Journal of Cellular Biochemistry 93:999–1010 (2004)

Modulation of O-GlcNAc Glycosylation During *Xenopus* Oocyte Maturation

Tony Lefebvre,[1]* Frédéric Baert,[2] Jean-François Bodart,[2] Stéphane Flament,[3] Jean-Claude Michalski,[1] and Jean-Pierre Vilain[2]

[1]Unité Mixte de Recherches 8576 du CNRS, Glycobiologie Structurale et Fonctionnelle, IFR 118, USTL, Bâtiment C9, 59655 Villeneuve d'Ascq, France
[2]UPRES-EA, 1033, Laboratoire de Biologie du Développement, IFR 118, USTL, Bâtiment SN3, 59655 Villeneuve d'Ascq, France
[3]UPRES-EA, 3442, Génétique, Signalisation, Différenciation, Faculté des sciences, Université de Nancy I, BP 239, 54506 Vandoeuvre-lès-Nancy cedex, France

Abstract O-linked *N*-acetylglucosamine (O-GlcNAc) glycosylation is a post-translational modification, which is believed antagonises phosphorylation. We have studied the O-GlcNAc level during *Xenopus* oocyte meiotic resumption, taking advantage of the high synchrony of this model which is dependent upon a burst of phosphorylation. Stimulation of immature stage VI oocytes using progesterone was followed by a 4.51 ± 0.32 fold increase in the GlcNAc content, concomitantly to an increase in phosphorylation, notably on two cytoplasmic proteins of 66 and 97 kDa. The increase of O-GlcNAc for the 97 kDa protein, which we identified as β-catenin was partly related to its accumulation during maturation, as was demonstrated by the use of the protein synthesis inhibitor—cycloheximide. Microinjection of free GlcNAc, which inhibits O-glycosylated proteins–lectins interactions, delayed the progesterone-induced maturation without affecting the O-GlcNAc content. Our results suggest that O-GlcNAc glycosylation could regulate protein–protein interactions required for the cell cycle kinetic. J. Cell. Biochem. 93: 999–1010, 2004.

Key words: oocyte; *Xenopus*; maturation; O-GlcNAc; beta-catenin

O-linked *N*-acetylglucosamine (O-GlcNAc) glycosylation is the major glycosylation type found within the nucleus and the cytosolic compartment of eukaryota [for review, see Wells et al., 2001]. O-GlcNAc consists in the addition of a single residue of *N*-acetylglucosamine to the hydroxyl groups of serine and threonine. This type of glycosylation shares similar features with protein phosphorylation [Haltiwanger et al., 1997], and the occurrence of an antagonism between phosphorylation and O-GlcNAc glycosylation was demonstrated. The existence of a direct competition between O-GlcNAc and phosphate to occupy the same sites on proteins was reported [Haltiwanger et al., 1998; Griffith and Schmitz, 1999; Lefebvre et al., 1999]. The emergence of an O-GlcNAc/phosphorylation balance leads to the question of its biological significance: if the role of phosphorylation is well documented and generally contributes to the regulation of the protein activity, the particular role of O-GlcNAc glycosylation is still not well understood. For example, whereas the reciprocity between O-GlcNAc and O-Phosphate on the carboxyl terminal domain (CTD) of RNA polymerase II was studied in detail, the phosphorylation of the CTD is associated with

Abbreviations used: O-GlcNAc, O-linked *N*-acetylglucosamine; GVBD, germinal vesicle breakdown; PNGase F, peptide *N*-glycosidase F; WGA, wheat germ agglutinin; PNA, peanut agglutinin; TBS, tris buffered saline; BSA, bovine serum albumin; AMP, adenosine 5′-monophosphate; MPF, maturation promoting factor; MAPK, mitogen activated protein kinase; CHX, cycloheximide.

Tony Lefebvre and Frédéric Baert are co-authors.

Grant sponsor: Genopole of Lille; Grant sponsor: Centre National de la Recherche Scientifique; Grant sponsor: Université des Sciences et Technologies de Lille; Grant sponsor: Comité du Nord de la Ligue contre le Cancer.

*Correspondence to: Tony Lefebvre, UGSF/CNRS, UMR 8576, cité scientifique, 59655 Villeneuve d'Ascq, France.
E-mail: tony.lefebvre@univ-lille1.fr

Received 28 August 2002; Accepted 11 June 2004
DOI 10.1002/jcb.20242

promoter clearance, stabilization of elongation complexes and with the involvement of the mRNA processing machinery—the function of O-GlcNAc glycosylation of the CTD remains speculative [Comer and Hart, 2001].

Changes in the level of glycosylation in several nuclear and cytoplasmic proteins were observed during the mitogenic activation of both T-lymphocytes and T-cell hybridoma. In T-lymphocytes, O-GlcNAc levels change rapidly after stimulation to enter the cell cycle [Kearse and Hart, 1991]. In the human colon cell line HT 29, mitotic arrest with either okadaic acid or the microtubule destabilizing agent nocodazole, causes an increase in the O-GlcNAc levels of keratins [Haltiwanger and Philipsberg, 1997]. These observations suggest that O-GlcNAc glycosylation might be involved in the cell cycle regulation, while no other data corroborated this assumption. Many of the nuclear pore complexes, which are modified by single series of O-GlcNAc, are phosphorylated in a cell cycle specific manner, though the levels of O-GlcNAc remain constant [Miller et al., 1999]. Other experiments were led to investigate into the role of O-GlcNAc during the cell division process through the microinjection of galactosyltransferase (GalT) into *Xenopus* oocytes arrested in prophase I. However, injected GalT was toxic for oocytes stimulated to resume meiosis [Fang and Miller, 2001].

Xenopus oocyte offers opportunities to study the M-phase regulation that is highly controlled by specific kinases and phosphatases. Full-grown *Xenopus* stage VI oocytes are arrested at the prophase of the first division of meiosis in a G_2-like state. In response to progesterone, oocytes undergo germinal vesicle breakdown (GVBD), chromosomes condensation, and spindle formation. The end of the first division of meiosis is attested by the extrusion of the first polar body. The second division resumes, but it is arrested at metaphase [Hausen and Riebesell, 1991]. This process, also called maturation, is characterized by the simultaneous activation of two pathways. On one hand, the M-phase Promoting Factor (MPF; p34Cdc2/Cyclin B) that regulates both the entry and exit of the M-phase is activated through the dephosphorylation of its catalytic subunit by a dual-specificity phosphatase Cdc25 [Masui, 2001]. On the other hand, the activation of the Mos/MEK/Xp42Mpk1 Mitogen Activated Protein Kinases (MAPK) cascade depends upon Mos accumulation; and the effects of this pathway are mainly mediated through p90Rsk [Gotoh and Nishida, 1995; Gross et al., 2000].

Since phosphorylation is well known as means to regulate the cell cycle progression and since O-GlcNAc could antagonize phosphorylation, we assumed that O-GlcNAc variations could also occur during the meiosis resumption and that O-glycosylated proteins–lectins interactions might influence the G_2/M transition in *Xenopus laevis* oocytes. Thus, we studied the O-GlcNAc level during *Xenopus* oocyte maturation, taking advantage of the high and natural synchrony of this model [Ferrell, 1999]. Moreover, we observed the O-GlcNAc glycosylation of several proteins during maturation. Among them, we identified β-catenin which is phosphorylated by the Glycogen synthase kinase-3 (GSK3) and lead to its degradation. In return, the stabilization of β-catenin through the inactivation of GSK3 is essential for the dorsal determination in *Xenopus* embryos [Heasman et al., 1994]. Moreover, we have shown that the microinjection of free GlcNAc delayed progesterone-induced maturation, thereby suggesting a role for O-GlcNAc mediated interaction in the cell cycle.

