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Yeast cells as an assay system for in vivo *O*-GlcNAc modification☆

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ABSTRACT

Background: *O*-GlcNAcylation is a reversible protein post-translational modification, where *O*-GlcNAc moiety is attached to nucleocytoplasmic protein by *O*-GlcNAc transferase (OGT) and removed by *O*-GlcNAcase (OGA). Although *O*-GlcNAc modification widely occurs in eukaryotic cells, the budding yeast *Saccharomyces cerevisiae* notably lacks this protein modification and the genes for the GlcNAc transferase and hydrolase.
Methods: Human OGT isoforms and OGA were ectopically expressed in *S. cerevisiae*, and the effects of their expressions on yeast growth and *O*-GlcNAc modification levels were assessed.
Results: Expression of sOGT, in *S. cerevisiae* catalyzes the *O*-GlcNAc modification of proteins in vivo; conversely, the expression of OGA mediates the hydrolysis of these sugars. sOGT expression causes a severe growth defect in yeast cells, which is remediated by the co-expression of OGA. The direct analysis of yeast proteins demonstrates protein *O*-GlcNAcylation is dependent on sOGT expression; conversely, the hydrolysis of these sugar modifications is induced by co-expression of OGA. Protein *O*-GlcNAcylation and the growth defects of yeast cells are caused by the *O*-GlcNAc transferase activity because catalytically inactive sOGT does not exhibit toxicity in yeast cells. Expression of another OGT isoform, ncOGT, also results in a growth defect in yeast cells. However, its toxicity is largely attributed to the TPR domain rather than the *O*-GlcNAc transferase activity.
Conclusions: *O*-GlcNAc cycling can occur in yeast cells, and OGT and OGA activities can be monitored via yeast growth.
General significance: Yeast cells may be used to assess OGT and OGA.

1. Introduction

O-GlcNAcylation is a form of post-translational modification wherein single *N*-acetylglucosamine (GlcNAc) is attached to serine or threonine residues on target proteins [1]. *O*-GlcNAc modification is a unique type of glycosylation because it is a reversible modification, which takes place in the cytosol, nucleus, and mitochondria [2,3]. The addition of an *O*-GlcNAc to specific serine or threonine residues can influence several aspects of protein function, such as enzymatic activity [4], stability [5], and subcellular localization [6]; various cellular processes are regulated through these modifications [7]. So far no consensus *O*-GlcNAc modification motifs have been reported; thus, sites for *O*-GlcNAc modification cannot be predicted [7].

The addition of *O*-GlcNAc moiety is mediated by *O*-GlcNAc transferase (OGT) [8–10]. OGT is encoded by a single gene; alternative splicing generates three distinctive isoforms: ncOGT, sOGT, and mOGT [11–13]. mOGT is a unique isoform because it contains a matrix targeting signal that targets it to the mitochondria [13]. The other isoforms, ncOGT and sOGT, are both localized to the nucleocytoplasm [9,14]. ncOGT and sOGT are mainly composed of two functional domains: the N-terminal tetratricopeptide repeat (TPR) and the C-terminal catalytic domains. These OGT isoforms contain an identical catalytic domain, but differ in the length of the TPR repeats; the number of TPR repeats in ncOGT and sOGT are 12.5 and 2.5, respectively [11]. Generally, the TPR domains mediate protein–protein interactions [15]. In ncOGT and sOGT, the difference in the length of the TPR domain may modulate their substrate specificities. Previous studies have showed that recombinant OGTs with three or fewer TPR repeats generally show poor or no *O*-GlcNAc transfer reactions to certain substrate proteins in vitro [14,16,17]. More recent activity assays using various short peptides as

Abbreviations: *S. cerevisiae*, *Saccharomyces cerevisiae*; GlcNAc, *N*-acetylglucosamine; TPR, tetratricopeptide repeat; OGT, *O*-GlcNAc transferase; sOGT, short OGT; ncOGT, nuclear and cytoplasmic OGT; mOGT, mitochondrial OGT; OGA, *O*-GlcNAcase; HAT, histone acetyltransferase; HA, hemagglutinin; GalNAz, azido-acetylgalactosamine; HRP, horseradish peroxidase.

