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ORIGINAL PAPER

Identification of new *O*-GlcNAc modified proteins using a click-chemistry-based tagging

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Abstract The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification is an abundant post-translational modification in eukaryotic cells. This dynamic glycosylation plays a fundamental role in the activity of many nuclear and cytoplasmic proteins and is associated with pathologies like type II diabetes, Alzheimer's disease or some cancers. However the exact link between *O*-GlcNAc-modified proteins and their function in cells is largely undefined for most cases. Here we report a strategy based on the 1,3-dipolar cycloaddition, called click chemistry, between unnatural *N*-acetylglucosamine (GlcNAc) analogues (substituted with an azido or alkyne group) and the corresponding biotinylated probe to specifically detect, enrich and identify *O*-GlcNAc-modified proteins. This bio-orthogonal conjugation confirms that only azido analogue of GlcNAc is metabolized by the cell. Thanks to the biotin probe, affinity purification on streptavidin beads allowed us to identify 32 *O*-GlcNAc-azido-tagged proteins by LC-MS/MS analysis in an MCF-7 cellular model, 14 of which were previously unreported. This work illustrates the use of the click-chemistry-based strategy combined with a proteomic approach to get further insight into the pattern of *O*-GlcNAc-modified proteins and the biological significance of this post-translational modification.

Keywords *O*-GlcNAc · Click chemistry · Azido sugar · Biotin probe · Protein labelling

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Introduction

The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) modification of serine or threonine residues of nuclear and cytosolic proteins is an abundant and dynamic post-translational modification [1, 2]. The enzymes that catalyse the dynamic addition and removal of *O*-GlcNAc on proteins are respectively the *O*-GlcNAc transferase (OGT) and a specific β-*N*-acetylglucosaminidase named *O*-GlcNAse [3]. This glycosylation plays a fundamental role in the modulation of the activity of proteins, including transcription factors, cytoskeletal proteins, signalling proteins and kinases. Although these roles are not fully understood, an increase of the cellular *O*-GlcNAc level has been tightly associated with type II diabetes phenotypes, while in the case of Alzheimer's disease, the hyperphosphorylation of Tau protein is linked to a decrease in *O*-GlcNAc modification [4, 5]. The specific detection and identification of such modified proteins is consequently of great interest in order to decipher the regulating functions of *O*-GlcNAc modification under physiological conditions and in pathological situations. For this purpose, Bertozzi and coworkers developed a strategy based on the Staudinger ligation which involves the highly specific reaction between a phosphine and an azide group [6]. This reaction has the feature that neither phosphine nor

azide occur naturally in biomolecules, which makes the approach particularly suitable for biological applications. They have demonstrated that the enzymes implicated in the hexosamine salvage pathway tolerate and metabolize the azido analogue (GlcNAz) of their natural substrate *N*-acetylglucosamine. Indeed, a fraction of the extracted protein was detected using a fluorescent phosphine probe, demonstrating the presence of *O*-linked GlcNAz on the proteins. Another bio-orthogonal reaction, click chemistry, has proven to be effective in detecting and purifying proteins targeted with azide-substituted affinity labels. This activity-based protein profiling (ABPP) developed by Cravat and coworkers uses the Cu^{I}-catalysed azide–alkyne cycloaddition reaction [7, 8]. This reaction is a variant of Huisgen's 1,3-dipolar cycloaddition between an azide group and a terminal alkyne to afford stable 1,2,3-triazoles. This chemical method represents a powerful tool in the field of biology research as the cycloaddition reaction operates in aqueous buffers compatible with biological investigations using copper(I) as catalyst [9, 10]. Like the azide group, alkynes are essentially inert towards most of the chemical groups encountered in biological molecules [11, 12]. This bio-orthogonality makes the click chemistry reaction suitable for functional proteomic studies. Moreover, comparative studies between the Staudinger ligation, the strain-promoted and the click chemistry cycloadditions have shown that the last of these brings the highest sensitivity of protein labelling in a complex protein extract [13].

Given these data we wondered whether a click-chemistry-based strategy could not only detect proteins modified by *O*-GlcNAc moieties but also enable their specific purification for subsequent identification. To address this question, two analogues of *N*-acetylglucosamine modified with an alkyne or azide group and their corresponding reactive biotinylated probes were synthesized. The reactivity of the two pairs of reacting partners in terms of click chemistry cycloaddition was first measured in vitro by mass spectrometry. The efficiency of in cellulo metabolic incorporation of these analogues was then carried out in the breast cancer cell line MCF-7. The specificity of the click chemistry coupling between the modified proteins and their probes was assessed by western blotting prior to their purification on a streptavidin column for further proteomic analysis by mass spectrometry. This strategy allowed us to identify 32 *O*-GlcNAz-modified proteins in MCF-7 cells.

