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O-Glycosylation of the Nuclear Forms of Pax-6 Products in Quail Neuroretina Cells

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Abstract Many transcription factors are demonstrated as being glycosylated with *O*-*N*-acetylglucosamine (GlcNAc) residue in transfected insect cell lines, but rarely in the original cells. For the first time, we demonstrate the *O*-GlcNAc modification of the p48/p46 Pax-6 gene (a developmental control gene involved in the eye morphogenesis) products in the quail neuroretina (QNR). In conjunction with a systematic PNGaseF treatment, we used wheat germ agglutinin (WGA) binding, in vitro labeling with bovine galactosyltransferase, and labeling of cultured QNR with [^{14}C]GlcNH$_2$. Glycosylated forms of Pax-6 proteins were found in the nucleus of the neuroretina cells. WGA-selected Pax-6 proteins produced in the reticulocyte lysate were able to bind a DNA target, as well as to the unglycosylated form. The *O*-GlcNAc may, however, modulate protein interactions, mainly with other factors involved in the transcription process. Characterization of products released after reductive alkaline treatment of the proteins clearly demonstrates that *N*-acetylglucosamine is directly linked to serine or threonine residues. Examination of Pax-6 primary sequence allowed us to determine potential *O*-GlcNAc attachment sites. Most of these expected glycosylation sites appear to be located on the two DNA binding domains and on the carboxyterminal transactivation domain, while experimental evidence taken from WGA-selected proteins experiment points in favor of a main localization on the paired-box domain. J. Cell. Biochem. 85: 208–218, 2002.

Key words: *O*-GlcNAc; nucleus; transcription factors; Pax-6 products; quail neuroretina

For a long time, glycosylation was thought to be restricted to proteins confined to the cell surface or within the lumen of intracellular organelles. This view was governed by our understanding on the biosynthetic pathways of *N*- or *O*-glycans. However, in the past 15 years, the existence of a major form of glycosylation found within the cytosol and the nucleus has been established: the *O*-linked *N*-acetylglucosaminylation (*O*-GlcNAc) [Torres and Hart, 1984; Wells et al., 2001]. *O*-GlcNAc is formed by the addition of a single *N*-acetylglucosamine (GlcNAc) on serine or threonine residues. More than hundreds of nuclear and cytoplasmic proteins are modified with *O*-GlcNAc, but only a few have yet been identified, including cytoskeletal proteins (keratins) [Chou et al., 1992], neurofilaments [Dong et al., 1993], nuclear pore proteins [Holt et al., 1987; Lubas et al., 1995], adenovirus fibers [Caillet-Boudin

Abbreviations used: QNR, quail neuroretina; BSA, bovine serum albumin; GlcNAc, *N*-acetylglucosamine; PBS, phosphate-buffered saline; TBS, tris-buffered saline; PNGaseF, peptide-*N*-glycosidase F; WGA, wheat germ agglutinin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; uridine diphospho *N*-acetylglucosamine; GST, glutathione S-transferase.

