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Preview

Enzyme-based assay for quantification of UDP-GlcNAc in cells and tissues

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In this issue of *Cell Reports Methods*, Sunden et al. develop an enzymatic assay to measure UDP-GlcNAc levels from cells and tissue.[1] By reporting on the level of the substrate itself, this approach can potentially enhance the fields' understanding of UDP-GlcNAc concentration under a variety of conditions.

UDP-GlcNAc plays an essential role in all forms of protein glycosylation including protein O-GlcNAcylation, where O-GlcNAc transferase (OGT) modifies intracellular proteins with a single O-linked-GlcNAc attached to serine and threonine residues. A key feature of O-GlcNAc protein modification is that, in contrast to other glycosylation modifications, it is a dynamic and readily reversible process. In addition to OGT, O-GlcNAc levels are regulated by O-GlcNAcase, which catalyzes its removal from proteins as well as UDP-GlcNAc availability, the substrate for OGT. UDP-GlcNAc is a product of the hexosamine biosynthesis pathway (HBP), a multi-step process in which glucose, glutamine, acetyl-CoA, and uridine-5'-triphosphate (UTP) are metabolized to synthesize UDP-GlcNAc.[2] Consequently, the availability of substrates used in its synthesis coupled with the activity of the HBP, and the resulting availability of UDP-GlcNAc, are considered critical regulators of protein O-GlcNAcylation.[3] One method for quantifying UDP-GlcNAc is via high pressure liquid chromatography (HPLC), but this typically requires two steps; the first using a phosphate gradient anion-exchange HPLC which separates the UDP-HexNAc fraction (i.e., UDP-GlcNAc plus UDP-GalNAc). This is followed by running the UDP-HexNAc fraction on an isocratic anion-exchange HPLC with a borate buffer, which separates the two nucleotide sugars.[4] UDP-GlcNAc levels can be quantified using liquid chromatography-tandem mass spectrometry (LC-MS)[5]; however, separation of UDP-GlcNAc from UDP-GalNAc is not always possible.[6] Given the technical challenges in separating UDP-GlcNAc from UDP-GalNAc, cellular UDP-GlcNAc levels are rarely reported, or are reported as UDP-HexNAc levels. As a result, changes in the availability of UDP-GlcNAc for OGT or other key enzymes that use UDP-GlcNAc are rarely determined. Therefore, the assumption that the rate of UDP-GlcNAc synthesis and its availability regulates protein O-GlcNAc levels is inferred indirectly based on changes in protein O-GlcNAcylation.

The lack of a robust assay that can be performed using readily available materials for determining UDP-GlcNAc levels in tissues and cells has been a major impediment in our understanding of both the regulation of the HBP and protein O-GlcNAcylation. In this issue of *Cell Reports Methods*, Sunden et al., describe an innovative method using readily available technology and reagents for quantifying UDP-GlcNAc levels in cells and tissues.[1] There was one earlier report of an enzyme assay for quantifying UDP-GlcNAc[7]; however, this has not been widely adopted, and Sunden et al. were unable to demonstrate its activity. Therefore, taking an alternative approach they used the high affinity of OGT for UDP-GlcNAc, as well as its selectivity for UDP-GlcNAc over UDP-GalNAc,[8] to develop a UDP-GlcNAc assay. The assay uses a commercially available human recombinant OGT and a custom synthesized GlcNAc acceptor peptide, derived from human casein kinase 2 (CK2), cross-linked to bovine serum albumin. Combined with dot blotting, it is used for immunodetection of the O-GlcNAcylated peptide with RL2, a monoclonal antibody for detecting O-GlcNAc modifications (Figure 1). The addition of an alkaline phosphatase removed free UDP, a reaction product that is a potent inhibitor of OGT. Even though the pH of the reaction is not optimal for alkaline phosphatase, it performed better than a phosphatase with a pH optimum closer to 7.0. The assay was shown to work with UDP-GlcNAc standards and, importantly, with liver tissue extracts.

To expand the utility of the assay, the authors adapted it to a 384-well microplate format and used the RL2 antibody coupled with peroxidase-conjugated secondary antibody enabling an ELISA-like detection approach. The authors estimated that the lowest limit of quantification, 110 fmol, is equivalent to 5.5 nM UDP-GlcNAc in the reaction mixture, indicating that only 1 mg of tissue or 50,000 cultured cells would be sufficient for reliable quantification of UDP-GlcNAc levels.

Following several optimizations of assay conditions, a critical next step was demonstrating that it worked reliably in biological samples and provided accurate results. The assay was used to quantify UDP-GlcNAc in liver, heart, and brain tissues and the results were comparable to previous reports of UDP-HexNAc in these tissues.