Materials and methods

Synthesis of GlcNAc analogues and biotin probes

See Electronic supplementary material

In vitro assessment of cycloaddition between the modified GlcNAc and the appropriate probe

Modified GlcNAc (5 mM) and the appropriate probe (5 mM, stock solution in DMSO) were mixed in phosphate buffer pH 8 at room temperature. The ligand (tris(triazolyl) amine) (2 mM, stock solution in DMSO/*t*BuOH 1:4, v/v) and freshly prepared reducing agent (2 mM) (tris(2-carboxyethyl)phosphine (TCEP) or sodium ascorbate) were added, followed by $CuSO_4$ (1 mM) in a final volume of 1 mL. As soon as $CuSO_4$ was added, the time course of the click chemistry reaction was followed by LC-MS. LC-MS analyses were performed on a triple quadrupole mass spectrometer (API 2000, Applied Biosystems) equipped with an electrospray source and operating in single ion monitoring (SIM) mode. The mass spectrometer was coupled to a high pressure liquid chromatography system (Agilent) equipped with a C_{18} HPLC column (50 mm×2.1 mm, dp=3.5 μm, Waters Symmetry C18).

Cell culture and metabolic protein labelling

MCF-7 cells were routinely cultured in DMEM (4.5 g L^{-1} Glc) supplemented with 10% fetal calf serum (FCS) and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. For labelling experiments, cells were seeded in 6-well dishes (Nunc, Fisher Bioblock Scientific, France) in Complete medium and when 80% confluency was obtained, the culture medium was replaced with low-glucose-containing DMEM (2 g L^{-1} Glc) supplemented with 10% FCS and containing either DMSO (1:1,000) or peracetylated GlcNAc ($GlcNAc_4$), peracetylated azido-GlcNAc (**2**, GlcNAz) or peracetylated alkyne-GlcNAc (**4**, GlcNAk) prepared at 250 mM in DMSO and diluted to the indicated concentrations (50–250 μM). Cells were labelled for 24 h, or for the indicated times.

Preparation of cytosolic and nuclear protein fractions from MCF-7 cells

Two hours before the end of the labelling time, 50 μM *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenyl carbamate (PUGNAc)/well was added into the medium. Cells were then washed twice with cold PBS and lysed for 30 min on ice in 200 μL $well^{-1}$ of lysis buffer I (20 mM Tris/HCl pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100) containing inhibitors of de-*O*-glucosaminylation and dephosphorylation (100 μM PUGNAc, 5 mM NaF, 1 mM orthovanadate) and a cocktail of protease inhibitors (Complete protease inhibitors cocktail tablets, Roche Diagnostics). Samples were collected and each well was rinsed once with 100 μL of buffer I. The lysates were centrifuged at 800 *g* for 10 min at 4 °C. The supernatant was

clarified by centrifugation (20,000 *g*, 20 min, 4 °C) and the resulting supernatant was considered as the cytosolic fraction. The pellet from the first centrifugation was rinsed once with 100 μL of buffer I, centrifugated one more time (800 *g*, 10 min, 4 °C) and then lysed for 30 min on ice in 100 μL of lysis buffer II (20 mM Tris/HCl pH 7.6, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20% glycerol, containing the same inhibitors as buffer I). After centrifugation (800 *g*, 10 min, 4 °C) the final supernatant was considered as the nuclear fraction.

