Communication

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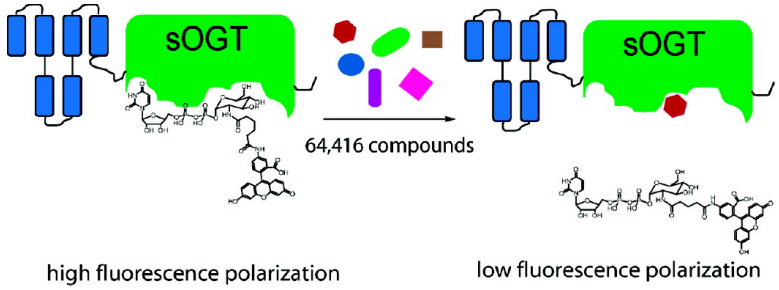

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Discovery of *O*-GlcNAc Transferase Inhibitors

Benjamin J. Gross,[‡] Brian C. Kraybill,[†] and Suzanne Walker*,[†]

Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, and Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

Received August 12, 2005; E-mail: suzanne_walker@hms.harvard.edu

Many nuclear and cytosolic proteins are transiently glycosylated by an enzyme known as *O*-GlcNAc transferase (OGT), which transfers *N*-acetylglucosamine from UDP-GlcNAc to selected serine and threonine residues. *O*-GlcNAcylation affects such diverse cellular processes as transcription, translation, organelle targeting, and protein−protein interactions,[1] and is believed to play a role in a variety of signaling cascades that mediate glucose homeostasis and stress responses.[2] Specific inhibitors of OGT could be valuable tools to probe the biological functions of *O*-GlcNAcylation, but the inability to obtain significant quantities of enzyme, combined with the lack of a high-throughput assay, has impeded efforts to identify such compounds.[3] We have developed conditions to express large quantities of the catalytic domain of active OGT for the first time, and we report a high-throughput donor displacement assay for the enzyme along with the discovery of a set of small-molecule inhibitors. This work lays the foundation for both structural and functional analysis of the catalytic domain of OGT.

The human, rat, and mouse *ogt* genes have previously been expressed in baculovirus,[4] several mammalian cell lines,[5,6] and *Escherichia coli*,[7] but good expression levels were not achieved. Therefore, our first goal was to develop conditions to produce large amounts of pure, active OGT. We chose to focus our efforts on expression in *E. coli* because the potential to obtain large amounts of protein is greater than in eukaryotic expression systems. OGT is a bipartite protein consisting of a C-terminal glycosyltransferase (Gtf) domain and an N-terminal protein−protein interaction domain comprised of 12 tetratricopeptide (TPR) repeats. To optimize expression, we synthesized the gene for human OGT using preferred *E. coli* codons, and then made constructs based on three known splice variants of OGT (ncOGT, mOGT, and sOGT; Figure 1).[8] These constructs were cloned into a modified pET-24b (Novagen) vector for expression as C-terminal His_8 fusions. Good expression for all constructs could be achieved by late log induction in BL21-(DE3) at low temperature (16 °C; OD = 1.2; 0.4 mM IPTG), but soluble sOGT was expressed at much higher levels than the other proteins. We obtained 10−12 mg of sOGT/L of culture in >95% purity after a single step Ni^{2+}−IDA IMAC purification. Activity was evaluated using a known peptide substrate. The K_m of UDP-GlcNAc was found to be 6.7 ± 0.5 μM, nearly identical to the value reported for a construct of the rat enzyme containing six TPRs.[4] The specific activity, however, is over 150-fold higher than that reported for the rat enzyme (161 nmol min^{-1} mg^{-1} vs 1.06 nmol min^{-1} mg^{-1}), which may reflect differences in phosphorylation or glycosylation states between OGT produced in *E. coli* and insect cells.[4,9] Nup62, a known ncOGT substrate, is also glycosylated by sOGT. We note that this is the first report of sOGT activity in vitro.