MATERIALS AND METHODS

Handling of Oocytes

Stage VI *Xenopus* oocytes were isolated and prepared as previously described [Flament et al., 1996]. Briefly, prophase I-arrested oocytes were obtained from animals that were not primed with any gonadotropins. Defolliculated oocytes were kept in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). Progesterone was added at a final concentration of 10 μM. Percentages of maturation were marked by the appearance of a white spot at the animal pole of the oocyte and GVBD was confirmed via microscopically examination after the dissection of heat-fixed oocytes. For biochemical analysis, oocytes were homogenized in β glycerophosphate, 60 mM; paranitrophenylphosphate, 15 mM; MOPS, 25 mM; EGTA, 15 mM; $MgCl_2$, 15 mM; DTT, 2 mM; sodium orthovanadate, 1 mM; NaF, 1 mM and proteases inhibitors; pH 7.2 [Flament et al., 1996] and centrifuged at 10,000 g for 10 min to eliminate yolk and membranous pellet. All the experiments were performed at least in duplicate.

Enucleation

Immature oocytes were pricked at the animal pole and placed in ND96 diluted four times. Under such conditions, the germinal vesicle that is located in the animal hemisphere went out of the cell. Enucleated oocytes were then replaced in normal ND96 for recovery, at least 1 h prior to treatment.

To check the efficiency of oocytes enucleation, we used an antibody raised against p62 (sc-1916, Santa Cruz Biotechnology, Santa Cruz, CA), a nuclear pore specific protein. After nitrocellulose saturation with milk, the antibody was used at a dilution of 1:1,000 overnight at 4°C, and the horseradish peroxidase-coupled anti-goat secondary antibody was used at a dilution of 1:2,000 for 1 h (Dako, Glostrop, Denmark).

Cytological Analysis

Oocytes were fixed overnight in Smith's fixative, dehydrated and embedded in paraffin. Sections (7 μm thick) were stained with nuclear red to detect nuclear structures and chromosomes and with picroindigo-carmine, which reveals cytoplasmic and microtubular structures. This method is precise enough to detect spindles and condensed chromosomes, even if they are not located near the plasma membrane [Flament et al., 1996].

Western Blotting

Analysis of the O-GlcNAc content. The proteins were run on a 10% SDS–PAGE and were electrophoretically transferred onto nitrocellulose membrane. Blots were saturated in 3% bovine serum albumin (BSA) in TBS-Tween (15 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween, pH 8.0) for 45 min and washed three times for 15 min with TBS-Tween. Before wheat germ agglutinin (WGA) staining, samples were systematically digested with PNGase F in order to remove N-linked oligosaccharides by direct incubation of the nitrocellulose sheets in 5–20 ml Phosphate Buffer (pH 7.5–50 mM) containing 2,500–10,000 U PNGase F overnight [Lefebvre et al., 1999]. Desialylation was performed with formic acid pH 2.0, for 30 min at 80°C. Proteins were analyzed for their O-GlcNAc status via Western blotting using horseradish peroxidase-coupled WGA (HRP–WGA) was incubated in TBS-Tween (1:10,000) for 1 h, and horseradish peroxidase was detected with ECL (Amersham Biosciences, Saclay, France). In the same way, the occurrence of O-glycans was assessed by the use of peanut agglutinin (PNA).

The anti-O-GlcNAc antibody (RL2, Affinity Bioreagents, Golden, CO) was used at a dilution of 1:1,000 over night at 4°C, after the nitrocellulose sheets were saturated with milk. The horseradish peroxidase-coupled anti-mouse secondary antibody was used for 1 h at a dilution of 1:10,000 (Amersham Biosciences). For immunoprecipitation, we used RL2 at a dilution of 1:200 for 2 h at 4°C, and protein G sepharose was finally added for 1 h at 4°C for the recovery of the O-GlcNAc proteins.

The phosphorylation status of proteins extracted from G_2 and M-phase arrested oocytes has been analyzed using an anti-phosphoserine (1:200 dilution, Sigma, Saint Quentin Fallavier, France). Alkaline phosphatase coupled anti-mouse antibody was used as the secondary antibody.

Analysis of β-catenin content. Oocyte homogenates were prepared and proteins were submitted to Western blot analysis as previously reported [Bodart et al., 1999]. β-catenin was detected using antibody H102 (Santa Cruz Biotechnology) (1:500). After washes in TBS, horseradish peroxidase-coupled goat anti-rabbit IgG antiserum (1:5,000 in 2.5% of non-fatty milk in TBS) was incubated for 1 h. In order to inhibit GSK-3β, oocytes were transferred to ND96 containing 20 mM LiCl [Fisher et al., 1999] before homogenization or progesterone addition.

Analysis of the O-GlcNAc content using gas phase chromatography. Mature and immature oocytes extracts were submitted to a reductive β-elimination in 0.1 M NaOH and 1 M potassium borohydride at 65°C overnight. The reaction was stopped by addition of ice-cold acetic acid under vigorous stirring until the pH value of 5.0 was reached. The β-eliminated material was dried several times under vacuum with anhydrous methanol in order to remove borate in its methyl ester form. The released saccharides were peracetylated in anhydride acetic for 4 h at 95°C, dried and finally extracted in chloroform (the extraction with chloroform was performed twice, and this volume was washed four times with water). After drying under nitrogen and adding 100 μl of chloroform, 2 μl of the peracetyled saccharides were analyzed using gas liquid chromatography

[Lefebvre et al., 2001]. Analysis was performed on a BPX column (30 m × 0.32 mm) at an initial temperature of 150°C, with a gradient of 3°C/min to 230°C, then with a gradient of 5°C/min to 250°C, and finally with a plateau of 5 min at 250°C.

Oocyte microinjection. Microinjections were performed in the equatorial region of the oocytes, using a positive displacement digital micropipette (Nichiryo). Free GlcNAc or Gal microinjections were performed in order to obtain a final concentration of 10 nmol per oocyte. To trigger the MPF activation and GVBD, GST-Cdc25A was injected into immature oocytes at a final concentration of 1.2 μM.