Click reaction on labelled proteins and detection by western blotting

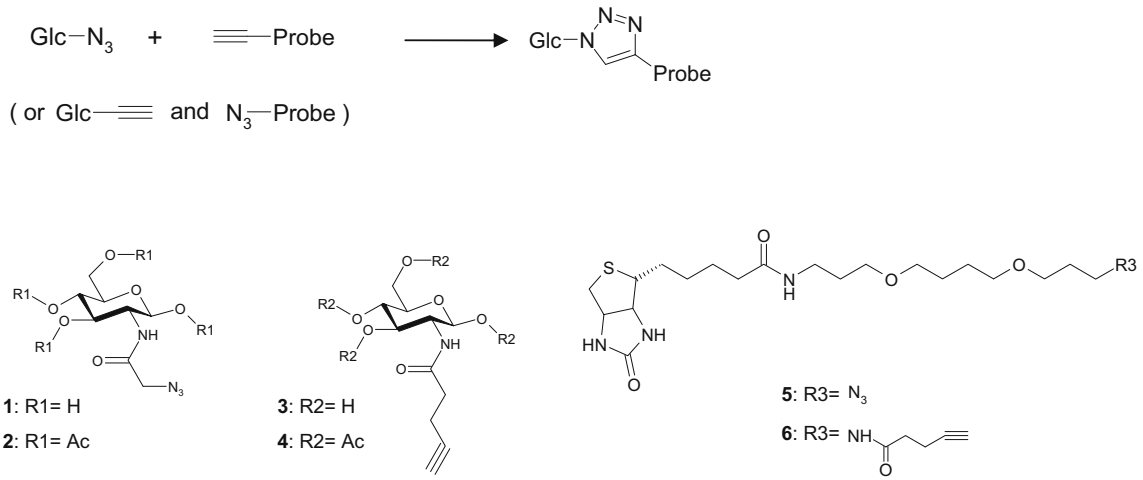
Cytosolic or nuclear fractions (50 μL) were treated with 100 μM of the appropriate biotin probe, freshly prepared 1 mM sodium ascorbate and 100 μM ligand. After gentle homogenisation, 1 mM $CuSO_4$ was added and the reaction mixture was gently homogenised. Click reaction was performed for 1 h at room temperature and then stopped by the addition of 15 μL sample^{-1} of 5× Laemmli buffer (50 mM Tris pH 6.8, 5% SDS, 5% beta-mercaptoethanol, 40% glycerol) and heating at 100 °C for 10 min. Denaturated proteins were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences). Membranes were then incubated for 1 h in blocking buffer (3% bovine serum albumin, BSA, fraction V, Sigma) prepared in TBST (50 mM Tris pH 8.0, 140 mM NaCl, 0.05% Tween-20) followed by incubation with horseradish peroxidase conjugated to avidin D in blocking buffer (HRP-avidin, Vector, France). After extensive washes in TBST, labelled proteins were revealed using the ECL system according to the manufacturer's instructions (Immobilon Western, Millipore, France).

Affinity purification of biotinylated GlcNAz-modified proteins

Cytosolic proteins from MCF-7 cells cultured for 24 h with or without 250 μM GlcNAz were submitted to affinity purification on streptavidin beads (Novagen) after click chemistry reaction which was stopped by chloroform/methanol precipitation. After centrifugation, the protein pellet was resuspended in the equilibration buffer (PBS with 1% SDS, 150 mM NaCl, 1 mM DTT and protease inhibitors cocktail), heated at 80 °C and sonicated to solubilise proteins. After centrifugation (to clarify the protein solution), sample was incubated on streptavidin beads for 2 h at room temperature. The flow-through was discarded, beads were washed successively in equilibration buffer, PBS, 8 M HCl guanidium pH 3.5, and labelled proteins were eluted with the elution buffer (8 M urea, 2% SDS, 30 mM biotin in PBS) by vortexing the beads vigorously for 15 min at room temperature followed by incubation for 15 min at 96 °C [14]. For 10% SDS-PAGE separation, 5× Laemmli buffer was added into the eluted fractions. After migration, the gel was silver-stained.

Identification of proteins by nano-HPLC–tandem mass spectrometry

Bands of interest were cut and submitted to in-gel trypsin digestion overnight at 37 °C in 25 mM ammonium bicarbonate buffer (Sequencing modified grade trypsin, Promega, France). Tryptic peptides were sequentially extracted with 45% acetonitrile/45% water/10% trifluoroacetic acid (TFA) (v/v/v) and 95% acetonitrile/5% TFA. The peptide samples were then dried in a speed-vac, dissolved in 5 μL water/ 5%



Scheme 1 1,3-Dipolar cycloaddition (click chemistry) between a modified GlcNAc analogue and the appropriate biotinylated probe. Synthesized molecules used for the click-chemistry-based detection of proteins modified by *O*-linked GlcNAc are **1** GlcNAz, **2** peracetylated GlcNAz, **3** GlcNAk, **4** peracetylated GlcNAk, **5** azido-biotin probe, **6** alkyne-biotin probe

