Analytical Biochemistry 532 (2017) 12–18

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Measuring O-GlcNAc cleavage by OGA and cell lysates on a peptide microarray

Suhela Sharif [a], Jie Shi [a], Mostafa Bourakba [b], Rob Ruijtenbeek [a, b], Roland J. Pieters [a, *]

[a] Department of Chemical Biology and Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P. O. Box 80082, 3508 TB Utrecht, The Netherlands
[b] PamGene International BV, 's-Hertogenbosch, The Netherlands

ARTICLE INFO

Article history:
Received 23 January 2017
Received in revised form
12 May 2017
Accepted 26 May 2017
Available online 29 May 2017

Keywords:
O-GlcNAcylation
O-GlcNAcase
O-GlcNAcylated peptide
Peptide microarray
Cell lysate

ABSTRACT

O-GlcNAcylation is a post-translational modification resulting from the addition of an N-acetylglucosamine moiety to the hydroxyl groups of serine and threonine residues of nuclear and cytoplasmic proteins. In addition, O-GlcNAcylated proteins can be phosphorylated, which suggests the possibility for crosstalk between O-GlcNAcylation and phosphorylation. Dysregulation of O-GlcNAcylation affects cell signaling, transcriptional regulation, cell cycle control and can e.g. lead to tumorigenesis and tumor metastasis. There is a strong demand for efficient analytical techniques to better detect and investigate this abundant modification and its role in cancer. Herein we demonstrated the utility of an O-GlcNAcylated peptide array to examine O-GlcNAcase (OGA) activity and substrate specificity of both purified protein as well cell lysates of different cancer cell lines. Using this microarray, we clearly observed OGA activity and also inhibition thereof by OGA inhibitor thiamet G. Interestingly, different levels of OGA activity were observed of lysates derived from different cancer cell lines. This suggests that the tool may be useful in cancer research and biomarker development.

Introduction

Cancer cells have a different metabolism than normal cells. Cancerous cells produce ATP by promoting a high rate of glycolysis followed by lactic acid fermentation (Warburg effect) rather than oxidative phosphorylation in mitochondria. These cells also need carbon- and nitrogen-rich nutrients for cell proliferation [1]. Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), is located at the centre of the glucose, nitrogen, fatty acid and nucleic acid metabolic pathways [2]. UDP-GlcNAc is the metabolic substrate for O-GlcNAc transferase (OGT). OGT uses UDP-GlcNAc to catalyze O-GlcNAc addition to the free hydroxyl group of serine or threonine residues of cytoplasmic and nuclear proteins. This modification is subsequently removed by the glycosidase enzyme O-GlcNAcase (OGA) [3]. Dysregulation of the O-GlcNAcylation modification affects cell signaling, transcriptional regulation and cell cycle control and can lead to tumorigenesis and tumor metastasis [4–6]. Most oncogenic factors such as p53, MYC, NF-κB, Snail, HCF-1, β-catenin and p27 are O-GlcNAcylated [6]. In addition, crosstalk between O-GlcNAcylation and phosphorylation was shown especially for oncogenic factors [7,8]. For example, the *in vivo* stability of the tumor suppressor protein p53 is regulated by phosphorylation. Phosphorylation at Thr155 leads to proteasomal degradation of this protein, which keeps it at a low abundance. O-GlcNAcylation of Ser149 decreases Thr155 phosphorylation and stabilizes p53 [9] protein expression. In hepatocellular carcinoma, a dynamic interplay between O-GlcNAcylation and phosphorylation of tumor suppressor p27 regulates cell proliferation [10].

A recent study showed a link between fatty acid synthase (FAS) and OGT [11]. Both FAS and OGT indirectly use the same substrate, glucose, which is highly increased in cancer cells. FAS is overexpressed is breast, colon, esophageal, lung, melanoma, ovarian, pancreatic, prostate and stomach cancer [11]. An elevated O-GlcNAcylation level has been observed in aggressive lung tumor [12], colon tumor [13], and chronic lymphocytic leukemia [14]. On the other hand, the O-GlcNAc level is decreased in breast cancer [15] and thyroid cancer [16].