The ability to obtain large amounts of protein allowed us to investigate possible high-throughput screens. Ligand displacement

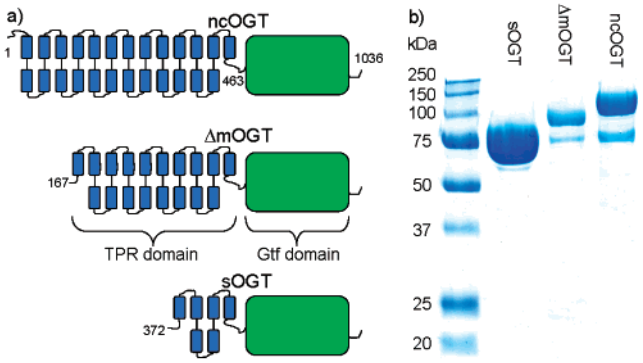

Figure 1. (a) OGT constructs expressed in *E. coli*. Blue columns represent α-helices, two of which comprise a TPR. ncOGT and sOGT are identical to known splice variants; ΔmOGT is 50 residues shorter than mOGT. (b) PAGE of these three constructs after IMAC purification.

assays in which fluorescence polarization (FP) is monitored are being used increasingly for high-throughput screening (HTS) because they are technically simple to implement if an appropriate ligand can be identified. Furthermore, they result in hits that are biased toward compounds that bind in the same location as the fluorescent probe, which can simplify the analysis of structure−activity relationships.[10−12] We have previously developed a fluorescent UDP-GlcNAc displacement assay for a Gtf involved in peptidoglycan biosynthesis (MurG),[10] and we wondered whether a similar assay could be used to screen OGT. If so, we wanted to know whether the hits obtained would be selective for OGT relative to MurG.

There is no structure of the Gtf domain of OGT to guide the design of a fluorescent UDP-GlcNAc analogue, but it has been proposed that OGT is structurally related to MurG.[13] Therefore, we evaluated whether the fluorescent probe used to screen MurG (**1**, Figure 2) could also be used in an OGT screen.[14,15] The FP of a 50 nM solution of **1** in the presence of increasing amounts of sOGT did not change significantly until high concentrations of protein were added. Hypothesizing that the short linker between the sugar and the fluorophore interfered with binding to the enzyme, we prepared two additional fluorescent UDP-GlcNAc analogues containing longer linkers (**2** and **3**, Figure 2). We observed significant FP changes for both **2** and **3** in the presence of sOGT, but the change for **3** was larger over a wider range of protein concentrations. We selected this compound as our probe. From the change in polarization as a function of sOGT concentration, we calculated a dissociation constant of 1.3 ± 0.1 μM for **3**. Addition of unlabeled UDP-GlcNAc or UDP to a pre-equilibrated mixture of sOGT and **3** resulted in a decrease in polarization, and both compounds completely displaced **3** from sOGT at high concentrations. Dissociation constants were calculated from the displacement curves and found to be 1.5 ± 0.4 μM for UDP-GlcNAc, which implies that the fluorophore on **3** does not affect binding, and 0.8

[†] Harvard Medical School.
[‡] Harvard University.

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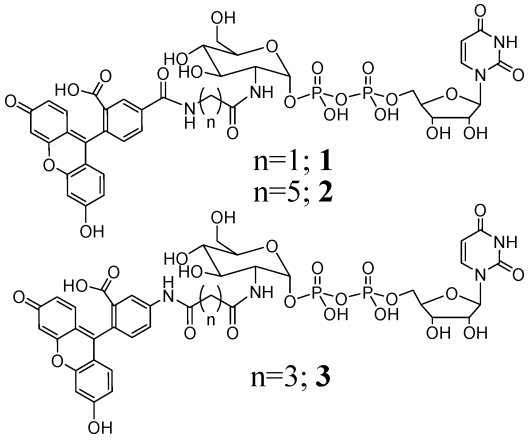

Figure 2. Donor analogue displacement probes used in this study.