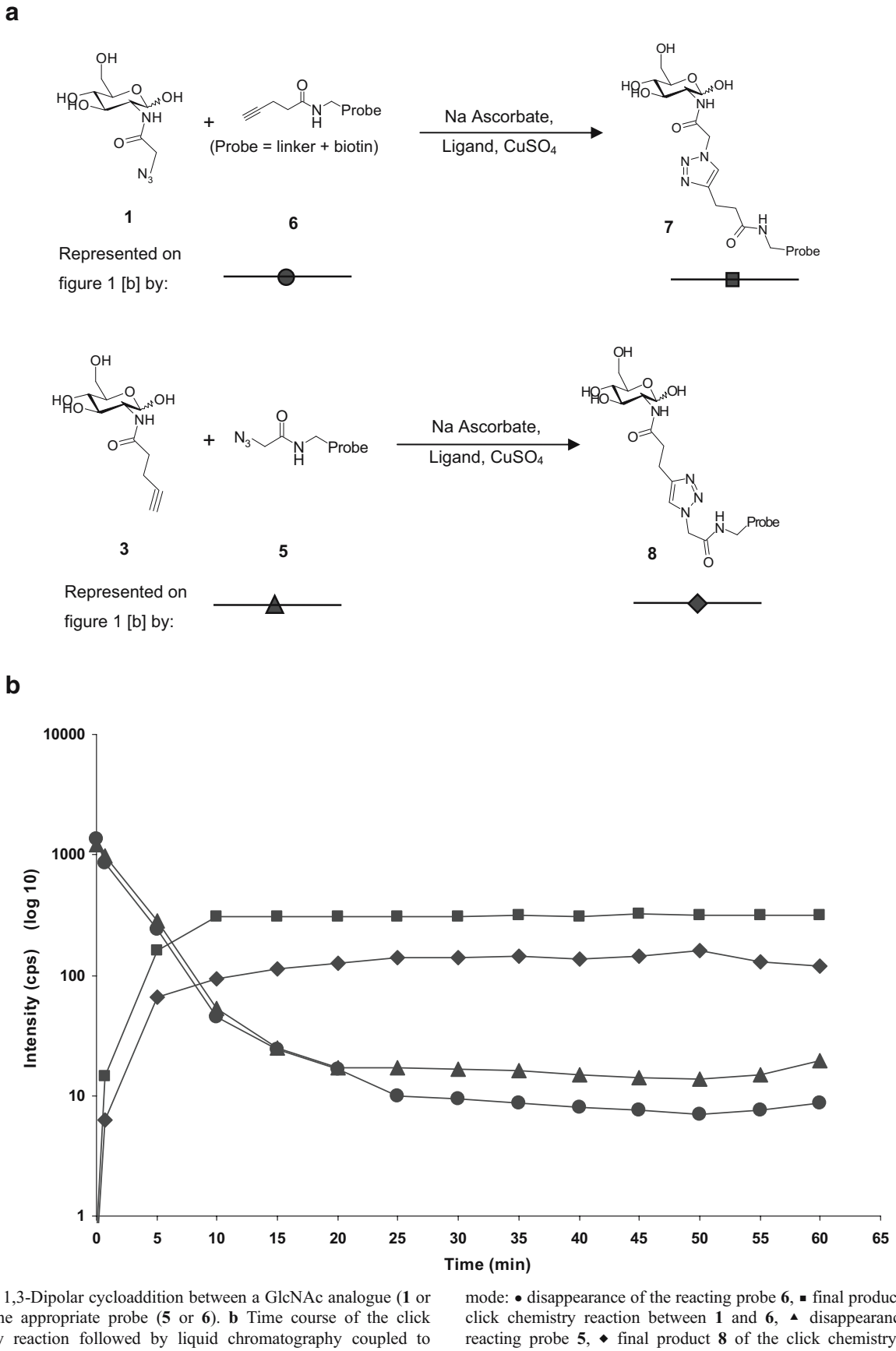


Fig. 1 **a** 1,3-Dipolar cycloaddition between a GlcNAc analogue (**1** or **3**) and the appropriate probe (**5** or **6**). **b** Time course of the click chemistry reaction followed by liquid chromatography coupled to electrospray mass spectrometry operating in single ion monitoring mode: • disappearance of the reacting probe **6**, ▪ final product **7** of the click chemistry reaction between **1** and **6**, ▲ disappearance of the reacting probe **5**, ◆ final product **8** of the click chemistry reaction between **3** and **5**

