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A mass-differentiated library strategy for identification of sugar nucleotidyltransferase activities from cell lysates

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ABSTRACT

Sugar nucleotidyltransferases, or nucleotide sugar pyrophosphorylases, are ubiquitous enzymes whose activities have been correlated to disease states and pathogen virulence. Here we report a rapid "one-pot" method to identify a range of sugar nucleotidyltransferase activities of purified proteins or in cell lysates using a mass-differentiated carbohydrate library designed for mass spectrometry-based analysis.
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Sugar nucleotidyltransferases (NTs)[1] are a ubiquitous class of enzymes that serve to activate sugars for most glycosyltransferases. These enzymes are central to primary and secondary metabolism, and their activities have been correlated to disease states such as diabetes and to the state of virulence of pathogens such as *Giardia* and *Neisseria meningitidis* [1,2]. For example, *N. meningitides*, an intracellular human-specific pathogen, encodes a CMP–sialic acid synthetase that transfers *N*-acetylneuraminic acid to bacterial and mammalian outer surfaces [2]. Sialic acid, found on the terminal ends of these glycoproteins, assists the immune system in recognizing self from non-self [3].Bacteria capable of this synthetase activity can mimic the hosts' cells by transferring sialic acid onto its surface and evade the host's immune system, resulting in the propagation of bacteria in undesirable places. Activity-dependent labeling of enzymes is a powerful method to identify proteins in a complex mixture with specific chemical functions [4]. However, this proteomics strategy is limited to enzymes such as metalloenzymes or proteases and glycosidases with key nucleophilic residues in their active sites and, therefore, cannot be readily applied to identifying the presence of sugar NTs. Here we report a rapid method to identify a range of sugar NT activities of purified proteins or in cell lysates using a library designed for mass spectrometry (MS)-based analysis.

Unlike the hydrolytic enzymes identified by activity-based labeling protocols, sugar NTs require two substrates: a sugar-1-phosphate and a nucleotide triphosphate (NTP). This class of enzymes is often assayed by monitoring of the reverse reaction—cleavage of a sugar nucleotide by pyrophosphate [5a]. However, a cell extract could contain other proteins that lead to the disappearance of sugar nucleotides. In addition, some sugar NTs can readily accept more than one sugar-1-phosphate or more than one NTP as substrates [6a,7a]; therefore, conditions to monitor the reaction in the synthetic direction are necessary for the facile characterization of sugar NT activities. Several methods have been developed to monitor the reaction in the synthetic direction: high-performance liquid chromatography (HPLC)-based assays [8], radioactivity-based assays [9a], coupled assays that require various other enzymes [5], and (most recently) MS-based assays [6]. Ideally, however, enzyme activity could be monitored quickly with a range of substrates in a limited number of reactions to conserve sample rather than in multiple parallel reactions [10]. MS appears to be best-suited for such a multiplexed approach. Our earlier work in developing an electrospray ionization (ESI)–MS-based general sugar NT assay for single use showed several advantages [6b]: (i) the simultaneous and direct detection of multiple substrates and products without their prior separation, (ii) circumventionof the use of additional enzymes as used in many indirect coupled assays, (iii) avoidance of radioactive compounds, (iv) avoidance of the development of new separation protocols for new substrates as is

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[1] *Abbreviations used:* NT, nucleotidyltransferase; MS, mass spectrometry; NTP, nucleotide triphosphate; HPLC, high-performance liquid chromatography; ESI, electrospray ionization.

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needed for HPLC-based assays, and (v) lower labor requirements. Given these advantages, we decided to look into the possibility of finding a way to expand this MS-based assay to test multiple substrates simultaneously, thereby further lowering the amounts of enzyme and labor required.

Results and discussion

The basic strategy of a chemical proteomics approach to detecting sugar NT activities (Fig. 1) relies on the differentiation of all possible substrates by mass to track their conversion in one pot to sugar nucleotide products. However, although some of the naturally occurring sugar-1-phosphates and sialic acid itself do not share molecular weights (Fig. 2), several potential library members cannot be distinguished using MS. In addition, the library cannot contain any inhibitors of sugar NT activity for the triumph of this one-pot approach.

