Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 369 (2008) 539–546
BBRC
www.elsevier.com/locate/ybbrc

Microinjection of recombinant *O*-GlcNAc transferase potentiates *Xenopus* oocytes M-phase entry

Vanessa Dehennaut [a,b], Xavier Hanoulle [a], Jean-François Bodart [b], Jean-Pierre Vilain [b], Jean-Claude Michalski [a], Isabelle Landrieu [a], Guy Lippens [a], Tony Lefebvre [a,*]

[a] *UMR-CNRS 8576, UGSF, USTL, IFR 147, 59655 Villeneuve d'Ascq, France*
[b] *EA 4020, Laboratoire de Régulation des Signaux de Division, USTL, IFR147, Villeneuve d'Ascq, France*

Received 12 February 2008
Available online 25 February 2008

Abstract

In order to understand the importance of the cytosolic and nuclear-specific *O*-linked *N*-acetylglucosaminylation (*O*-GlcNAc) on cell cycle regulation, we recently reported that inhibition of *O*-GlcNAc transferase (OGT) delayed or blocked *Xenopus laevis* oocyte germinal vesicle breakdown (GVBD). Here, we show that increased levels of the long OGT isoform (ncOGT) accelerate *X. laevis* oocyte GVBD. A N-terminally truncated isoform (sOGT) with a similar *in vitro* catalytic activity towards a synthetic CKII-derived peptide had no effect, illustrating the important role played by the N-terminal tetratrico-peptide repeats. ncOGT microinjection in the oocytes increases both the speed and extent of *O*-GlcNAc addition, leads to a quicker activation of the MPF and MAPK pathways and finally results in a faster GVBD. Microinjection of anti-OGT antibodies leads to a delay of the GVBD kinetics. Our results hence demonstrate that OGT is a key molecule for the timely progression of the cell cycle.

Keywords: OGT; *O*-GlcNAc; *Xenopus laevis*; Oocyte; Cell cycle

O-GlcNAc is a dynamic post-translational modification (PTM) abundantly found in metazoans (for review see [1]). *O*-GlcNAc addition and removal are, respectively, catalyzed by two enzymes, the *O*-GlcNAc transferase (OGT) and the *O*-GlcNAcase. Although three different OGT isoforms have been described [2], their number remains largely inferior to the number of kinases that promote the phosphate moiety addition on Ser, Thr or Tyr hydroxyl functions. Study of *O*-GlcNAc began in the middle of the eighties, leading to a total of over 400 *O*-GlcNAc-bearing proteins presently identified. Nevertheless, despite the yin–yang model describing the reciprocal nature of *O*-GlcNAc addition and phosphorylation, the exact functions performed by *O*-GlcNAc remain elusive.

An involvement of this modification in cell cycle progression was suggested two decades ago [3] and was recently further detailed [4]. Different chemical inhibitors of both molecular players—alloxan [5] and a benzoxazolinone derivative [6] for OGT and PUGNAc [7] for *O*-GlcNAcase—have been described. Incubation of *Xenopus laevis* oocytes with them allowed to establish a direct link between *O*-GlcNAc and cell cycle control [8]. These studies demonstrated that OGT was involved in oocyte maturation and that it affects activation of both p42MAPK and MPF (Cdk1-CyclinB) pathways induced by hormonal stimulation. In *Xenopus* oocytes or egg extracts it was also shown that microinjection of a galactosyltransferase and the resulting elongation of GlcNAc residues with galactose, inhibited M-phase entry and blocked S to M-phase transition [9]. Altogether, these results strongly suggest the pivotal role of the *O*-GlcNAc modification in the control of cell cycle progression.