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et al., 1989], and several transcription factors including Sp1 [Jackson and Tjian, 1988], c-myc [Chou et al., 1995a], and SV40 large-T antigen [Medina et al., 1998]. Interestingly, all of the *O*-GlcNAc modified proteins that undergo reversible phosphorylation/dephosphorylation, form heteromeric complexes with other proteins, and most translocate between the cytosol and the nucleus. Particularly, it has been speculated that this type of glycosylation shares common features with protein phosphorylation [Haltiwanger et al., 1997]. For example, it has been demonstrated that c-myc, a helix-loop leucine zipper phosphoprotein that heterodimerizes with Max and regulates gene transcription, is modified with *O*-GlcNAc on a known in vivo glycogen synthase kinase-3 phosphorylated site (Thr 58) [Chou et al., 1995b]. In a previous report, we demonstrated that okadaic acid, an inhibitor of serine/threonine phosphatases (PP1, PP2A, and 2B), induces a decrease of the protein *O*-GlcNAc level [Lefebvre et al., 1999]. In addition, using the potent peptide *O*-GlcNAc-β-*N*-acetylglucosaminidase inhibitor, *O*-(2-acetamido-2-deoxy-D-glucosaminopyranosylidene)-amino-*N*-phenylcarbamate (PUGNAc), Haltiwanger et al. [1998] have demonstrated a two-fold increase of the *O*-GlcNAc level and a reciprocal decrease in the phosphate incorporation concomitant to an increase on GlcNAc on the Sp1 transcription factor. These results could be interpreted by a competition between *O*-GlcNAc and phosphate for the same attachment sites on proteins. Using a metabolic pulse-chase labeling of U373-MG astroglioma cells, Roquemore et al. [1996] showed that the turnover of the *O*-GlcNAc carbohydrate is dynamic and proceeds much more rapidly than the turnover of the protein backbone itself, consistent with a regulatory role for *O*-GlcNAc on αβ-crystallin a newly recognized member of the small heat shock protein family. Other authors hypothesize that *O*-GlcNAc may play a role in the nuclear transport of cytosolic proteins: Duverger et al. [1993, 1995, 1996] have demonstrated a sugar-dependent transport of proteins, and Finlay et al. [1987] have shown a specific inhibition of the nuclear transport by wheat germ agglutinin (WGA) and not by other lectins. *O*-N-acetylglucosaminylation has also an effect on transcriptional regulation by RNA polymerase II, which is itself modified with this type of glycosylation on the carboxyterminal tandem repeats [Kelly et al., 1991]. This post-translational modification similarly to phosphorylation, could modulate protein–protein interactions. The hypothesis was made that removal of *O*-GlcNAc from an interaction domain could be a signal for protein association. Roos et al. [1997] have demonstrated that *O*-GlcNAc inhibits interactions between Sp1 and Drosophila TAF110 or holo-Sp1.

The paired-class transcription factor, Pax-6, is a master control gene for eye morphogenesis in both invertebrates [Halder et al., 1995] and vertebrates [Chow et al., 1999]. Mutations in the Pax-6 gene are associated with the mouse mutant small eye [Hill et al., 1991] and the corresponding human gene (AN) has been found deleted or mutated in aniridia [Glaser et al., 1994]. Pax-6 is reported to be expressed in the developing central nervous system [Gruss and Walther, 1991], in the endocrine pancreas and to be critical for α-cell development [St-Onge et al., 1997]. The quail homologue of Pax-6 termed Pax-quail neuroretina (QNR) [Martin et al., 1992], encodes five proteins through alternative splicing and internal initiations [Carrière et al., 1993]. Three proteins of 48, 46, and 43 kDa contain the paired domain, while two proteins of 33 and 32 kDa are devoid of this DNA-binding domain. All these proteins contain another DNA-binding motif, the homeodomain. The homeobox encodes the homeodomain, a conserved DNA-binding domain with a helix-turn-helix motif [Kissinger et al., 1990]. The p46 encodes a transcription factor able to positively regulate the Pax-6 promoters [Plaza et al., 1993; Carrière et al., 1995] and glucagon gene [Ritz-laser et al., 1999]. Pax-6 encodes serine and threonine phosphoproteins [Carrière et al., 1993].

In this study, using a combination of different techniques, we demonstrate that the Pax-6 gene products are modified with *O*-GlcNAc, more probably in the paired box domain, and that the modified protein are present in neuroretina nuclear extract. After in vitro binding experiments with a target DNA, we demonstrate that the *O*-GlcNAc motifs are not directly implicated in the DNA binding, but we speculate that these residues may remain available for interaction with other transcription factors.

MATERIALS AND METHODS

Cells

Neuroretinas were dissected from quail embryos from day 4 to hatching. Quail embryo

cell cultures were maintained and passed in Dulbecco's modified eagle's medium supplemented with 10% fetal calf serum.