To address the first problem, the mass redundancy of the three common sugar phosphates based on glucose, galactose, and mannose was broken by introduction of deuterium labels (Fig. 3). Lithium aluminum deuteride reduction of the known aldehyde [11] or carboxylic acid [12] produced monodeuterated galactose and dideuterated glucose analogs. Protection of the resulting alcohols, followed by hydrolysis of the anomeric methyl group, allowed the selective installation of an anomeric phosphate via the phosphite [13]. Finally, the phosphorylated compounds were deprotected by hydrogenation with $Pd(OH)_2$, and the resulting compounds were converted into their sodium salts using an ion exchange resin to provide the new deuterium labeled sugar-1-phosphates **5** and **6**.

Sugar NTs are known to operate by a bi–bi mechanism in which the NTP is bound first to the active site, followed by the sugar-1-phosphate [7]. Some sugar NTs display promiscuity with regard to the NTP and, therefore, can produce more than one product. However, many enzymes can at least bind alternate NTPs even if they are not turned over and, therefore, can be inhibited by the presence of NTPs. Indeed, incubation of the library containing the sugar-1-phosphates and sialic acid with the five NTPs used to make activated sugar nucleotides (Fig. 2) and with a well-characterized sugar NT [7a] did not result in detection of the expected range of products in the mass spectrum. Even the presence of the correct

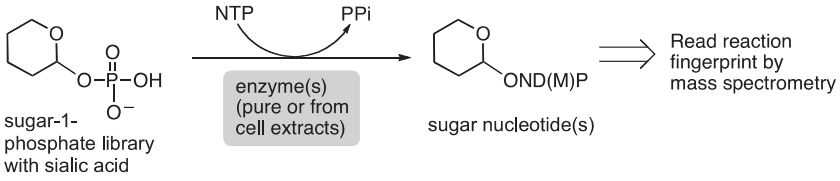

Fig.1. Strategy for identification of multiple sugar NT activities in a cell extract or purified protein preparation.

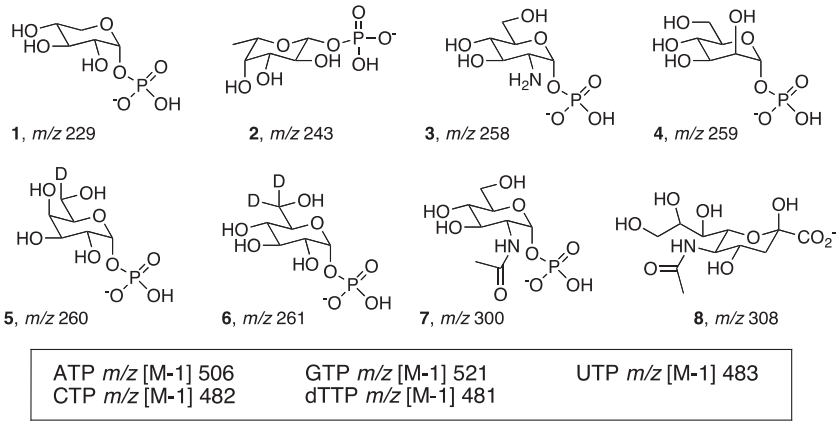

Fig.2. Mass-differentiated library of substrates for sugar NTs.

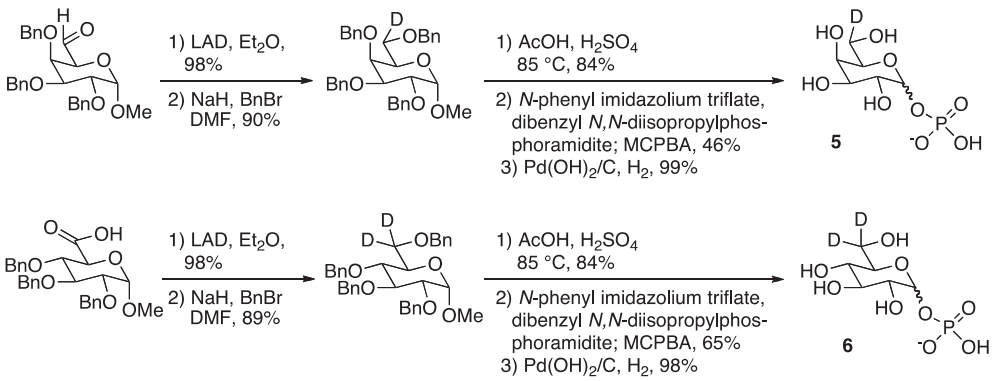

Fig.3. Synthesis of deuterium-labeled glucose- and galactose-1-phosphates.

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