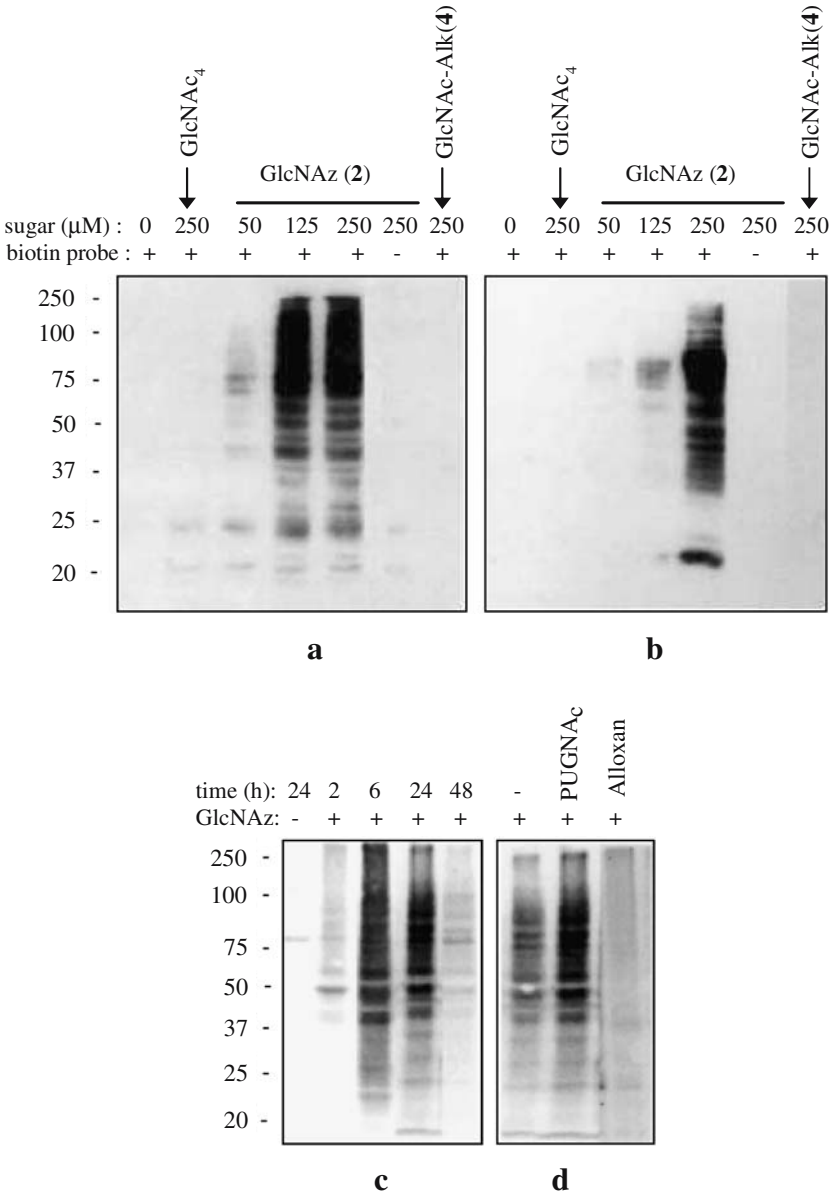


Fig. 2 Detection of metabolically labelled *O*-GlcNAz proteins in MCF-7 cells. **a** Cytosolic proteins and **b** nuclear proteins extracted from cells cultured for 24 h in the presence of increasing concentrations of peracetylated analogue of GlcNAc were conjugated to the corresponding biotinylated probe. Peracetylated GlcNAc ($GlcNAc_4$, 250 μM) was used as a control. The resulting tagged proteins were separated by SDS-PAGE and detected by western blotting using the HRP-conjugated avidin. **c** For the time course experiment, cells were labelled with 250 μM GlcNAz for 2–48 h, followed by conjugation with the alkynyl-biotinylated probe on the cytoplasmic fractions. Protein detection was performed as described above. **d** PUGNAc (100 μM) or alloxan (5 mM) were added respectively in the last 2 h and 20 h of the labelling period (24 h) in the presence of 250 μM GlcNAz. Labelled proteins were detected as previously described. Molecular masses of prestained protein standards are indicated in kDa

HCOOH (v/v) and submitted to nano-HPLC–tandem mass spectrometry analysis.

Nano-HPLC-nanoESI-MS/MS analyses were performed on an ion trap mass spectrometer (LCQ Deca XP^+, Thermoelectron, San Jose, CA) equipped with a nano-electrospray ion source coupled to a nano flow high-pressure liquid chromatography system (LC Packings Dionex, Amsterdam, The Netherlands). Peptides were loaded in buffer A (95% water/5% acetonitrile/0.08% HCOOH (v/v/v)) onto a C_{18} HPLC column (15-cm length, 75-μm ID, C18 Pepmap column, Dionex) and were eluted from the column with a linear gradient from 5 to 100% solvent B (20% water/80% acetonitrile/0.08% HCOOH (v/v/v)) in buffer A for 45 min. The eluted peptides were directly electrosprayed into the LCQ mass spectrometer operating in a data-dependent mode. Fragment ion spectra were searched against the NCBInr Homo sapiens database using the Mascot software (Matrix Science, London, UK). The search parameters were 1.5-Da

Table 1 *O*-GlcNAz-labelled proteins identified by the click-chemistry-based strategy