For [^{35}S]Cys/Met labeling, 10^7 cells were incubated in a medium depleted for Cys/Met for 10 min and then incubated for 1 h at 37°C with 250 μCi of Mix [^{35}S]Cys/Met. For [^{14}C]-glucosamine labeling cells are incubated in a glucose poor medium (1/3) with 25 μCi [^{14}C]glucosamine overnight at 37°C.

Antibodies

Different rabbit anti-Pax-6 sera were used: serum 11 is directed against the paired domain, serum 13 is directed against the homeodomain, serum 14 is directed against the carboxyl terminus; the preimmune serum 13 is named p13.

Lysate and Immunoprecipitation

Cells were washed with phosphate-buffered saline (PBS) (20 mM phosphate, 150 mM NaCl, pH 7.5) and then incubated in the lysate buffer (10 mM Tris-HCl, 150 mM NaCl, EDTA 1 mM, 1% v/v Triton X-100, 0.5% sodium desoxycholate, 0.1% sodium dodecyl sulfate, 0.5% aprotinin, pH 7.4) for 5 min. The extract was centrifuged at 100,000*g* for 30 min at 4°C.

The lysate was incubated overnight at 4°C with the different rabbit anti-Pax-6 sera described above. Then protein A sepharose was added at a 50% v/v for 30 min at 4°C, and the complex was successively washed with lysate buffer (RIPA), RIPA/NaCl (lysate buffer with 350 mM NaCl), RIPA/TNE and TNE (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.5% aprotinin, pH 7.4).

Nuclear Extracts Preparation

All experiments were performed in ice-cold baths. Neuroretina cells pellet was resuspended in ten volumes of buffer (300 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, and 15 mM HEPES, pH 7.9). After 5 min, 0.3% NP40 was added and the suspension was maintained in ice for 5 min. The cells were centrifuged at 10,000*g* for 1 min. The supernatant was carefully discarded and the pellet was resuspended in 9 volumes of buffer (1.1 M urea, 330 mM NaCl, 1.1% NP40, 27.5 mM HEPES, pH 7.6) and 15 min in ice. The suspension was centrifuged for 15 min at 1,000*g*, and the resulting supernatant was exhaustively dialysed for 4 h against buffer DB (DNA binding buffer) (20 mM HEPES, pH 7.9, 60 mM KCl, 20% glycerol, 0.25 mM EDTA, and 0.125 mM EGTA). All the buffers were supplemented with 0.5 mM PMSF and 1 mM DTT.

Peptide-*N*-Glycosidase F (PNGase F) Digestion

Samples were systematically digested with PNGase F (BioLabs) according to manufacturer's recommendations. SDS was added at a final concentration of 0.5%, and samples were boiled 5 min at 100°C. Nonidet P40 was added at a final concentration of 1% (v/v) in order to neutralize SDS. Buffer G7 (phosphate buffer pH 7.5) was finally added to a final concentration of 0.05 M. After addition of the PNGaseF, samples were incubated overnight at 37°C.

Labeled Galactose Transfer on GlcNAc Residues With Galactosyltransferase

Galactosyltransferase is a specific and sensitive probe frequently used in the detection of *O*-GlcNAc on cytosolic and nuclear proteins [Whiteheart et al., 1989; Kearse and Hart, 1991]. Samples were added to an equal volume of sample buffer (56.25 mM HEPES, 11.25 mM $MnCl_2$, 250 mM galactose, and 12.5 mM AMP, pH 7) with proteases inhibitors (1 mM PMSF and 5 μg/ml of leupeptin and pepstatin, Sigma). 0.025 U of bovine milk GlcNAc β-1,4-galactosyltransferase (Sigma) and 5 μCi of UDP-[6-^{3}H]gal (Amersham) were finally added to initiate the reaction. The samples were incubated at 37°C for 2 h.