RESULTS

O-GlcNAc Content in Immature Versus Mature Oocytes

In a first set of experiments, immature oocytes (G_2-like phase-arrested) and mature oocytes (metaphase II-arrested) obtained after treatment with 10 μM progesterone during 16 h, were compared for their O-GlcNAc content. After treatments with PNGase F for deglycosylation of the N-linked glycans and formic acid for desialylation, proteins were separated on a 10% SDS–PAGE and checked for the presence of O-GlcNAc using WGA staining (Fig. 1Aa). A competition assay with an excess of free GlcNAc was done in conjunction with WGA (Fig. 1Ab). In this case, staining by WGA disappeared, demonstrating that it was specifically bound to O-GlcNAc proteins. We showed that the progesterone treatment induced an increase in the O-GlcNAc content of several different proteins in the *Xenopus* oocyte. Two proteins of 97 and 66 kDa were mainly concerned (Fig. 1Aa). PNA was used after formic acid desialylation to assess the absence of contaminant O-Glycans (Fig. 1Ac). Finally, the confirmation that the glycosylation borne by the proteins of 97 and 66 kDa was actually O-GlcNAc was done with a specific anti-O-GlcNAc antibody (RL2, Fig. 1Ae). It is to be noted that WGA stained more bands than the anti-O-GlcNAc antibody RL2 (compare panels a and e). This observation is not new and has already been reported [Comer et al., 2001]. The WGA lectin and the RL2 monoclonal antibody did not stained exactly the same motif: the lectin only needs the saccharide moiety for the binding, whereas the RL2 antibody needs not only the saccharide, but also a peptidic backbone. In these conditions, if the peptidic environment of the protein is too different from the peptidic environment against which this antibody was originally raised [Snow et al., 1987], the recognition might not occur.

We performed the characterization and the quantification of the GlcNAc release after the β-elimination of the two extracts and the analysis of the peracetylated released saccharides via gas phase chromatography (Fig. 1B). The integration of the relevant GlcNAc peak areas (Fig. 1Ba) showed a 4.51 ± 0.32 fold increase in the O-GlcNAc content in mature oocytes (the calculated values were normalized to an equal protein amount assayed using the Bradford method).

In order to compare the O-GlcNAc profile with the phosphorylation status of immature and matured oocytes, we performed Western blotting using WGA, anti-phosphoserine and anti-O-GlcNAc antibodies. These profiles show that the induction of oocyte maturation with progesterone induces an increase both on the O-GlcNAc (Fig. 1Cc,Cd) and on the phosphorylation (Fig. 1Cb) status of proteins. We must note that interestingly, the two proteins of particular interest, i.e., 66 and 97 kDa, also appear to be phosphorylated and that this phosphorylation was increased by the G_2/M transition, especially for the 97 kDa one (arrowheads).

WGA staining on mature enucleated oocytes extracts demonstrated that a majority of proteins exhibiting O-GlcNAc modulation during maturation were localized in the cytoplasm (Fig. 2A). Indeed, we did not observe any difference in the O-GlcNAc proteins profile between the total oocyte and the enucleated oocyte. This result is interesting as many kinases/phosphatases that are involved during meiosis are also cytosolic components. The enucleation efficiency was demonstrated using an antibody raised against a specific nuclear pore protein, p62. The latter was not detected in enucleated oocytes extracts, confirming the absence of any nuclear components (Fig. 2C). Then, O-GlcNAc glycosylation observed in enucleated oocytes as well as in intact cells are representative of cytosolic proteins, and not nuclear proteins.

O-GlcNAc Modified Proteins During the Maturation Process

In a second set of experiments, modifications of cellular proteins' O-GlcNAc levels during

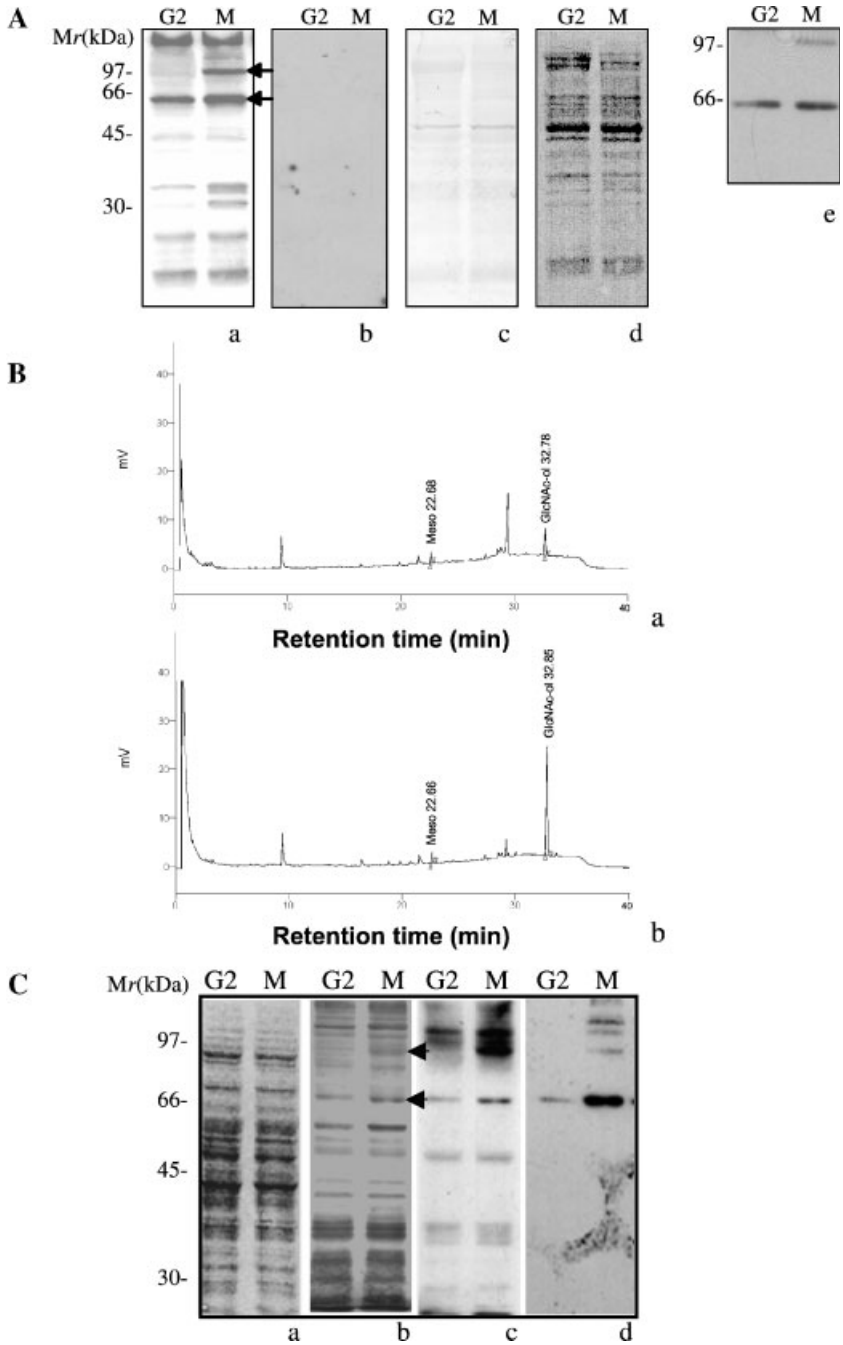

Fig. 1. O-linked *N*-acetylglucosamine (O-GlcNAc) content analysis of immature and mature oocytes. **A**: Immature (G_2) and mature (M) oocytes were homogenised in lysis buffer. After PNGase F and formic acid treatment, proteins were run on a 10% SDS–PAGE. **Aa**: The blot was then analyzed for the O-GlcNAc content by wheat germ agglutinin (WGA) staining. It revealed an increase in the O-GlcNac content and especially on two proteins of 97 and 66 kDa (Aa, arrows). **Ab**: In the presence of an excess of free GlcNAc, no more staining was observed with WGA, which demonstrated the specificity for O-GlcNAc. **Ac**: Peanut agglutinin (PNA) staining was done to assess the absence of contaminant O-Glycans. **Ad**: Red ponceau staining showing equality of the quantity of proteins in the lanes. **Ae**: To definitely assert the presence of O-GlcNAc glycosylation, we also used RL2, a specific antibody raised against O-GlcNAc. **B**: The same extracts were submitted to a reductive β-elimination and the peracetylated released saccharides were analyzed and quantified by gas phase chromatography (**Ba**, immature oocytes; **Bb**, mature oocytes). The peak corresponding to peracetylated GlcNAc-ol is indicated. Mesoinositol (Meso) was used as an internal standard. After integration, the GlcNAc peak area of mature oocytes appeared to be 4.51 ± 0.32 times more important than the peak area of immature oocytes. **C**: The phosphorylation status of immature and mature oocytes was tested using an anti-phosphoserine directed antibody (**Cb**) in conjunction with peroxidase-labeled WGA (**Cc**) and with the anti-O-GlcNAc antibody (**Cd**). The panel Ca shows the ponceau red staining.