* Corresponding author. Fax: +33 3 20 43 65 55.
E-mail address: tony.lefebvre@univ-lille1.fr (T. Lefebvre).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.02.063

In the present report, we further exploit the possibility to microinject recombinant proteins into oocytes to detail the relationship between cell cycle and *O*-GlcNAc. Microinjection of the OGT long isoform into immature oocytes was found to accelerate progesterone-induced GVBD kinetics. This effect critically depends on the presence of the full-length N-terminal TPRs, as a shorter isoform devoid of these repeats had no significant effect. OGT neutralization through the use of anti-OGT antibodies corroborated these observations, since a concomitant decrease in OGT activity was found to delay the GVBD/M-phase entry.

Materials and methods

Expression and purification of sOGT and ncOGT. Plasmids for the recombinant production of human sOGT and ncOGT in *Escherichia coli* were obtained from the laboratory of S. Walker (Harvard Medical School). The expression and the first step of purification were done as previously described [6]. Following the Ni^{2+}-NTA IMAC purification, recombinant enzymes were purified by gel filtration chromatography using a Superdex200 prep grade column equilibrated in 20 mM KH_2PO_4/K_2HPO_4, 150 mM NaCl, 1 mM EDTA, pH 7.5. Fractions from the size exclusion chromatography were selected based on SDS–PAGE analysis, pooled, supplemented with 0.5 mM Tris(hydroxypropyl)phosphine (THP) and stored at −80 °C. Starting from 1.5 L cultures and following the purification scheme described above we obtained 12 mg of pure sOGT and 4.9 mg of ncOGT (Fig. 1A).

In vitro activity of sOGT and ncOGT. The enzymatic activity of the recombinant sOGT and ncOGT was assessed on a peptide substrate (Pep-CKII: KKKYPGGSTPVSSANMM) derived from Casein kinase-II which is a known natural substrate for OGT [6]. The reaction mixtures contained 200 μM Pep-CKII, 500 μM UDP-GlcNAc, 12.5 mM $MgCl_2$ in buffer 50 mM KH_2PO_4/K_2HPO_4, 150 mM NaCl, 1 mM EDTA, 0.5 mM THP, pH 7.5. The assays were done in a final volume of 50 μL, with 2 μM of either sOGT or ncOGT for 45 min at 37 °C. The reactions were stopped with TFA and stored at −80 °C until analyzed.

The reaction mixtures were analyzed by RP-HPLC on a μRPC C2/C18 ST 4.6/100 column (GE Healthcare) with a TFA-acetonitrile system buffer then by mass spectrometry on a MALDI-TOF (Applied Biosystem, Voyager DE Pro). The addition of *O*-GlcNAc induces a different behaviour of the Pep-CKII on the reverse phase column. Indeed, the modified peptide elutes earlier (16.15 mL) compared to the non-modified one (16.80 mL), enabling a rapid quantification of the degree of modification on the peptide substrate. The *in vitro* enzymatic addition of *O*-GlcNAc on the peptide Pep-CKII was almost quantitative with both sOGT and ncOGT (Fig. 1B). MALDI-TOF analyses of the RP-HPLC peaks confirmed that the modification corresponds to the addition of a single *O*-GlcNAc residue per peptide. The modified peptide was further analyzed by NMR spectroscopy. Spectra were recorded on 200 μM samples in buffer 100 mM NaH_2PO_4/Na_2HPO_4, 10 mM NaCl, 1 mM EDTA, pH 6.1, on a Bruker Avance 600 MHz spectrometer equipped with a cryogenic triple resonance probe head, at 25 °C (Bruker, Karslruhe, Germany). The proton chemical shifts were referenced using the methyl signal of TMSP (sodium 3-trimethyl-silyl-[2,2,3,3-d.4]propionate) at 0 ppm. The spectra were processed with the Bruker TOPSPIN software package.

Handling of oocytes. After anesthetizing *Xenopus* females (purchased from the University of Rennes I, France) by immersion in 1 g L^{-1} MS222 solution (tricaine methane sulfonate; Sandoz), ovarian lobes were surgically removed and placed in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes–NaOH, pH 7.5). Fully grown stage VI oocytes were isolated and follicle cells were partially removed by 1 mg mL^{-1} collagenase A (Roche Applied Science) treatment for 30 min followed by a manual microdissection. Oocytes were stored at 14 °C in ND96 medium until experiments. Meiotic resumption was induced by incubating oocytes at 19 °C in ND96 medium containing 10 μM of progesterone (Sigma–Aldrich). GVBD which reflects oocyte entry into the maturation process was scored by the appearance of a white spot at the animal pole of the oocyte.