Using this assay, Sunden et al. investigated how different culture conditions affected UDP-GlcNAc levels in AML12 cells, a hepatocyte cell line.[1] When grown in 5 or 25 mM glucose, cells had similar UDP-GlcNAc levels and it was only when glucose was withdrawn for 16 h that a significant decrease in UDP-GlcNAc levels was observed. Only two of the conditions tested significantly increased UDP-GlcNAc levels: the addition of

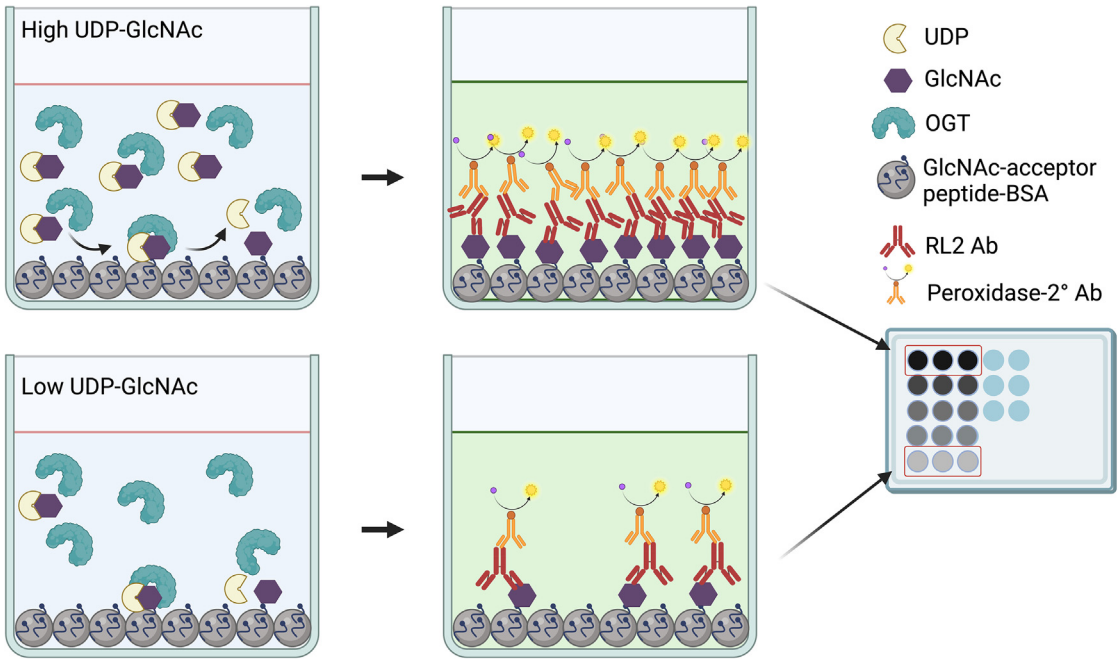

Figure 1. Schematic of the enzyme-based UDP-GlcNAc quantification assay

The UDP-GlcNAc quantification method utilizes the enzymatic role of O-GlcNAc transferase (OGT) and BSA-conjugated GlcNAc-acceptor peptide. The UDP-GlcNAc-containing samples react with the GlcNAc-acceptor peptide-BSA and OGT. Following immunodetection with RL2 antibody, O-GlcNAc can be detected. Figure created with BioRender (https://biorender.com/).

1 mM GlcNAc, which is directly phosphorylated to GlcNAc-6-phosphate by N-acetylglucosamine kinase, and serum deprivation. Importantly, azaserine, an amidotransferase inhibitor that inhibits glutamine fructose-6-phosphate amidotransferase (GFAT), which regulates the HBP, decreased UDP-GlcNAc levels by ~50%, demonstrating that the assay was responsive to changes in HBP activity. Interestingly, O-GlcNAc levels were unchanged under all conditions except serum deprivation where they decreased, despite the increase in UDP-GlcNAc concentrations.

Furthermore, overexpression of wild-type or a gain-of-function mutant of GFAT resulted in 5- or 10-fold increases in UDP-GlcNAc levels, respectively, but only a 1.7-fold increase in protein O-GlcNAcylation in both cases. Knockout (KO) of GFAT in the absence of GlcNAc decreased UDP-GlcNAc by 90%, and this was associated with almost a complete lack of O-GlcNAcylation. Both UDP-GlcNAc and O-GlcNAc levels were rescued by the addition of GlcNAc. The almost complete loss of O-GlcNAcylation in GFAT KO cells, even though some UDP-GlcNAc remained, combined with the rescue by the addition of GlcNAc highlights the importance of salvage pathways in contributing to regulating cellular UDP-GlcNAc levels. This is consistent with a study that demonstrated that when UDP-GlcNAc availability was limiting, the salvage pathways are sufficient to maintain endoplasmic reticulum (ER)- and Golgi-mediated glycosylation at the expense of protein O-GlcNAcylation.[9]

Since antibodies, such as the RL2 O-GlcNAc antibody, recognize a well-characterized O-GlcNAcylation site on a peptide from human CK2α, concerns common to pan-*O*-GlcNAc antibodies, such as epitope specificity, are not relevant. On the other hand, this raises the question as to whether a site-specific O-GlcNAc antibody might provide increased sensitivity. However, given the challenges associated with developing site-specific O-GlcNAc antibodies, it is unclear whether such an effort would be worthwhile.