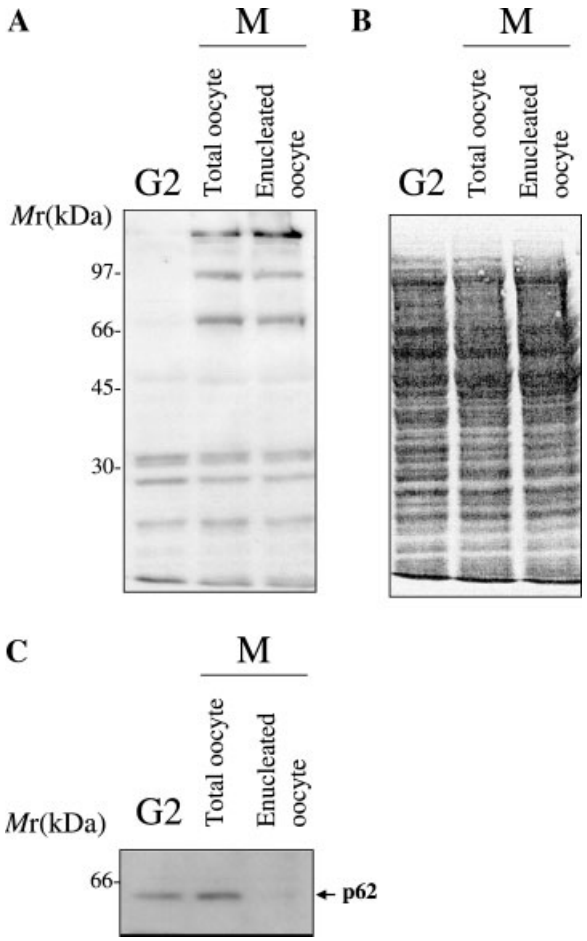

Fig. 2. Localization of the proteins that have their O-GlcNAc modulated during maturation. **A**: WGA staining and (**B**) ponceau red staining. Stage VI oocytes were manually enucleated before treatment with progesterone. The lysates were further analyzed for their O-GlcNAc content as described in Figure 1. Following progesterone stimulation, changes in O-GlcNAc glycosylation occurred in these enucleated oocytes (compare with immature oocytes: G_2) as was already observed in Figure 1Aa. The profile was not different from those observed in mature nucleated oocytes (total oocyte). **C**: We assessed for correct enucleation of oocytes using an antibody raised against p62, a specific nuclear pore protein. Lack of p62 staining confirmed the absence of any nuclear components.

meiosis were monitored. Kinetic maturation experiments were performed on oocytes after a hormonal stimulation using progesterone: oocytes were taken out at different times and homogenized. Numerous proteins (indicated with arrows in Fig. 3) showed modifications of their O-GlcNAc content during the meiosis resumption. For example, proteins at 150 and at 97 kDa reached a maximum in O-GlcNAc glycosylation 6–8 h after the progesterone addition. Then, their O-GlcNAc level decreased at the end of the maturation. The 66 kDa protein was already O-GlcNAc glycosylated before the progesterone treatment, but this O-glycosylation increased 2 h after the progesterone application and then sharply decreased until the end of meiosis. Lastly, the O-GlcNAc glycosylation of a 30 kDa protein decreased during maturation and increased at the end of the maturation process.

Free GlcNAc Injection Delays Maturation in *Xenopus* Oocytes

Since oocyte maturation was accompanied by a modulation in O-GlcNAc glycosylation, the potentially inhibiting effect of free GlcNAc on maturation stages was evaluated after the microinjection of this monosaccharide into oocytes (Fig. 4). GlcNAc could not enter the hexosamine biosynthetic pathway, contrary to glucose or glucosamine, and thus could not take part in the synthesis of UDP-GlcNAc for the O-GlcNAc events. Besides, it is well-known that O-GlcNAc transferase is unaffected by GlcNAc [Haltiwanger et al., 1990]. After the microinjection of free GlcNAc, we observed that maturation was delayed. From the three experiments reported in Figure 4, we concluded that $GVBD_{50}$ (the time required for 50% of the oocytes to undergo GVBD) occurred 4 h later than in control oocytes injected with water (Fig. 4A). As a negative control, free galactose was microinjected before the progesterone treatment and it did not delay maturation, and such treated oocytes exhibited the same kinetics of GVBD than water-injected oocytes. As was expected, WGA staining did not shows any significant differences in the O-GlcNAc content among oocytes in the three batches (Fig. 4B).

In order to determine the position in the cell cycle of GlcNAc injected oocytes, a cytological analysis was performed 4 h after the $GVBD_{50}$ of control oocytes injected with galactose. The latter exhibited a metaphase II spindle with condensed chromosomes, located on the plasma membrane, and a polar body (Fig. 5A), as water-injected oocytes did (data not shown). At the same time, cytological sections of GlcNAc-injected oocytes showed either oocytes with a typical metaphase II block (Fig. 5B) or oocytes with a metaphase I spindle and no polar body (Fig. 5C). Thus, though GlcNAc injection delayed progesterone-induced maturation, it did not seem to alter the condensation of chromosomes or the meiotic spindle morphogenesis.