PAGE and Western Blotting

The immunoprecipitated proteins were separated on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [Laemmli, 1970] without 2-mercaptoethanol, Coomassie blue stained, dried under vacuum and then exposed on Biomax film® for the radiolabeled proteins, or electrophoretically transferred on nitrocellulose membrane (Schleicher & Schuell) and analyzed by Western blot for the non-radiolabeled proteins. Western blots were performed with nuclear extracts or WGA-selected nuclear extract proteins boiled in loading buffer, subjected to SDS–PAGE and electrophoretically transferred to Immobilon membranes (Millipore). Filters were incubated for 60 min in blocking buffer (5% non-fat dry milk in PBS). Filters were then placed overnight in blocking buffer containing rabbit serum diluted

1:200 (v/v) in tris-buffered saline (TBS)-Tween, followed by 30 min washing in blocking buffer, 0.1% Tween 20. Bound antibodies were revealed with an ECL kit (Amersham).

Western Blots with WGA were saturated in 3% bovine serum albumin (BSA) in TBS-Tween (15 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween) for 45 min and washed three times for 15 min with TBS-Tween [Datta et al., 1989]. Horseradish peroxidase-labeled WGA was incubated in TBS-Tween (1/10,000) for 1 h. Blots were washed three times with TBS-Tween for 15 min and detection was done with the ECL Western blotting detection reagent (Amersham).

In order to control WGA specificity and avoid non-specific lectin binding, control chase experiments were performed, where 0.2 M free GlcNAc was added together with WGA for 1 h.

β-Elimination and Carbohydrate Analysis of Pax-6 Products

After labeling with 50 μCi [^{14}C]glucosamine and immunoprecipitation of the Pax-6 products, β-elimination was performed on the samples using 0.1 M NaOH solution containing 1 M sodium borohydride at 37°C for 72 h. The reaction was stopped by dropwise addition of ice-cold acetic acid, under stirring, until pH 5.0. After vacuum evaporation (three times in a rotavapor) with anhydrous methanol in order to remove borate as its methyl ester, the residue was dissolved in 0.5 ml water and further desalted by descending paper chromatography on Whatman 3 MM paper with n-butanol/ethanol/water (4/1/1) (v/v/v) as a solvent. Radioactivity was detected after cutting the lanes into 1-cm pieces and counting. Radioactive fractions were eluted from corresponding regions with water, lyophylisated and then finally analyzed by high pH anion exchange chromatography (HPAEC) [Diekmann-Schuppert et al., 1993] using a Dionex HPLC system equipped with a model PAD2 pulsed amperometric detector (Dionex Corp., Sunnyvale, CA). The column was a Carbopac -PA-1 (4 mm × 250 mm) pellicular anion exchange column with a PA-1 guard column (4 mm × 50 mm). The column was eluted at a flow rate of 1 ml/min with 15 mM NaOH. Fractions were collected, and radioactivity was measured by liquid scintillation counting.

In Vitro Transcription

The different Pax-6 coding sequences cloned in pSG5 [Carrière et al., 1993] were transcribed in vitro using T7 polymerase according to the manufacturer's specifications (Promega). Proteins were translated in vitro using a rabbit reticulocyte lysate in the presence of L-[^{35}S] methionine (1,000 Ci/mmol, Amersham). Ten microliters of extracts containing labeled proteins were incubated for 1 h at 4°C in 0.5 ml of 25 mM HEPES, pH 7.5, 12.5 mM $MgCl_2$, 150 mM KCl, 0.1% Nonidet P40, 20% glycerol with WGA sepharose beads. The beads were washed four times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris/HCl, pH 8, 0.5% Nonidet P40) and bound proteins were released by heat denaturation, analyzed on a 10% SDS gel incubated in 16% sodium salicylate, dried, and subjected to autoradiography. The percentage of bound radioactivity was calculated using PhosphorImager (Molecular Dynamics).

Pax-6-DNA Binding Assays

The DNA-binding procedure was carried out as previously described [Hay et al., 1989]. The DNA probe used was the P6CON double-strand oligonucleotide (5′-GGATGCAATTTCACG-CATGAGTGCCTCGAGGGATC-3′) [γ^{32}P]ATP-labeled with the polynucleotide kinase T4. The P6CON probe is a specific DNA sequence known to be recognized by p46 [Epstein et al., 1994].