Name of identified proteins	NCBI accession number	Theoritical MW (Da)	Mascot score	Number of peptides	% covered	Biological function (GO)	Previously reported
Protein synthesis							
Elongation factor 1 alpha 1	NP_001393	50,451	72	1	2	Translational elongation	[19]
Eukaryotic translation elongation factor 2	NP_001952	96,246	703	13	14	Protein biosynthesis	[19]
Ribosomal protein L8	NP_000964	28,235	59	1	6	Protein biosynthesis	
Ribosomal protein L17	NP_000976	21,611	117	2	13	Protein biosynthesis	
Ribosomal protein L21	AAA80462	17,703	82	1	9	Protein biosynthesis	
Ribosomal protein S2	NP_002943	31,590	42	1	3	Protein biosynthesis	
Ribosomal protein S8	NP_001003	24,475	76	1	6	Protein biosynthesis	
Signal transduction							
14–3–3, zeta	NP_003397	27,899	173	3	13	Anti-apoptosis signalling	
14–3–3, theta	NP_006817	28,032	100	2	8	Control of cell cycle progression	
Annexin A2 isoform 2	NP_004030	38,808	68	1	2	Phospholipase inhibitor activity	
Protein folding							
Chaperonin containing TCP1, subunit 2	NP_006422	57,794	64	1	2	Unfolded protein binding	
Heat shock protein 27	AAA62175	22,427	42	1	8	Anti-apoptosis, cell motility, regulation of translational initiation, response to unfolded protein	[19]
Heat shock-induced protein (HSP70–1)	AAA63226	70,294	54	1	2	Anti-apoptosis, mRNA catabolic process, response to unfolded protein	[19]
Heat shock protein	AAD11466	70,237	52	1	2	ND	[19]
Cytoskeleton/structural proteins							
Actin,alpha	AAA51577	42,480	52	1	4	Cell motility	
Actin, beta	NP_001092	42,052	198	5	13	Cell motility	[19]
Cytokeratin 18	NP_000215	48,029	161	3	7	Anatomical structure morphogenesis, negative regulation of apoptosis	[23]
Filamin A, alpha	NP_001447	282,581	165	2	1	Actin cytoskeleton organization and biogenesis	[19]
Tubulin, alpha	CAA25855	50,810	135	4	12	Cell motility	[24]
Tubulin, beta	AAH20946	50,096	187	3	9	Cell motility	
Tubulin, beta 2	NP_006079	50,255	174	3	8	Cell motility	[19]
Metabolism							
Alpha-enolase	NP_001419	47,421	61	1	2	Glycolysis, negative regulation of cell growth and transcription	[19, 25]
C-1-tetrahydrofolate synthase	P11586	102,180	245	4	4	Methenyltetrahydrofolate cyclohydrolase activity	[19]
Fatty acid synthase	NP_004095	275,877	337	7	4	Fatty acid metabolic process	
Glutathione transferase M3	AAA60964	27,127	101	3	12	Glutathione transferase activity, response to estrogen stimulus	[19]
Glucose-6-phosphate dehydrogenase	CAA39089	59,708	105	1	3	Glucose 6-phosphate utilization, pentose-phosphate shunt	[19]

Glyceraldehyde-3-phosphate dehydrogenase	CAA25833	36,202	362	8	24	Glycolysis	[19, 25]
M2-type pyruvate kinase	1T5A_A	62,570	124	3	9	Glycolysis	[19]
Proteasome beta 5 subunit	NP_002788	28,633	78	1	5	Ubiquitin-dependent protein catabolism	
Solute carrier family 7 member 5	NP_003477	55,659	71	1	3	Neutral amino acid transport	
Others							
Cellular apoptosis susceptibility protein	AAC50367	111,108	44	1	2	ND	[19]
Clathrin heavy chain 1	NP_004850	193,260	331	5	3	Intracellular protein transport	[19]
Heterogeneous nuclear ribonucleoprotein K	CAI16019	47,756	65	2	6	RNA splicing	[19]

ND not determined
Metabolically *O*-GlcNAz-labelled proteins from MCF-7 cells were purified on a streptavidin column. Eluted proteins were separated by SDS-PAGE and nano-LC-MS/MS analyses were performed after trypsin digestion as described in the Materials and methods section. Biological functions of the identified proteins are indicated according to gene ontologies (GO) obtained from the UniProtKB accession number on the Expasy server (www.expasy.org). References in which the *O*-GlcNAc-modified proteins were first reported are indicated in the last column

tolerance for the parent ion mass and 0.8 Da for the MS/MS fragment ions, one missed cleavage allowed, carbamidomethylcysteine as fixed modification, and methionine oxidation as possible modification. Only the proteins with a significant Mascot score (>41) were considered and reported after manual verification of the fragmentation spectra.