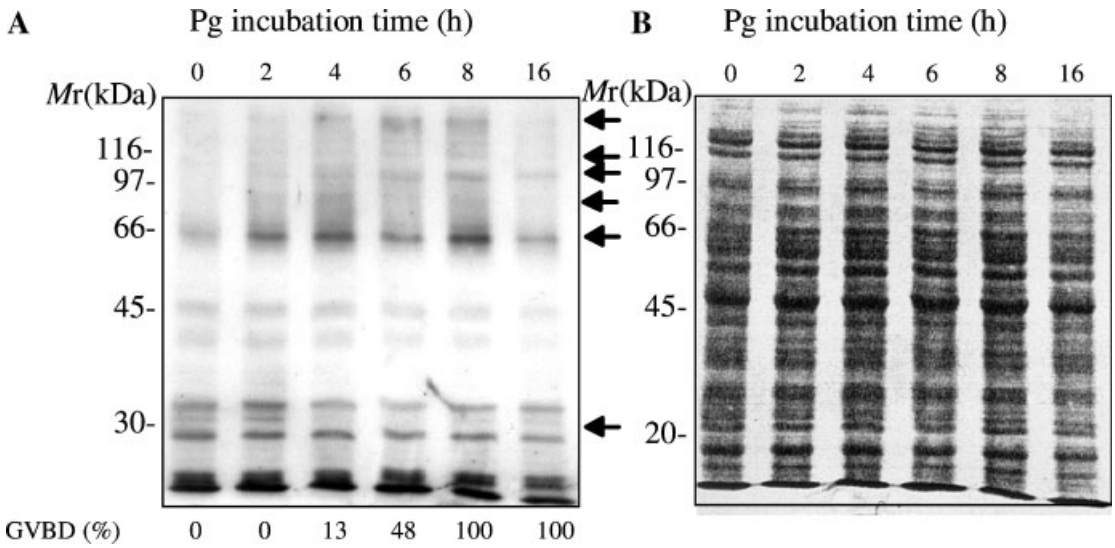

Fig. 3. O-GlcNAc glycosylation during the maturation process. The O-GlcNAc content of oocytes at different times of the maturation was analyzed as indicated in Figure 1. The WGA staining is shown in Figure 3A, and ponceau red staining in Figure 3B. Arrows indicate the main profile changes observed by WGA staining. The evolution of O-GlcNAc glycosylation was not similar for all proteins. The 97 kDa protein showed a maximum in O-GlcNAc glycosylation 6–8 h after the progesterone addition. Then, the latter decreased but remained higher than in immature oocytes. The 66 kDa protein was already glycosylated in immature oocytes and this glycosylation increased as early as 2 h after the progesterone application; it sharply decreased at the end of the maturation. The 30 kDa protein showed an O-glycosylated profile in immature oocytes, and this glycosylation decreased after the progesterone treatment.

Synthesized β-catenin Is O-GlcNAc Modified During *Xenopus* Oocyte Maturation

In order to check whether the increase in the O-GlcNAc content observed during maturation was related to an increase in the synthesis of the corresponding proteins, oocytes were induced to mature in the presence of cycloheximide (CHX, 50 μg/ml) a protein synthesis inhibitor. Since progesterone-induced maturation is dependent upon protein synthesis, maturation was triggered by micro-injection of GST-Cdc25A in this set of experiments. Cdc25 is a dual specificity phosphatase that directly dephosphorylates and activates $p34^{Cdc2}$, the catalytic subunit of the M-phase promoting factor. Results are

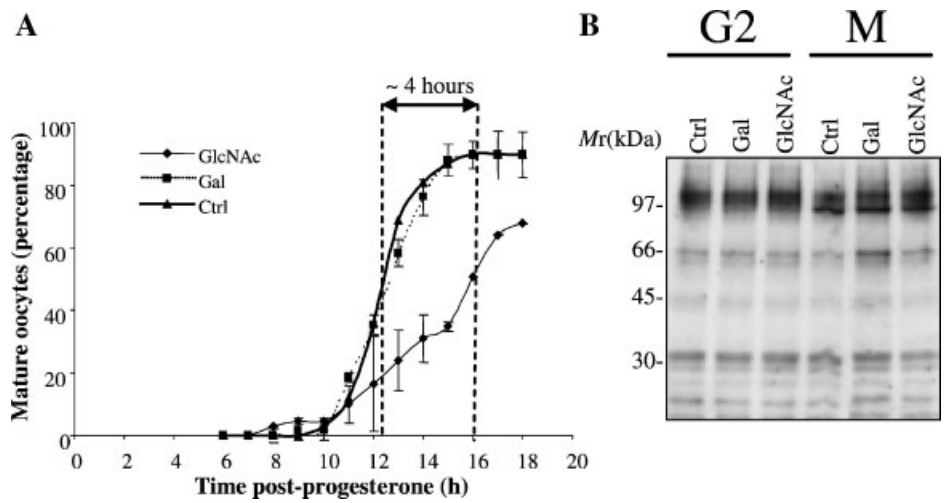

Fig. 4. Effect of GlcNAc microinjection on oocyte maturation. **A**: The progesterone treatment was performed after the control microinjection of either water (-▲-) or free galactose (-■-) or after the microinjection of free GlcNAc (-◆-). Maturation was assessed using white spot examination. The results are from three different experiments. **B**: WGA staining of oocytes extracts. The injection of either Gal or GlcNAc did not modify the O-GlcNAc profile in immature oocytes (G_2). This did not inhibit the occurrence of the changes of O-GlcNAc glycosylation usually observed in mature oocytes (M).

described in Figure 6A. In the absence of CHX, the injection of Cdc25A induced the changes in O-GlcNAc usually observed following progesterone stimulation (see Fig. 1Aa). The same changes were still observed in the presence of CHX for most of the proteins, except for the protein with an apparent molecular weight of 97 kDa, which was just slightly glycosylated. So, based on the observations that the O-GlcNAc glycosylation of the 97 kDa protein was related in part to its accumulation during maturation (i.e., its synthesis) and according to its apparent molecular weight, we assumed that the protein was β-catenin. This hypothesis was reinforced by the description of the O-GlcNAc modification of β-catenin [Zhu et al., 2001]. In fully grown immature oocytes, the glycogen synthase kinase-3β (GSK-3β) is active, and it must be inactivated to allow maturation [Fisher et al., 1999]. This kinase is responsible for β-catenin phosphorylation, which is the signal for its degradation using the proteasome pathway. The inactivation of GSK-3β allows β-catenin to accumulate in mature oocytes [Fisher et al., 1999]. O-GlcNAc proteins were enriched on agarose beads-coupled WGA, run on SDS–PAGE, transferred and stained with the anti-β-catenin antibody. As shown with the RL2 antibody (Fig. 6B), after WGA-enrichment, β-catenin was better detected in mature oocytes (M-phase) compared to immature oocytes (G_2-phase). LiCl, also known as a GSK-3β inhibitor, was used to accumulate β-catenin [Hedgepeth et al., 1997; Davies et al., 2000; Davies et al., 2001]. When oocytes were treated with LiCl, we found greater amounts of glycosylated β-catenin both in mature and immature oocytes. To confirm that the identity of the 97 kDa-protein was actually β-catenin, we performed immunoprecipitations with the anti-O-GlcNAc RL2 antibody on immature and mature extracts. The immunoprecipitated proteins were separated on a 10% SDS–PAGE, electrotransfered on nitrocellulose and stained with an anti-β-catenin antibody (Fig. 6C). These immunoprecipitations clearly showed the accumulation of β-catenin during the maturation process and