± 0.3 μM for UDP, which agrees well with previously reported values.[16]

The above experiments established the feasibility of a donor displacement assay for HTS of OGT, and we adapted the assay to a 384-well microplate format and screened 64 416 commercial library compounds at the Institute of Chemistry and Cell Biology (ICCB) at Harvard Medical School. The libraries were screened in duplicate at a final concentration of 25 μg/mL using a Perkin-Elmer Envision microplate reader. Included in the compounds screened were 12 390 molecules that had previously been screened against MurG.[10] This subset contained 58% of the hits that were identified in the MurG screen. Each plate contained a positive control well containing sOGT, **3**, and 1 mM UDP-GlcNAc and a negative control well containing sOGT and **3**. Compounds that reproducibly caused a significant decrease in FP without a corresponding change in fluorescence intensity were scored as hits. Using this criterion, 102 compounds were scored as positives, for a hit rate of 0.2%.

The positive compounds were then evaluated for OGT inhibition using a radiometric assay that involves monitoring transfer of ^{14}C-GlcNAc to an OGT acceptor peptide containing an N-terminal $(Lys)_3$ tag that enables capture on phosphocellulose filter disks.[4] Nineteen of these 102 compounds inhibited sOGT >40% at 25 μM. These molecules do not share obvious common structural features. However, this may be a consequence of library diversity since fewer than five compounds with the same core are present in the screened libraries for almost all of the 19 inhibitors. IC_{50} values were determined for several compounds, and the mode of inhibition was determined for two of the best; both were found to be competitive with respect to UDP-GlcNAc (see Supporting Information). All of the compounds examined also inhibited the full-length construct, ncOGT.

Remarkably, none of compounds that were identified as hits in the MurG screen, which was based on displacement of UDP-GlcNAc analogue **1**, were found to displace UDP-GlcNAc analogue **3** from OGT. Furthermore, none of the OGT inhibitors identified in this screen were found to inhibit MurG. Thus, there is no overlap in the compounds selected in the two high-throughput screens, even though both screens were based on displacement of the same glycosyl donor, UDP-GlcNAc, and led to the discovery of compounds that compete with this donor. We conclude that there are substantial differences in the binding pockets for UDP-GlcNAc in these enzymes that can be exploited to develop specific inhibitors. The ability to use the same screening strategy against different Gtfs could have clear advantages for the rapid discovery of orthogonal inhibitors for enzymes that use similar substrates.

We are currently investigating the effects of these compounds in cell culture. If they reduce *O*-GlcNAcylation in cells, they could be useful tools for probing the biological functions of OGT. In the meantime, the ability to obtain large quantities of the catalytic domain of OGT enables structural analysis of this biologically important enzyme.

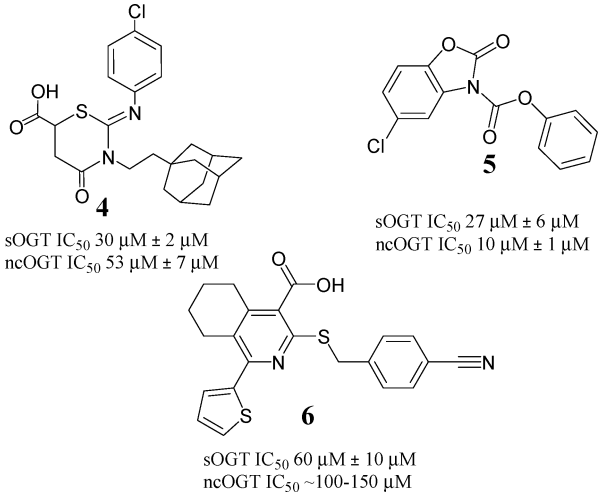

Figure 3. Validated OGT inhibitors found in this HTS.

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Supporting Information Available: OGT expression, purification, and kinetic characterization of ncOGT and sOGT with peptide and protein substrates; synthetic scheme for **2** and **3**; experimental details for primary and secondary screening; assay conditions; IC_{50} values and inhibition pattern for **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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