The p46 protein produced in reticulocytes lysates was bound to WGA beads as described above. The beads were rinsed in DB buffer and recovered with 10 μl of the same buffer. DNA binding was then performed by incubating the beads with [1 μg poly (dI) poly (dC) (1 mg/ml); 5 ng of ^{32}P labeled P6CON probe (5 μl); 4 μl H_2O] for 10 min at room temperature. Beads were then washed two times in washing buffer (10 mM Tris/HCl, pH 8, 50 mM NaCl, 0.5 mM EDTA, and 0.1% NP40) and recovered with 20 μl of the same buffer. The labeled DNA was recovered by phenol/chloroform extraction and loaded on a polyacrylamide gel in Tris/borate/EDTA buffer.

Fifty nanograms of bacterially expressed glutathione S-transferase (GST)-p46 fusion protein (10 μl) were incubated with [1 μg poly (dI) poly (dC) (1 mg/ml); 5 ng of ^{32}P labeled P6CON probe (5 μl); 16 μl buffer DB] for 10 min at room temperature. The binding product was then immunoprecipitated for 1 h at 4°C with a rabbit antiserum directed against the paired domain linked to protein A sepharose beads in 200 μl of washing buffer. As for WGA beads, protein A sepharose beads were then washed two times in

washing buffer and recovered with 20 μl of the same buffer. The labeled DNA was also recovered by phenol/chloroform extraction.

RESULTS

All experiments were performed at least in duplicate, except for the [^{14}C]GlcNH$_2$ labeling.

Pax-6 Products Are Specifically Bound by WGA

In order to detect the Pax-6 products, QNRs were isolated and labeled with [^{35}S]Cys/Met for 1 h. SDS–PAGE analysis of Pax-6 products immunoprecipitated with serum 11 (directed against the paired domain) revealed a predominant doublet with an apparent molecular weight of 48 and 46 kDa, respectively, and a minor protein of 43 kDa (Fig. 1a). The same proteins were recognized with immune serum 13 (directed against the homeodomain), but no recognition was observed with the pre-immune serum named p13. Serum 13 also revealed a band of approximately 32/33 kDa. These immunoprecipitated were electrotransferred on a nitrocellulose sheet and checked for the presence of GlcNAc by Western blotting using peroxidase-labeled WGA. Since GlcNAc residues recognized by WGA may be either *N*- or *O*-glycans linkages, the samples were digested with PNGaseF before electrophoresis.

The 48/46 kDa double band was stained by WGA, but no staining neither of the 43 nor of the 32/33 kDa minor bands (Fig. 1b) was observed. The WGA binding was inhibited by co-incubation with 0.2 M-free GlcNAc, a competitive inhibitor of WGA (Fig. 1b). These experiments demonstrate that the 48/46 kDa sized Pax-6 products express *O*-linked terminal GlcNAc.

Pax-6 Products Are Specifically Recognized by WGA in the Neuroretina Nuclear Extracts

We previously reported that the different Pax-6 proteins exhibit distinct nuclear localization signals and distinct subcellular localization and that p48 and p46 were exclusively found into the nucleus, whereas the p43 and paired-less p32/33 proteins were localized both in the nucleus and the cytoplasm [Carrière et al., 1993, 1995]. We thus address the question whether the glycosylated form of this transcription factor could be found in the nucleus. As shown in Figure 1B, neuroretina nuclear extract incubated with WGA-sepharose contains the p48 and p46 Pax-6 isoforms. In contrast, the p43 is only very faintly detected,