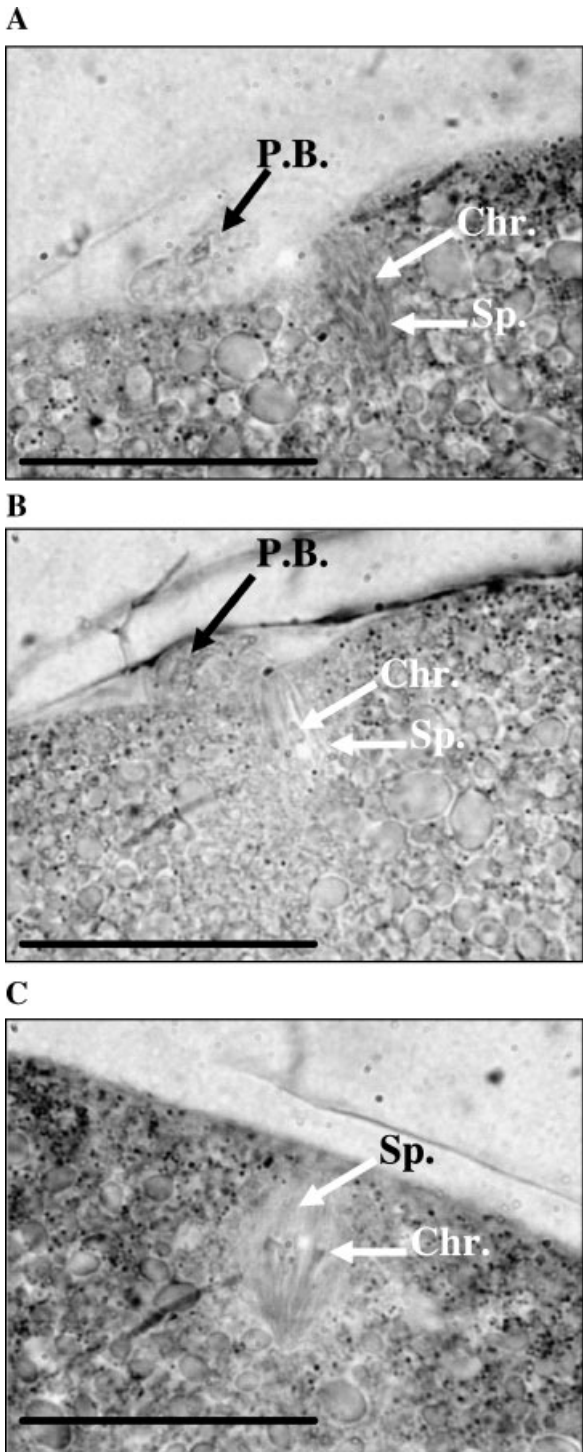

Fig. 5. Effects of free GlcNAc injection on the cytological events of the maturation. In this experiment, oocytes were fixed 4 h after control oocytes reached the $GVBD_{50}$ stage. **A**: Galactose-injected oocytes showing the usual aspect of mature oocytes with a polar body and a metaphase spindle with condensed chromosomes (arrows). **B**: Free GlcNAc injected oocytes showing a metaphase II status as in A. This was observed in 50% oocytes. **C**: Free GlcNAc injected oocytes with an unachieved maturation. Here is shown an example with a metaphase I spindle with condensed chromosomes but no polar body. Chr., condensed chromosomes; P.B., polar body; Sp., spindle. bar represents 50 μm.

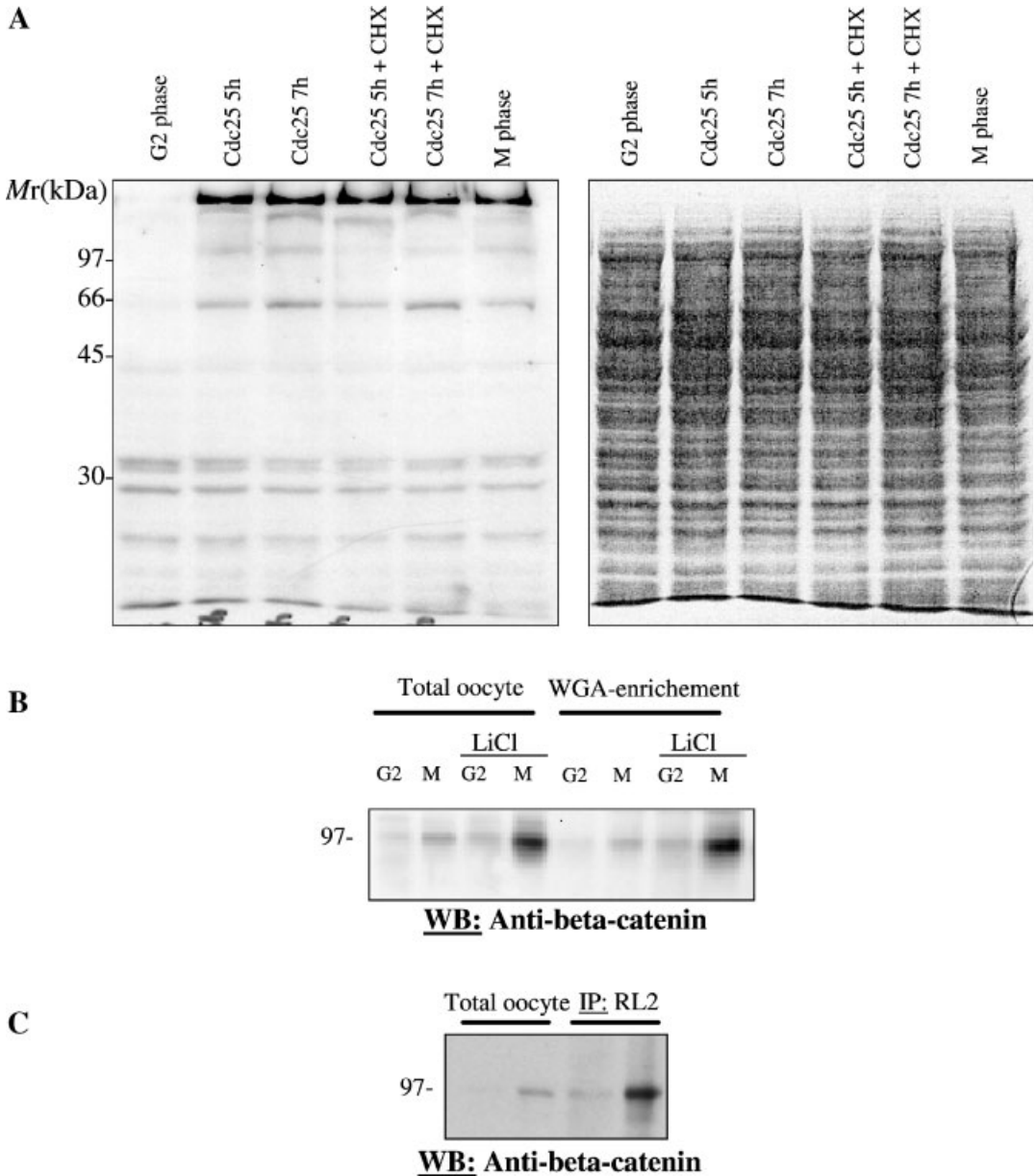

Fig. 6. Inhibition of protein synthesis and identification of the 97 kDa protein as β-catenin. **A**: Maturation was performed in the presence of the protein synthesis inhibitor, cycloheximide (CHX) and was triggered using microinjection of GST-Cdc25A. Oocytes were analyzed 5 and 7 h after the injection (**A**). A: **Left panel** represents the WGA staining as described in Figure 1A whereas the **right panel** represents the ponceau red staining. Cdc25 injection induced an increase in the O-GlcNAc glycosylation as was previously observed with progesterone. Except for the 97 kDa protein, the glycosylation was independent from the protein synthesis as CHX had no effect on it. **B**: Anti-β-catenin staining. β-catenin was strongly detected in the WGA-enriched fraction, proving that β-catenin was still present after WGA precipitation. The GSK-3β inhibitor, LiCl, induced an increase in the amount of β-catenin which was observed not only in total homogenates but also following WGA enrichment (G_2, immature oocytes; M, mature oocytes). **C**: Immunoprecipitation was performed with the anti-O-GlcNAc antibody (RL2) on the two extracts (G_2 and M), the bound proteins were separated on a 10% SDS–PAGE, electroblotted onto nitrocellulose and then stained with the anti–β-catenin.

concomitantly its O-GlcNAc glycosylation. This identification of β-catenin prompted us to make a further experiment. It seemed particularly interesting to investigate into the inhibition of the β-catenin phosphorylation on the O-GlcNAc glycosylation of the protein. These points unambiguously demonstrate that the 97 kDa glycosylated protein is β-catenin.

