyltransferases), to a variety of acceptor substrates. Some of their glycan products can be detected using specific antibodies[4] or lectins,[5] but no single detection method can be used with all members of the glycosyltransferase family.

A common feature of glycosyltransferases is their ability to utilize modified nucleotide sugar donors bearing unnatural substituents on the monosaccharide.[6] Thus, it is possible to adorn glycosyl donors with bioorthogonal chemical handles that can be covalently reacted with probes and detected in an array format. The azide is ideal for this purpose, as it can be readily installed synthetically and is a minimal structural perturbation to the glycosyl donor substrate. By contrast, larger probes such as biotin may undermine enzyme recognition, although some glycosyltransferases can tolerate biotinylated nucleotide sugars.[6b] More importantly, the azide can be detected by conjugation with phosphine probes via the Staudinger ligation,[7] or alkyne probes via cycloaddition reactions.[8] Thus, the transfer of an azidosugar from a nucleotide sugar donor to a biotinylated acceptor substrate can be monitored by capture in a microtiter plate and detection by Staudinger ligation. This method, which we term the "azido-ELISA" (*e*nzyme-*l*inked *i*mmunosorbent *a*ssay), can be generalized to any glycosyltransferase capable of transferring an azide-modified sugar, and is depicted in Figure 1. Here we report an azido-ELISA for the polypeptide *N*-α-acetylgalactosaminyltransferases (ppGalNAcTs) that can be used to probe their substrate specificities in a high-throughput format.

The ppGalNAcTs play a critical role in mucin-type *O*-linked glycoprotein biosynthesis by attaching the initial GalNAc to Ser or Thr residues of the polypeptide scaffold using UDP-*N*-acetylgalactosamine (UDP-GalNAc) as a glycosyl donor (Figure 2). Analysis of the human and mouse genomes predicts ~24 ppGalNAcT isoforms. For the most part, their precise peptide substrate specificities are undefined.[9] Furthermore, some members of the ppGalNAcT family prefer peptide substrates previously modified with GalNAc on nearby Ser or Thr residues, thus classifying them as glycopeptide GalNAcTs (gppGalNAcTs).[9] The identification of the preferred

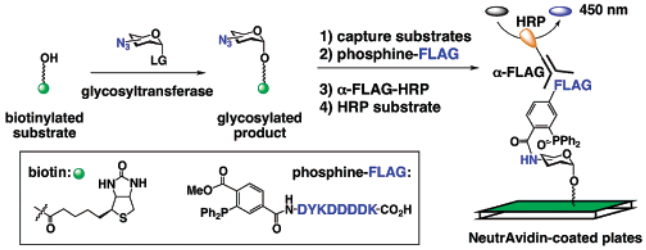

Figure 1. Schematic diagram of azido-ELISA. LG = nucleotide leaving group.

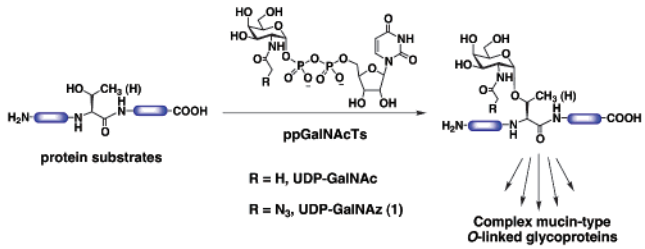

Figure 2. Initiation of mucin-type *O*-linked glycosylation by ppGalNAcTs. The product of the reaction is elaborated by a number of downstream glycosyltransferases to give complex mucin-type *O*-linked glycoproteins. Experimental details for the synthesis of **1** are provided in the Supporting Information.

(glyco)peptide substrates of each (g)ppGalNAcT would help elucidate the biological roles of this complex enzyme family. Accordingly, we designed an analogue of UDP-GalNAc, UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz, **1**) bearing an azido group on the *N*-acyl side chain, as an unnatural substrate for the (g)ppGalNAcTs (Figure 2). Transfer of GalNAz from UDP-GalNAz to acceptor substrates could be detected and quantified with the azido-ELISA shown in Figure 1.

Prior to its execution with ppGalNAcTs, we determined the sensitivity of the azido-ELISA using biotinylated azide **2** as a model substrate for capture and detection (Figure 3A). The compound was immobilized onto 96-well NeutrAvidin-coated plates from solutions that included various percentages of biotin analogue **3** lacking an azide (Figure 3A). In this fashion, the percentage of capture sites occupied by an azide-containing molecule could be controlled and the signal associated with those azides quantified. After capture on the plates, the immobilized azides were reacted with phosphine-FLAG[7b] and detected with an α-FLAG monoclonal antibody−horseradish peroxidase (HRP) conjugate. A chromogenic substrate for HRP was added, and absorbance at 450 nm was monitored. As shown in Figure 3B, the signal increased in an azide-dependent manner, enabling the generation of a standard curve that correlates absorbance with the number of immobilized azides in each well. The signal observed when 20% of the NeutrAvidin sites were occupied by compound **2** was approximately 8-fold higher than the background observed with immobilized **3** alone. This represents

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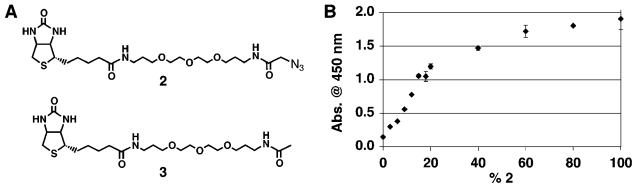

Figure 3. Sensitivity of the azido-ELISA. (A) Biotinylated substrates for azido-ELISA standard curve. (B) Signal as a function of immobilized azide. Experimental details for syntheses of **2** and **3** and assays are in the Supporting Information. Error bars represent the values of two data points.