There is a major challenge to detect and study O-GlcNAcylation. This is due to its dynamic, substiochiometric and labile O-glycosidic linkage. The traditional methods such as the wheat germ agglutinin (WGA) lectin [17], pan-specific O-GlcNAc antibodies [18,19], or

* Corresponding author.
E-mail address: r.j.pieters@uu.nl (R.J. Pieters).

http://dx.doi.org/10.1016/j.ab.2017.05.027
0003-2697/

radiolabeling using β-1,4-galactosyltransferase (GalT), which transfers [^{3}H]Gal from UDP-[^{3}H]galactose to terminal GlcNAc groups [20] have limited sensitivity. Proteins with weakly expressed or few O-GlcNAc sites are not detected by the WGA lectin [17]. Furthermore, the WGA lectin also detects GlcNAc on both N- and O-linked GlcNAc moieties and does so with higher sensitivity. For a definitive identification of O-GlcNAc, WGA lectin must be used in conjunction with other treatments [20]. The success of β-1,4-galactosyltransferase (GalT) labeling for detection is dependent on the accessibility of O-GlcNAc residues to the transferase which is provided by denaturation of the substrate protein by SDS. In some cases, O-GlcNAc is not fully accessible to the enzyme even after SDS treatment [20]. Recently, the biotinylated GalNAz labeling using GalT^{Y289L}allowed the enrichment of the GalNAz labelled peptides. Although this approach improved the sensitivity of the detection [21], the harsh conditions required to disrupt the biotin-streptavidin interaction may hydrolyze the labile O-GlcNAc moiety [22]. In the present study, a novel and dynamic serine O-GlcNAc peptide microarray is developed which includes immobilized O-GlcNAcylated peptides on a chip. This chip is able to identify OGA activity in both purified protein as well as in cell lysates of different cancer cell lines. The chip method has the potential to simultaneously evaluate hundreds of glycopeptides, may lead to new specificity insights and the role of other cellular proteins in this process. Furthermore, it might be a promising tool for the study of cancer and biomarkers, diagnostics and personalized medicine.

Material and method

Materials

Isopropyl 1-thio-β-D-galactopyranoside (IPTG) and OGA inhibitor (3aR,5R,6S,7R,7aR)-2-(ethylamino)-3a,6,7,7a-tetrahydro-5-(hydroxymethyl)-5H-Pyrano [3,2-d]thiazole-6,7-diol (Thiamet G) were purchased from sigma Aldrich (Zwijndrecht, The Netherlands). The mouse monoclonal Anti-O-Linked N-Acetylglucosamine antibody [RL2] (ab2739) was obtained from Abcam (London, England). A FITC-conjugated goat anti-mouse secondary antibody was purchased from Thermo Scientific (Bleiswijk, Netherland). All PamChip4 microarray chips were provided by PamGene (PamGene International, The Netherlands) and printed at their facilities.

Overexpression and purification of O-GlcNAcase

Plasmid encoding O-GlcNAcase cDNA (GenBank accession number AB014579) was kindly provided by Dr. Gerald Warren Hart from the Johns Hopkins University School of Medicine. *Escherichia coli* BL21 (DE3) transformed with the expression plasmid was grown in Luria–Bertani (LB) broth supplemented with 50 μg/mL ampicillin at 37 °C at 200 rpm. Protein expression was initiated by the addition of IPTG (Isopropyl-L-thio-β-D-galactopyranoside) to a final concentration of 0.1 mM when the optical density at 600 nm was reached around 0.6. The culture was further grown for 10 h at 16 °C with shaking at 200 rpm. Cells were harvested by centrifugation and suspended in 50 mM phosphate buffer, pH 7.5, 300 mM NaCl, 0.1% triton, 5% glycerol and complete mini EDTA-free protease inhibitor cocktail for 10 min on ice. The lysate was then disrupted by sonication. Cell debris was removed by centrifugation (13,000 g, 30 min, 4 °C), and the supernatant was loaded onto a His60 Ni Gravity column (Clontech). After several times washing, the bound protein was eluted using 50 mM phosphate buffer pH 7.5, 300 mM NaCl, and 300 mM imidazole. The eluted fraction was concentrated using a 100 kDa molecular weight cut off Amicon unit by centrifugation at 4 °C for 10 min at 5000 rpm. Protein concentration was measured by the BCA method. The purity and apparent molecular mass were determined by SDS-PAGE.