DISCUSSION

The aim of this study was to investigate into the O-GlcNAc level of cellular proteins during *Xenopus* oocyte maturation. *Xenopus* oocytes were widely used to study the regulation of the M-phase entry that is triggered by well-characterized and well-conserved pathways of phosphorylations and dephosphorylations [Nebreda and Ferby, 2000]. Several reports have suggested that phosphorylation could be antagonized on the same site, or on an adjacent site, through O-GlcNAc glycosylation [Torres and Hart, 1984; Comer and Hart, 2000; Comer and Hart, 2001]. Hence, resumption of meiosis in oocytes, according to the phosphorylation/dephosphorylation cascade, might be accompanied by a modulation of the O-GlcNAc content of individual proteins, which could play a part in the cell cycle progression.

Unexpectedly, we observed that progesterone-induced oocyte maturation is correlated with an increase of O-GlcNAc glycosylation. Indeed, when Western blots were analyzed after WGA staining, several proteins exhibited an increase in their O-GlcNAc glycosylation in M-phase (mature) oocytes versus G_2-phase (immature) ones. The major changes were mainly shown by two proteins of 66 and of 97 kDa (the O-GlcNAc glycosylation for these two proteins was confirmed with the RL2 antibody). These observations were confirmed by gas phase chromatography, which showed a global increase in the O-GlcNAc content in mature oocytes ($\times 4.51 \pm 0.32$). This increase may be partly due to an increase in the O-GlcNAc transferase (OGT) activity: measurements performed on the OGT activity have shown a slight increase in this activity during the maturation process (data not shown). Similar changes in the O-GlcNAc content were observed when the M-phase entry was triggered by the microinjection of Cdc25 that is a direct activator of $p34^{Cdc2}$. The importance of the O-GlcNAc glycosylation-mediated phenomenon for oocyte maturation was strongly suggested by the noticeable delay observed in the maturation process following the microinjection of free GlcNAc (4 h delay).

The conclusion drawn from these results seems to disagree with a competition between phosphorylation and O-GlcNAc for the same sites. Indeed, since maturation is mainly associated with a burst in phosphorylation due to the activation of many kinases (Mos, MEK1, $p42^{Erk2}$, $p90^{rsk}$, $p34^{Cdc2}$, Cdc25C, Plx1, Plkk1; for review, see [Nebreda and Ferby, 2000]), we could have expected to observe a decrease in the O-GlcNAc content. However, such a conclusion should be qualified. First, because the analyses that were performed during time-course experiments showed that the changes in O-GlcNAc were rather complex. For instance, a protein of 30 kDa showed a transient decrease in its O-GlcNAc glycosylation. Secondly, even if a burst of phosphorylation accompanies G_2/M transition in *Xenopus* oocytes, kinases such as PKA or GSK-3β have to be inhibited for cell cycle progression [Maller and Krebs, 1977; Fisher et al., 1999]. Interestingly, we characterized the protein of 97 kDa as β-catenin. In immature oocytes, when β-catenin is phosphorylated by GSK-3β, this protein is degraded by an ubiquitin ligase-proteasome system [Aberle et al., 1997; Orford et al., 1997]. Following the progesterone stimulation, GSK-3β is inhibited, β-catenin is synthesized but no longer proteolysed and it consequently accumulates [Fisher et al., 1999]. Later, β-catenin accumulates on the future dorsal side of the *Xenopus* embryo due to the two-cell stage [Larabell et al., 1997]. We found high amounts of glycosylated β-catenin in mature oocytes as well as following treatments with the GSK3 inhibitor, LiCl. This result confirmed the O-GlcNAc glycosylation of β-catenin recently reported in MCF-7, MDA-MB-468, and MDCK cells as well as in 3T3-L1 adipocytes [Zhu et al., 2001; Vosseler et al., 2002]. In the oocyte, the increase in the O-GlcNAc content resulted from the accumulation and stabilization of the protein as was demonstrated by experiments using cycloheximide. This apparent correlation between the glycosylation of β-catenin and its stabilization is in line with the O-glycosylation of the Sp1 transcription factor since it was demonstrated that reduced O-GlcNAc of Sp1 increased the proteasome susceptibility [Han and Kudlow, 1997]. So, at least in the case of β-catenin, the increase in O-GlcNAc might be correlated to a decrease in phosphorylation.

We assumed that O-GlcNAc could promote interactions between several components required for the G_2/M transition and subsequent cytological events during oocyte meiosis. These interactions could be disturbed by the presence of high amounts of free GlcNAc in the oocyte and a similar approach was used by Fang and Miller [2001]. Such a role for the modulation of the

protein–protein interaction by O-GlcNAc has already been suggested. For example proteins that link the cytoskeleton to cellular membranes are O-GlcNAc modified but the exact function of O-GlcNAc in these interactions remains obscure [Hagman et al., 1992; Cole and Hart, 1999]. The best-known example is the interaction between Sp1, the TATA-binding-protein-associated factor (TAF110) and holo-Sp1. It was suggested that the removal of O-GlcNAc from an interaction domain was a signal for the protein association [Roos et al., 1997]. In this example O-GlcNAc may thereby prevent untimely and ectopic interactions. Conversely, our findings suggest that O-GlcNAc promotes protein–protein interactions that are involved in the cell cycle kinetic but not in spindle morphogenesis, nor chromosomes condensation.

As a conclusion, this study provides new evidence of the dynamic feature of the O-GlcNAc glycosylation during a cellular process and particularly during the cell cycle. Further experiments are required to investigate into the role of the O-glycosylation-phosphorylation balance in the cell cycle control. The role of O-GlcNAc might be important not only in the resumption of meiosis but also in the events that follow maturation: fertilization and early development. So, the role of the O-glycosylation of β-catenin is interesting since it is an important factor in the Wnt signaling pathway [Akiyama, 2000].

ACKNOWLEDGMENTS

The authors would like to thank Dr. Yann Guerardel and Mr. Yves Leroy for their technical assistance with gas liquid chromatography. We also thank Ms. Arlette Rousseau for her help in the handling and preparation of oocytes. We are grateful to Dr. Willy Morelle for the final reading of the article and for English corrections.

REFERENCES

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. 1997. Beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J 16:3797–3804.

Akiyama T. 2000. Wnt/β-catenin signaling. Cytokine Growth Factor Rev 11:273–282.

Bodart JF, Béchard D, Bertout M, Gannon J, Rousseau A, Vilain JP, Flament S. 1999. Activation of *Xenopus* eggs by the kinase inhibitor6-DMAP suggests a differential regulation of cyclin B2 and $p39^{Mos}$ proteolysis. Exp Cell Res 253:413–421.