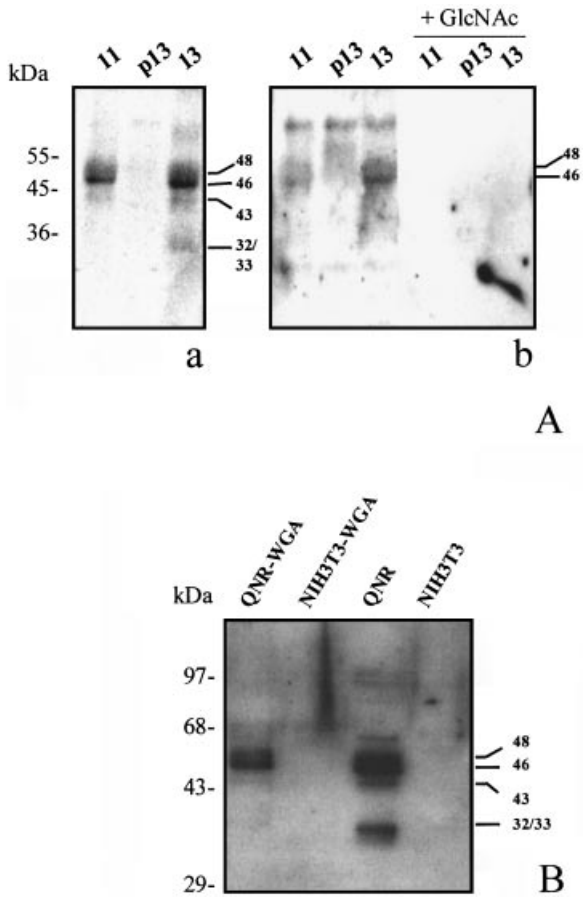

Fig. 1. **A**: Pax-6 products are specifically recognized by WGA. Pax-6 products were immunoprecipitated with serum 11 (directed against the paired domain), immune serum 13 (directed against the homeodomain), and a control was done with pre-immune serum named p13. These immunoprecipitated were submitted to a 12.5% SDS–PAGE, electrotransferred on a nitrocellulose sheet, and analyzed by autoradiography (**a**) for [^{35}S]Cys/Met-labeled QNRs or by Western blotting using peroxidase labeled-WGA according to their terminal GlcNAc content (**b**). A control chase experiment was realized by co-incubation of WGA with 0.2 M GlcNAc (b). Prior to the analysis, samples were systematically hydrolyzed with PNGaseF in order to remove possible *N*-glycans. **B**: Pax-6 products recognized by WGA in neuroretina nuclear extracts. Neuroretina nuclear extracts (**lane QNR**), neuroretina cell extracts purified on WGA-agarose beads (**lane QNR–WGA**) or as a control, NIH 3T3 nuclear extract (**lane NIH3T3**) or nuclear extracts purified on WGA-agarose beads (**lane NIH3T3–WGA**) were allowed to react with rabbit serum 14 directed against the Pax-6 carboxyl terminus. Bound antibodies were detected with an ECL detection kit.

and the paired-less proteins observed in the total nuclear extract are undetected after WGA-enrichment suggesting that in the nucleus the Pax-6 proteins were essentially glycosylated on the paired domain. NIH3T3 cells nuclear extracts were used as negative controls. According to Xu and Saunders [1997], NIH3T3 cells did not contains Pax-6 products.

In Vitro Labeling of Pax-6 Products With Galactosyltransferase

In order to confirm the *O*-*N*-acetylglucosaminylation of Pax-6 products, an in vitro labeling approach was used. Bovine galactosyltransferase specifically transfers a [^{3}H]galactose residue from UDP-[^{3}H]galactose on terminal GlcNAc. After PNGase F treatment, the different immunoprecipitates (sera 11 and 13, and pre-immune serum 13) were incubated with galactosyltransferase for 4 h. These immunoprecipitates were further analyzed on a 12.5% SDS–PAGE gel and visualized by fluorography (Fig. 2). Galactose incorporation was observed for the 46/48 kDa band, but not for the 43 and 33/32 kDa isoforms.

This experiment confirms the previous WGA staining results, and strongly asserts the occurrence of covalently attached terminal GlcNAc on QNR-Pax products *O*-glycans.

Cell Labeling With [^{14}C]GlcNH$_2$

QNR were specifically radiolabeled with [^{14}C]GlcNH$_2$ overnight. After immunoprecipitation with serum 11 and pre-immune serum 13, and PNGaseF treatment, QNR-Pax products were analyzed on a 12.5% SDS–PAGE gel and visualized by fluorography (Fig. 3). A staining was observed for the 48/46 kDa band, but the p43 band remained undetectable confirming the previous experiments.