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substrates have demonstrated that sOGT can mediate the reaction with some specificity [18,19]. ncOGT and sOGT exhibit distinct tissue and developmental expression patterns, thereby suggesting their specialized functions [9,20,21]. However, the basis for their functional differences is not understood.

O-GlcNAc modification is tightly regulated by the proper addition and removal of the *O*-GlcNAc moiety (*O*-GlcNAc cycling) [22]. Hydrolytic cleavage of the *O*-GlcNAc moiety is catalyzed by *O*-GlcNAcase (OGA) [23,24]. Two major alternatively spliced OGA isoforms are present in mammals [25]. The longer isoform or OGA-L is composed of three domains: the N-terminal *O*-GlcNAcase catalytic domain, the OGT-binding domain, and the C-terminal histone acetyltransferase (HAT)-like domain [7,25]. The shorter isoform or OGA-S lacks the HAT-like domain; instead this isoform has a unique 15-amino acid C-terminal extension [7]. The OGT-binding domain of OGA interacts with ncOGT via its TPR domain [26].

Extent of *O*-GlcNAc modification in cell can be an indicator of nutrient flux [27]. The affinity of OGT to target proteins is largely dependent on the concentration of its donor substrate, UDP-GlcNAc [17]. Given that the cellular concentration of UDP-GlcNAc is closely linked to the flux of nutrients via the hexosamine biosynthetic pathway [28], the levels of *O*-GlcNAcylated proteins are altered in response to cues from environmental nutrients [29]. However, due to this regulatory mechanism, malfunctioning of nutrient homeostasis, such as chronic hyperglycemia, leads to abnormal increases in *O*-GlcNAc levels [29]. Aberrant *O*-GlcNAcylation causes insulin resistance and type-2 diabetes [27,30]. Aside from diabetes, the dysregulation of *O*-GlcNAcylation is implicated in several diseases, including neurodegeneration and cancer [31–34]. OGT and OGA have attracted attention for their biological importance and potential as therapeutic targets.

The budding yeast *S. cerevisiae* is a well-established eukaryotic model. Although the *O*-GlcNAc cycling is highly conserved among eukaryotes, OGT and OGA are absent in the yeast [9,10]. In the present study, we described the development of a yeast cell-based OGT assay system. This system will be useful for various analyses, including genetic and high-throughput screening for inhibitors or modifiers of the *O*-GlcNAc cycling.

2. Material and methods

2.1. Yeast strains, growth media and cell culture

The *S. cerevisiae* strain used in this study was YPH499 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his1-Δ200 leu2-Δ1*). YPAD media (10 g/l yeast extract, 20 g/l peptone, 30 mg/l adenine, 20 g/l glucose) were used to grow yeast cells without plasmids. Yeast cells harboring plasmids were grown in SD media (6.7 g/l yeast nitrogen base, 2 g/l dropout mix without appropriate selectable supplements, 20 g/l glucose or galactose). 20 g/l agar was added for making plates. The drop out mix was prepared by mixing the following supplements: 0.5 g adenine, 2 g L-alanine, 2 g L-arginine, 2 g L-asparagine, 2 g L-aspartic acid, 2 g L-cysteine, 2 g L-glutamine, 2 g L-glutamic acid, 2 g glycine, 2 g L-histidine, 2 g *myo*-inositol, 2 g L-isoleucine, 10 g L-leucine, 2 g L-lysine, 2 g L-methionine, 0.2 g para-aminobenzoic acid, 2 g L-phenylalanine, 2 g L-proline, 2 g L-serine, 2 g L-threonine, 2 g L-tryptophan, 2 g L-tyrosine, 2 g uracil, 2 g L-valine.

2.2. Growth assay

To compare the effect of OGT and OGA on yeast growth, we employed serial dilution assays. Yeast cells cultured in SD glucose media were collected by centrifugation and resuspended in water at concentrations of 1×10^7, 10^6, 10^5 and 10^4 cells/ml. 5 μl of each cell suspensions were plated onto SD glucose or galactose plate and incubated at 30 °C or 37 °C.