Cole RN, Hart GW. 1999. Glycosylation sites flank phosphorylation sites on synapsin I: O-linked *N*-Acetylglucosamine residues are localized within domains mediating synapsin I interactions. J Neurochem 73: 418–428.

Comer FI, Hart GW. 2000. O-glycosylation of nuclear and cytosolic proteins. Dynamic interplay between O-GlcNAc and O-phosphate. J Biol Chem 275:29179–29182.

Comer FI, Hart GW. 2001. Reciprocity between O-GlcNAc and O-phosphate on the carboxyl terminal domain of RNA polymerase II. Biochemistry 40:7845–7852.

Comer FI, Vosseler K, Wells L, Accavitti MA, Hart GW. 2001. Characterization of a mouse monoclonal antibody specific for *O*-linked *N*-acetylglucosamine. Anal Biochem 293:169–177.

Davies SP, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105.

Davies G, Jiang WG, Mason MD. 2001. The interaction between beta-catenin, GSK3beta and APC after motogen induced cell-cell dissociation, and their involvement in signal transduction pathways in prostate cancer. Int J Oncol 18:843–847.

Fang B, Miller MW. 2001. Use of galactosyltransferase to assess the biological function of O-linked *N*-acetyl-D-glucosamine: A potential role for O-GlcNAc during cell division. Exp Cell Res 263:243–253.

Ferrell JE, Jr. 1999. *Xenopus* oocyte maturation: New lessons from a good egg. BioEssays 21:833–842.

Fisher DL, Morin N, Doree M. 1999. A novel role for glycogen synthase kinase-3 in *Xenopus* development: Maintenance of oocyte cell cycle arrest by a beta-catenin-independent mechanism. Development 126:567–576.

Flament S, Browaeys E, Rodeau JL, Bertout M, Vilain JP. 1996. *Xenopus* oocyte maturation: Cytoplasm alkalization is involved in germinal vesicle migration. Int J Dev Biol 40:471–476.

Gotoh Y, Nishida E. 1995. The MAP kinase cascade: Its role in *Xenopus* oocytes, eggs, and embryos. Prog Cell Cycle Res 1:287–297.

Griffith LS, Schmitz B. 1999. O-linked *N*-acetylglucosamine levels is upregulated in Alzheimer brains. Eur J Biochem 262:824–831.

Gross SD, Schwab MS, Taieb FE, Lewellyn AL, Qian YW, Maller JL. 2000. The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by p90 (Rsk). Curr Biol 10:430–438.

Hagman J, Grob M, Burger MM. 1992. The cytoskeletal protein talin is O-glycosylated. J Biol Chem 267:14424–14428.

Haltiwanger RS, Philipsberg GA. 1997. Mitotic arrest with nocodazole induces selective changes in the level of O-linked *N*-acetylglucosamine and accumulation of incompletely processed N-Glycans on proteins from HT-29 cells. J Biol Chem 272:8752–8758.

Haltiwanger RS, Holt GD, Hart GW. 1990. Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins, identification of a uridine diphospho-*N*-acetylglucosamine: Peptide β-*N*-acetylglucosaminyltransferase. J Biol Chem 265:2563–2568.

Haltiwanger RS, Busby S, Grove K, Li S, Mason D, Medina L, Moloney D, Philipsberg G, Scartozzi R. 1997. O-glycosylation of nuclear and cytoplasmic proteins:

Regulation analogous to phosphorylation? Biochem Biophys Res Commun 231:237–242.

Haltiwanger RS, Grove K, Philipsberg GA. 1998. Modulation of O-linked *N*-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide O-GlcNAc-b-*N*-acetylglucosaminidase inhibitor *O*-(2-acetamido-2-deoxy-D glucosaminopyranossylidene) amino-*N*-phenylcarbamate. J Biol Chem 273:3611–3617.

Han I, Kudlow JE. 1997. Reduced O-glycosylation of Sp1 is associated with increased proteasome susceptibility. Mol Cell Biol 17:2550–2558.

Hausen P, Riebesell M. 1991. The early development of *Xenopus laevis*. An atlas of histology. Berlin: Springer-Verlag.

Heasman J, Crawford A, Goldstone K, Garner-Hamrick P, Gumbiner B, McCrea P, Kintner C, Yoshida-Noro C, Wilie C. 1994. Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. Cell 79:791–803.

Hedgepeth CH, Conrad LJ, Zhang J, Huang HC, Lee VMY, Klein PS. 1997. Activation of the Wnt signaling pathway: A molecular mechanism for lithium action. Dev Biol 185: 82–91.

Kearse KP, Hart GW. 1991. Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins. Proc Natl Acad Sci USA 88:1701–1717.

Larabell CA, Torres M, Rowning BA, Yost C, Miller JR, Wu M, Kimelman D, Moon RT. 1997. Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in β-catenin that are modulated by the Wnt signaling pathway. J Cell Biol 136:1123–1136.

Lefebvre T, Alonso C, Mahboub S, Dupire MJ, Zanetta JP, Caillet-Boudin ML, Michalski JC. 1999. Effect of okadaic acid on O-linked *N*-acetylglucosamine levels in a neuroblastoma cell line. Biochim Biophys Acta 1472: 71–81.

Lefebvre T, Cieniewski C, Lemoine J, Guerardel Y, Leroy Y, Zanetta JP, Michalski JC. 2001. Identification of *N*-acetyl-D-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as Heat-shock proteins Biochem J 360:179–188.

Maller JL, Krebs EG. 1977. Progesterone-stimulated meiotic cell division in *Xenopus* oocytes. Induction by regulation subunit and inhibition by catalytic subunit 3′, 5′-monophosphate dependent protein kinase. J Biol Chem 252:1712–1718.

Masui Y. 2001. From oocytes maturation to the in vitro cell cycle: The history of discoveries of maturation-promoting factor (MPF) and cytostatic factor (CSF). Differentiation 69:1–17.

Miller MW, Caracciolo MR, Berlin WR, Hanover JA. 1999. Phosphorylation and glycosylation of nucleoporins. Arch Biochem Biophys 367:51–60.

Nebreda AR, Ferby I. 2000. Regulation of the meiotic cell cycle in oocytes. Curr Opin Cell Biol 12:666–675.

Orford K, Crockett C, Jensen JP, Weissman A, Byers SW. 1997. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J Biol Chem 272:24735–24738.

Roos MD, Su K, Baker JR, Kudlow JE. 1997. O-glycosylation of an Sp1-derived peptide blocks the removal of O-GlcNAc. Mol Cell Biol 17:6472–6480.

Snow CM, Senior A, Gerace L. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J Cell Biol 104:1143–1156.

Torres CR, Hart GW. 1984. Topography and distribution of terminal *N*-acetylglucosamine residues on the surface of intact lymphocytes, evidence of O-linked GlcNAc. J Biol Chem 259:3308–3317.

Vosseler K, Wells L, Lane MD, Hart GW. 2002. Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes. Proc Natl Acad Sci USA 99: 5313–5318.

Wells L, Vosseler K, Hart GW. 2001. Glycosylation of nucleocytoplasmic proteins: Signal transduction and O-GlcNAc. Science 291:2376–2378.

Zhu W, Leber B, Andrews DW. 2001. Cytoplasmic O-glycosylation prevents cell surface transport of E-cadherin during apoptosis. EMBO J 20:5999–6007.