Results and discussion

Herein, we report a click-chemistry-based strategy devoted to the purification and identification of proteins modified by the post-translational *O*-GlcNAc glycosylation. For this purpose, two analogues of *N*-acetylglucosamine i.e. the azido (GlcNAz) (compounds **1** and **2**) and the alkyne (GlcNAk) analogues (compounds **3** and **4**), and their corresponding reactive biotinylated probes bearing respectively an alkyne or an azido group (compounds **5** and **6**) were synthesized (Scheme 1). The reactivity of the two pairs of reacting partners was assessed by the time course of the click chemistry cycloaddition which was followed in vitro by reversed-phase liquid chromatography coupled to electrospray ionisation mass spectrometry. The relative signal intensities of the following ions were monitored in single ion monitoring: $[M+H]^+$ and $[M+Na]^+$ corresponding to the biotinylated probes (**5** and **6**) (respectively at *m*/*z* 457.6, 479.3, 511.3, 533.2) and to the formed triazole-containing cycloadducts (compounds **8** and **7**) (respectively at *m*/*z* 717.4, 739.4, 773.4, 795.4). The signals corresponding to the GlcNAc analogues (**1** or **3**) were not monitored since both compounds eluted in the column void volume and were consequently subjected to ion suppression phenomena. The GlcNAc analogue was mixed in an equimolar ratio with the biotinylated probe, followed by 0.2 equivalents of tris (triazolyl)amine (ligand), 0.2 equivalents of sodium ascorbate and 0.1 equivalents of $CuSO_4$ as catalytic reagents [7, 8, 15–17]. Under these experimental conditions, the cycloaddition reaction proceeded to apparent completion in less than 20 min for both pairs of reacting partners (Fig. 1). Indeed, adding $CuSO_4$ and/or ascorbate after 30 min of reaction did not result in any further evolution of the reaction mixture (data not shown). These results suggest that both pairs could be appropriate for the proteomic investigation of proteins modified by *O*-GlcNAc.

In order to evaluate the ability of the click chemistry reaction to specifically detect the nucleocytoplasmic fraction of glycosylated proteins, breast cancer MCF-7 cells were incubated for 24 h either with GlcNAz (**2**) or GlcNAk (**4**) at several concentrations (50, 125, 250 μM). A low-glucose-containing culture medium (2 g L^{-1}) was used to favour incorporation of the modified GlcNAc into the proteins. The GlcNAz (**2**) has been previously demonstrated to be a substrate of the hexosamine salvage pathway and

to be subsequently incorporated into proteins [6, 18]. The coupling between the azido compound and the alkyne-biotinylated probe (**6**) was thus tested first. After cell lysis, the cycloaddition reaction was carried out both on the cytosolic and nuclear fractions obtained from MCF-7 cells. Proteins were then separated by 10% SDS-PAGE. Figures 2a and b show the western blot analysis of these fractions when cells were cultured either with peracetylated GlcNAc (control), peracetylated GlcNAz (**2**) or peracetylated GlcNAk (**4**). As illustrated, the GlcNAz-modified proteins can be selectively detected after conjugation with the alkynyl-biotinylated probe (**6**) and western blot detection by avidin peroxidase. Indeed, no chemiluminescence signal was observed when cycloaddition was performed with the same biotinylated probe on protein extracts from cells cultured with peracetylated GlcNAc. This feature underlines the lack of cross reactivity between the alkyne group and the chemical diversity of the amino acid side chains of proteins. Similarly, omitting the alkyne probe during the click chemistry step fully abolished the chemiluminescent signal despite the presence of *O*-linked GlcNAz proteins. This observation highlights the specificity of the HRP-avidin detection towards the biotinylated probe. No signal was observed with the other reacting pair i.e. GlcNAk (**4**) and the azido probe (**5**). Since we have previously shown in Fig. 1 that the two pairs of reacting partners exhibit similar reactivity, we can postulate that the alkyne analogue of GlcNAc is not metabolized by the MCF-7 cells. It is unlikely that *O*-linked GlcNAk does not react with the azido probe since it has been recently shown that an alkyne analogue of fucose allowed in vivo fluorescence imaging of glycans based on a click chemistry reaction [19].

For both the cytosolic and nuclear subcellular fractions, a concentration of 250 μM of GlcNAz was optimum to get an intense and specific signal, without affecting the cellular viability compared with control cells (i.e. culture medium with or without peracetylated GlcNAc). Moreover, a time course of GlcNAz incorporation into cellular proteins was performed and revealed that this GlcNAc analogue is metabolized in MCF-7 cells as rapidly as with 2 h, with the most intense signal obtained after 24 h of incorporation (Fig. 2c). After 48 h of incorporation, a decrease of chemiluminescence intensity was noted, which may reflect the natural turnover of proteins during this time and the possible degradation of the GlcNAz in the culture medium at 37 °C. *O*-GlcNAc dynamics are controlled by the interplay between *O*-GlcNAc transferase and *O*-GlcNAcase. Moreover it has been demonstrated that both enzymes tolerate the azido group in their respective catalytic activity [6]. The effect of alloxan, an inhibitor of *O*-GlcNAc transferase and *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenyl carbamate (PUGNAc), an *O*-GlcNAcase inhibitor [20], were both tested towards the incorporation rate of GlcNAz in MCF-7 cells. Western blotting (Fig. 2d) shows that the addition of PUGNAc in the last 2 h of the 24-h incorporation significantly increases the detection of *O*-GlcNAz-modified proteins, indicating that *O*-GlcNAz-labelled proteins can be a substrate for the *O*-GlcNAcase. In contrast, the lack of signal in the presence of alloxan indicates that the functionality of OGT is required for the metabolic incorporation of the azido analogue in living cells.