2.3. Plasmids

All plasmids and their relevant features are listed in Table 1. Primers used to construct the plasmids were listed in Table 2. sOGT, ncOGT and OGA were amplified by PCR using human cDNA library (Takara, Dalian, China) as a template, and cloned into pRS424GAL1 [35] which contains an inducible *GAL1* promoter. ncOGT^{TPR} is a truncated ncOGT which only contains the TPR domain (amino acids 1–505). This gene was amplified by PCR using pRS424GAL1-ncOGT as a template. Site directed mutations were introduced by the PCR-based mutagenesis. Primers named HXO420 and HXO421 were used to introduce H508A mutation into ncOGT, and H127A mutations into sOGT. Primers HXO522 and HXO523 were used to introduce D174A mutation into OGA. pRS424GAL1-ncOGT-HA was used to express ncOGT carrying a C-terminal three hemagglutinin tandem repeats (3 × HA) tag under the *GAL1* promoter. To construct this plasmid, first 3 × HA fragment was amplified by PCR using HXO356 and HXO357 as primers and pFA6a-3HA-His3MX6 [36] as a template. Fusion PCR was performed to produce ncOGT-3 × HA fusion gene where HXO324 and the PCR fragment containing 3 × HA were used as primers and pRS424GAL1-ncOGT as a template. The resulting fragment was digested by *Spe*I and *Sal*I, and cloned into similarly digested pRS424GAL1. pRS424GAL1-ncOGT^{TPR}-HA was used to express a 3 × HA tag onto C-terminus of ncOGT^{TPR}. To construct this plasmid, 3 × HA gene was amplified by PCR using HXO419 and HXO357 as primers and pFA6a-3HA-His3MX6 as a template. Then, resulting 3 × HA PCR fragment and HXO324 were used as primers to produce the ncOGT^{TPR}-3 × HA gene. The PCR fragment was digested by *Spe*I and *Sal*I, and cloned into similarly digested pRS424GAL1. pRS424GAL1-HA-sOGT was used to express sOGT carrying a N-terminal 3 × HA tag. This plasmid was constructed as follows. 3 × HA PCR fragment was amplified with primers HBX14 and HBX15. sOGT PCR fragment was amplified with primers HBX16 and HXO325. These PCR fragments were ligated into *Spe*I and *Sma*I, and *Sma*I and *Sal*I sites of pRS424GAL1, respectively. pRS426TEF-OGA-FLAG was used to express OGA carrying a C-terminal three FLAG tandem repeats (3 × FLAG) tag under the *TEF2* promoter. This plasmid was constructed by cloning OGA gene without stop codon into pRS426TEF-FLAG. pRS426TEF-FLAG was constructed by a DNA fragment containing a 3 × FLAG gene and the yeast *ADH1* terminator into *Xho*I and *Kpn*I sites of pRS426TEF [37]; in this plasmid the *CYC1* terminator originally included in pRS426TEF was replaced with the *ADH1* terminator. The DNA fragment containing the 3 × FLAG gene and yeast *ADH1* terminator (188 nucleotides) were synthesized by Sangon Biotech (Shanghai, China).

2.4. Protein analyses

Expression of OGT was induced by culturing yeast cells as follows. Cells were pre-cultured in 5 ml SD liquid media supplemented with glucose at 30 °C overnight. The culture was transferred into new 20 ml SD glucose media and grown at 30 °C until OD_{660} reached 1.0. The cells were collected by centrifugation, and resuspended into 30 ml SD liquid media supplemented with galactose to make OD_{660} of around 0.4. The cells were cultured at 30 °C or 37 °C until OD_{660} reached 0.8 to 1.0. After harvesting, cells were suspended in 200 μl of an 8 M urea buffer (25 mM Tris-HCl pH 8.0, 8 M urea, 0.15 M NaCl) supplemented with 2.5 mM PMSF (final concentration), and proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) as suggested in the manufacture's guide. For *O*-GlcNAcylated protein chemoenzymatic labeling experiment, cells were suspended in a 200 μl HEPES buffer (20 mM HEPES pH 7.9, 0.15 M NaCl, 1% NP-40) instead of 8 M urea buffer. Glass beads were added to the cell suspension and cells were lysed with vortexing. After centrifugation at 215000 ×*g* for 5 min to remove insoluble debris, cell free lysates were collected, and protein concentration was measured. 100 μg protein (for detection of HA and FLAG fusions, and *O*-GlcNAcylated proteins) or 50 μg (for detection of actin) were subjected to SDS-PAGE (5% stacking gel, and 8% or 10% separating gel). Rabbit