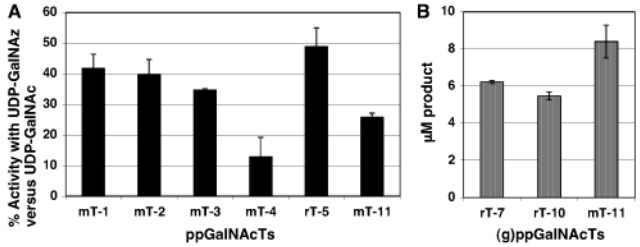

Figure 4. Activities of (g)ppGalNAcTs with UDP-GalNAz and UDP-GalNAc. (A) Relative activities of ppGalNAcTs (1−5 and 11) with UDP-GalNAz vs UDP-GalNAc; biotinylated EA2 peptide was at 250 μM. (B) Activities of (g)ppGalNAcTs (7 and 10) with UDP-GalNAz; biotinylated MUC5AC-3,13 glycopeptide substrate was at 250 μM. In all assays, the nucleotide sugar donor was at 500 μM, and reactions were performed at 37 °C for 16 h. Error bars represent the values of two data points. m = murine, r = rattus.

the scenario in which a glycosyl transfer reaction has proceeded to 20% completion, typical for kinetic analyses and inhibitor screens. On the basis of the binding capacity of the NeutrAvidin-coated plates (60 pmol/well), we calculated that the azido-ELISA can detect low picomolar amounts of immobilized azido compounds.

Having established the parameters of the assay, we applied the azido-ELISA to studies of the (g)ppGalNAcTs. The biotinylated peptide EA2 (H_2N-PTTDSTTPAPKK(biotin)-CO_2H) was prepared as a substrate for a panel of six ppGalNAcTs that are known to act on this sequence (the precise site of modification depends on the isoform).[9] The biotinylated glycopeptide MUC5AC-3,13 (H_2N-GTTPSPVPTTSTTSAPK(biotin)-CO_2H, GalNAc-modified residues are underlined) was used as a substrate for two gppGalNAcTs known to modify this sequence.[9] The enzymes were incubated in solution with the (glyco)peptide substrates and UDP-GalNAz. After capture on the NeutrAvidin-coated plates, the amount of product was quantified using the procedure shown in Figure 1. For comparison, the native substrate UDP-GalNAc was assayed with the six ppGalNAcTs under identical conditions using a previously reported *e*nzyme-*l*inked *l*ectin *a*ssay (ELLA).[5e]

The activities of ppGalNAcTs with UDP-GalNAz relative to their activities with UDP-GalNAc (both under saturating conditions) are shown in Figure 4A. These results demonstrate that UDP-GalNAz is utilized as a substrate by all of the ppGalNAcTs tested at roughly one-third the efficiency of UDP-GalNAc. The reaction of the gppGalNAcTs with glycopeptides and UDP-GalNAc could not be monitored using the ELLA since the detection relies on a GalNAc-specific lectin that would bind both the substrate and the product.[5e] This precluded the direct comparison of UDP-GalNAc and UDP-GalNAz as substrates for the two gppGalNAcTs tested. Nonetheless, the azido-ELISA demonstrated that the gppGalNAcTs utilize UDP-GalNAz as efficiently as ppGalNAcT-11 (Figure 4B). Furthermore, we determined the K_M and V_{MAX} values of UDP-GalNAz with ppGalNAcT-1 to be 7.68 ± 0.81 μM and 7.22 ± 0.16 μM/min, respectively, compared to 7.22 ± 0.16 μM and 36.17 ± 0.67 μM/min, respectively, for UDP-GalNAc determined with the ELLA. The V_{MAX}/K_M value for UDP-GalNAz was 0.2 relative to that for UDP-GalNAc.

In summary, the azido-ELISA provides a generalizable platform for rapid screening of glycosyltransferase activities in a microtiter-plate format. The azide serves to distinguish the transferred residue from related structures that might be present on the glycosyl acceptor, as in the case of the gppGalNAcT assays. The tolerance of the eight (g)ppGalNAcTs tested for the azide modification suggests that this approach can be used to profile the substrate specificity of the entire family. Finally, other enzymes that catalyze group-transfer reactions and tolerate modified substrates might be probed using the azido-ELISA.

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Supporting Information Available: Experimental procedures for chemical synthesis and enzymatic reactions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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