The demonstration that proteins bearing *O*-GlcNAz moieties are specifically detected by western blotting opens the way for affinity purification on a streptavidin column for their subsequent identification by mass spectrometry. A cytosolic protein extract treated with GlcNAz for 24 h was purified on streptavidin beads after tagging with the alkyne probe. In order to limit the nonspecific binding, the streptavidin affinity column was preferred to monomeric avidin as it exhibits a stronger affinity (K_d of 10^{-15} versus 10^{-7} M) therefore permitting harsh washes before the elution step [14].

The protein fractions specifically retained by streptavidin were separated by SDS-PAGE. After silver-staining, the protein bands were subjected to trypsin digestion and the resulting peptide mixtures were analysed by nano-LC–tandem mass spectrometry. Protein identification was performed using the Mascot search engine against the NCBInr protein database which allowed the identification of 32 proteins (Table 1). Control experiments towards affinity purification were carried out in parallel in order to eliminate the possible contaminants resulting from residual unspecific interaction. As a control, cytosolic proteins from MCF-7 cells treated with peracetylated GlcNAc (and tagging with the alkyne-biotinylated probe) or GlcNAz (without tagging with the alkyne-biotinylated probe) were subjected to similar affinity chromatography. Two proteins were detected in all purified eluted fractions: they were identified as fatty acid synthase (accession number NP_004095), a multienzymatic complex involved in fatty acid biosynthesis, and pyruvate carboxylase which is involved in gluconeogenesis (accession number AAB31500). They are both biotin-dependent enzymes [21, 22], that might explain why they are present in the eluted fraction of control samples. Nonetheless, we cannot exclude that they might also be OGT targets such as many metabolic enzymes which have been reported already.

As reported in Table 1 the 32 identified proteins have been classified according to their cellular function i.e. protein synthesis (7), signal transduction (3), protein folding (4), cytoskeletal and structural proteins (7), metabolism (8) and other functions (3). This classification reflects the wide regulatory role of this nucleocytoplasmic post-translational glycosylation and underlines the necessity to dispose of reliable and robust tools to study the dynamics of *O*-GlcNAc modification. Among these proteins, 18 have already been reported as *O*-GlcNAc-modified proteins, using either the

tagging via substrate strategy (Staudinger ligation) or usual biochemical techniques based on ^{3}H-Gal transfer or immunoprecipitation/immunorevelation [19, 23–26]. Fourteen proteins are new identified targets of OGT, 11 of which belong to protein families from whom some members are already known to be *O*-glycosylated. This is the case, for instance, for the proteins of the 14–3–3 family (14–3–3 zeta and theta), the ribosomal proteins (S2 and L8) or TCP1 chaperons [23, 27].

Conclusion

The present work demonstrates that the nucleocytoplasmic cellular proteins bearing *O*-linked GlcNAc residues are able to be specifically detected using a click-chemistry-based strategy that constitutes an alternative to the commonly used immunodetection with monoclonal antibodies. In our hands, only the azido analogue of GlcNAc seems to be efficiently metabolized by the enzymatic pathway driving this post-translational modification. Furthermore, after specific purification on a streptavidin column and subsequent analysis by LC coupling to tandem mass spectrometry we identified 32 *O*-GlcNAc-substituted proteins among which 14 were not previously reported to be a target of OGT. In the meantime, a commercial kit based on a similar click chemistry reaction (ClickIT™ by Invitrogen) has been proposed for western blot analysis of such glycosylated proteins. Nevertheless, the structure and accurate mass of the commercial biotinylated probe has up to now been unknown, thus preventing identification of the peptides modified by an *O*-GlcNAc residue in the database. Considering the biology community's growing interest in deciphering and understanding the cellular events governed by this dynamic glycosylation, we believe that it is of great interest to provide access to easily synthesised and cheaper reagents than those commercially available.

The accurate localization of the *O*-GlcNAc modification site is still challenging. We are currently developing a chemical alternative to the streptavidin affinity purification technique based on covalent tagging of *O*-GlcNAc-modified peptides. Enriching and selecting only the modified peptides would thus facilitate not only the localization of the *O*-GlcNAc on the peptide sequence but also the dynamics of this post-translational modification at each site.

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