The further application of fluorescence-conjugated secondary antibodies in this assay might reduce experimental errors; for example, the total amount of peptide-BSA coated on the plate could be simultaneously measured using antibodies with different fluorescence colors to reduce the variation between wells. The fluorescent probes could also be directly conjugated to the primary antibody, reducing a step in the assay. These approaches could also limit the impact of buffer-induced peroxidase activity in peroxidase-conjugated secondary antibody, indicating the potential for further assay optimization.

OGT activity is central to the reliable and precise functioning of the assay, but it is also a potential variable in assay performance. In the initial development of the assay, a commercially available recombinant OGT was used, but for the optimization in 384-well plates an in-house produced enzyme was used instead. The latter, likely more cost-effective, provided more control over the generation of the protein and its subsequent purification. As the authors note, a further refinement of the assay would be the determination of the specific activity of each OGT preparation required for optimal assay performance. This will be key for wider use of the assay given the

variability in the quality of recombinant OGT protein, and will facilitate comparisons across laboratories and different batches of protein. Several different tissue extraction methods are commonly used beyond the one used here, such as trichloroacetic and perchloric acid extractions, and removal of potential interfering reagents for each will need optimization. Nucleotides other than UDP also inhibit OGT,[10] but the presence of the phosphatase should be sufficient to account for this. High levels (~50 mM) of cations such as Na^+ and K^+ have also been reported to inhibit OGT activity.[10] Therefore, a better understanding of potential reagent contaminants or sample-related interference in the assay performance will be valuable when establishing this assay for the first time.

Overall, the authors have developed a sensitive, enzyme-based assay for the quantification of UDP-GlcNAc levels in different murine tissues and a range of different human and mouse cell lines. They have taken advantage of the selectivity of OGT for UDP-GlcNAc over UDP-GalNAc to minimize contributions from the latter, which is a major limitation in HPLC- and MS-based assays. Although the assay is a time-intensive multi-step process, it can be implemented using readily available reagents and supplies. In addition, the studies using this new assay highlight the importance of quantification of UDP-GlcNAc levels in the study of the HBP and all forms of protein glycosylation, including O-GlcNAcylation. The observations here demonstrating a dissociation between UDP-GlcNAc and O-GlcNAc levels, except under extreme conditions, in contrast to the widely accepted notion that changes in UDP-GlcNAc levels are reflected in changes in O-GlcNAcylation, challenging the concept that HBP pathway is directly regulated by nutrient availability. The ability to reliably quantify tissue and cellular levels of UDP-GlcNAc provided by this assay, is an important resource for understanding the physiological regulation of the HBP and protein glycosylation. The latter may give us new insights into how protein O-GlcNAcylation contributes to physiological states as well as to the dysfunction that occurs in response to stress or other pathological conditions.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Sunden, M., Upadhyay, D., Banerjee, R., Sipari, N., Fellman, V., Kallijärvi, J., and Purhonen, J. (2023). Enzymatic assay for UDP-GlcNAc and its application in the parallel assessment of substrate availability and protein O-GlcNAcylation. Cell Reports Methods *3*.
2. Chatham, J.C., Zhang, J., and Wende, A.R. (2021). Role of O-Linked N-Acetylglucosamine Protein Modification in Cellular (Patho)Physiology. Physiol. Rev. *101*, 427–493.
3. Hart, G.W. (2019). Nutrient regulation of signaling and transcription. J. Biol. Chem. *294*, 2211–2231.
4. Weckbecker, G., and Keppler, D.O. (1983). Separation and analysis of 4'-epimeric UDP-sugars by borate high-performance liquid chromatography. Anal. Biochem. *132*, 405–412.
5. Olson, A.K., Bouchard, B., Zhu, W.Z., Chatham, J.C., and Des Rosiers, C. (2020). First characterization of glucose flux through the hexosamine biosynthesis pathway (HBP) in ex vivo mouse heart. J. Biol. Chem. *295*, 2018–2033.
6. Garcia, A.D., Chavez, J.L., and Mechref, Y. (2013). Sugar nucleotide quantification using multiple reaction monitoring liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. *27*, 1794–1800.
7. Namboori, S.C., and Graham, D.E. (2008). Enzymatic analysis of uridine diphosphate N-acetyl-D-glucosamine. Anal. Biochem. *381*, 94–100.
8. Ma, X., Liu, P., Yan, H., Sun, H., Liu, X., Zhou, F., Li, L., Chen, Y., Muthana, M.M., Chen, X., et al. (2013). Substrate specificity provides insights into the sugar donor recognition mechanism of O-GlcNAc transferase (OGT). PLoS One *8*, e63452.
9. Boehmelt, G., Wakeham, A., Elia, A., Sasaki, T., Plyte, S., Potter, J., Yang, Y., Tsang, E., Ruland, J., Iscove, N.N., et al. (2000). Decreased UDP-GlcNAc levels abrogate proliferation control in EMeg32-deficient cells. EMBO J. *19*, 5092–5104.
10. Haltiwanger, R.S., Blomberg, M.A., and Hart, G.W. (1992). Glycosylation of nuclear and cytoplasmic proteins. Purification and characterization of a uridine diphospho-N-acetylglucosamine:polypeptide beta-N-acetylglucosaminyltransferase. J. Biol. Chem. *267*, 9005–9013.