OGT microinjection. Immature oocytes were microinjected using a positive displacement digital micropipette (Nichiryo, Tokyo, Japan) either with 50 nL of injection-buffer alone (50 mM KH_2PO_4/K_2HPO_4 pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM THP), or with 50 nL of injection-buffer containing 0.57 mg mL^{-1} sOGT or 0.27 mg mL^{-1} ncOGT. Before stimulation of meiotic resumption, oocytes were kept in ND96 at 14 °C for 1–2 h to allow them to heal. For each time-point, 5–10 oocytes were taken respecting the white spot ratio and stored at −20 °C until further biochemical analysis.

Anti-OGT antibody microinjection. Immature oocytes were microinjected either with 40 nL of anti-OGT antibody (DM17; Sigma–Aldrich) or with 100 nL of rabbit control IgG (Santa Cruz Biotechnologies). Before stimulation of meiotic resumption, oocytes were kept in ND96 at 14 °C overnight. For each time-point, 5–10 oocytes were taken respecting the white spot ratio and stored at −20 °C until further biochemical analysis.

SDS–PAGE and western blotting. Proteins (the equivalent of one oocyte was loaded per lane) were run on a 17.5% modified SDS–PAGE [8]—this kind of gel allows a better discrimination between protein isoforms of phosphorylation- and electroblotted onto nitrocellulose sheet. Blots were saturated with 5% (w/v) non-fatty milk in TBS (Tris-buffered saline)-Tween (15 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween, pH 7,5) for 45 min. Primary antibodies were incubated overnight at 4 °C. Mouse monoclonal anti-*O*-GlcNAc (RL-2; Affinity Bioreagents), mouse monoclonal anti-Erk2 (D-2; Santa Cruz Biotechnologies), rabbit polyclonal anti-β-catenin (H-102; Santa Cruz Biotechnologies), rabbit polyclonal anti-cyclin B2 (generously provided by Dr. John Gannon from the ICRF, South Mimms, UK) were used at a dilution of 1:1000. Rabbit polyclonal anti-actin (I-19; Santa Cruz Biotechnologies) antibody was used at a dilution of 1:10,000. Nitrocellulose membranes were washed in TBS-Tween and incubated with either an anti-mouse horseradish peroxidase-labeled secondary antibody or an anti-rabbit horseradish peroxidase-labeled secondary antibody (GE healthcare) at a dilution of 1:10,000. Finally, membranes washes were performed with TBS-Tween and the detection was carried out with enhanced chemiluminescence (GE Healthcare).

Results and discussion

Recombinant sOGT and ncOGT are both active on a casein kinase II-derived peptide

To date, three OGT isoforms have been described [2]: two nucleocytoplasmic forms, namely ncOGT (nucleocytoplasmic OGT) and sOGT (short OGT), and a mitochondrial form, mOGT (mitochondrial OGT). These three isoforms exhibit an identical catalytic domain localised in the second half of the protein but differ in the N-terminal part and more particularly in the number of tetratrico-peptide repeats (TPR) they contain. The TPR motif is a stretch of thirty four amino-acids that is found in numerous protein interacting domains: ncOGT possesses 12 TPR, sOGT only 2 and mOGT possesses 9 repeats in addition to a mitochondrial target sequence.

We recombinantly produced human sOGT and ncOGT in *E. coli*, and purified the resulting enzymes to purity, as assessed by SDS–PAGE (Fig. 1A). *In vitro* enzymatic activities for sOGT and ncOGT were tested using a CKII-derived peptide. After 45 min of incubation with a catalytic amount of enzyme (enzyme:substrate=1:100), *O*-GlcNAc