Synthesis of peptides and O-GlcNAcylated peptides

Synthesis of all peptides including the O-GlcNAcylated peptides (Fig. 1A) was achieved by following a standard Fmoc SPPS strategy on a Symphony Multiple Peptide Synthesizer starting from a Rink amide resin. The Fmoc amino acids and a Fmoc-Ser(β-D-GlcNAc(Ac)$_3$)–OH were used. Deprotection was performed using 20% piperidine in DMF, and coupling was performed using 1:0.9:2 amino acid/HBTU/DIPEA in DMF. The N-terminus of the peptides was then acetylated with Ac_2O while the O-GlcNAcylated peptides were still attached to the resin. On-Resin acetyl deprotection of GlcNAc was carried out with 80% hydrazine in methanol. The resins then were incubated with a 10 mL mixture of TFA (trifluoroacetic acid): H_2O: triisopropylsilane (TIPS): 1, 2-ethanedithiol (EDT) (9:0.5:0.25:0.25, v/v/v/v) and allowed to stir for 2 h at room temperature under a nitrogen atmosphere. The product was filtered and the resin was washed with TFA (2 mL) and DCM (4 mL). The residue was precipitated by the addition of pre-cooled diethyl ether and centrifugation. The precipitated peptides were dissolved in water, frozen, and overnight lyophilized. All products were stored at −20 °C. Crude peptide analysis was carried out by LC-MS.

Immobilization of peptides and O-GlcNAcylated peptides on PamChip® microarray

The synthesized peptides were immobilized in 3 different spotting concentrations (1.0, 0.6, 0.3 mM) (Fig. 1B) inside a porous membrane made of aluminium oxide which allows flow-through of the sample and thereby enhances assay sensitivity. Four arrays are combined into a PamChip 4 and were measured simultaneously. The reaction mixture was pumped up and down through the array, giving the OGA maximal opportunity to hydrolyze the O-GlcNAc from the O-GlcNAcylated peptides on each array. When the solution is temporarily placed underneath the array, the CCD camera in the workstation takes an image of each array. Functional readout is based on statistical analysis of disappearing O-GlcNAc signal on the array using fluorescently labelled anti-GlcNAc-antibody. Image analysis was performed using BioNavigator 6 software (PamGene International, The Netherlands). Each image was quantified by automated array grid finding and subsequent quantification of the signal (after local background subtraction) for each individual spot.

Cell cultures and cell lysis

The human breast carcinoma MCF7 cell line (ATCC HTB22), human HT29 (ATCC® HTB-38™) colon cancer cells, heterogeneous human epithelial colorectal adenocarcinoma Caco-2 (ATCC HTB 37) cells and fibroblasts WT ATCC were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10%(v/v) fetal calf serum (FCS) (Lonza) and 10 μg/mL streptomycin at 37 °C in a humidified atmosphere enriched with 5% CO_2. Cells were allowed to reach a confluency of about 75%. Cells were lysed on ice for 15 min using RIPA lysis buffer with protease inhibitor (complete mini EDTA-free protease inhibitor). The cell lysate was centrifuged at 4 °C for 15 min at 15,000 × g and the supernatant was collected. The BCA Protein Assay kit was used to determine the total protein concentration. The supernatant was then aliquoted and kept at −20 °C.

Preparation of the primary/secondary antibody mixture

A 1:1 fresh mixture of the mouse monoclonal anti-O-GlcNAc antibody (RL2) and a FITC-conjugated goat anti-mouse secondary