Determination of the Nature of Glycosidic Linkage

To clearly establish the nature of the glycosidic linkage occurring on Pax-6 products, the [^{14}C]glucosamine-labeled Pax-6 products were subjected to alkaline treatment in the presence of an excess of sodium borohydride in order to release the *O*-linked glycans from the proteins. The released carbohydrates were analyzed by HPAEC. Figure 4 (A = pre-immune serum 13 and B = immune serum 11) shows that when compared to the different standards, a single peak corresponding to [^{14}C]*N*-acetylglucosaminitol was detected. This clearly indicates that glycosylation consists of single GlcNAc residues directly *O*-linked to serine or threonine

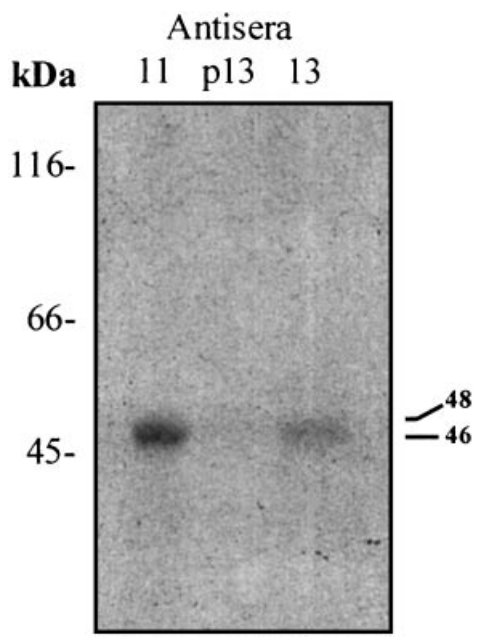

Fig. 2. In vitro labeling of Pax-6 products with galactosyltransferase. [^{3}H]galactose was specifically transferred from UDP-[^{3}H]galactose on external GlcNAc using bovine galactosyltransferase. After treatment with PNGase F, the different immunoprecipitates (sera 11 and 13, and pre-immune serum 13) were incubated with galactosyltransferase for 4 h and further analyzed on a 12.5% SDS–PAGE and visualized by fluorography.

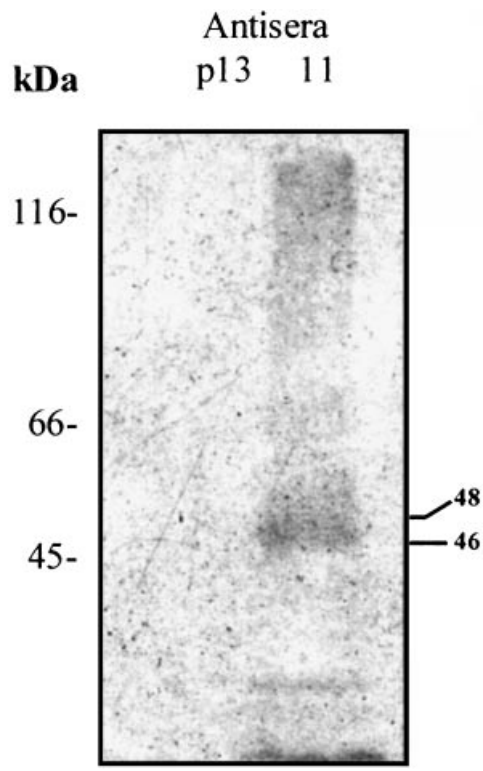

Fig. 3. Incorporation of [^{14}C]GlcNH$_2$ in the culture cells. QNRs were specifically radiolabeled with [^{14}C]GlcNH$_2$ overnight in a glucose poor medium. After immunoprecipitation with serum 11 and pre-immune serum 13, treatment with PNGase F, QNR-Pax products were analyzed on a 12.5% SDS–PAGE gel and visualized by fluorography.

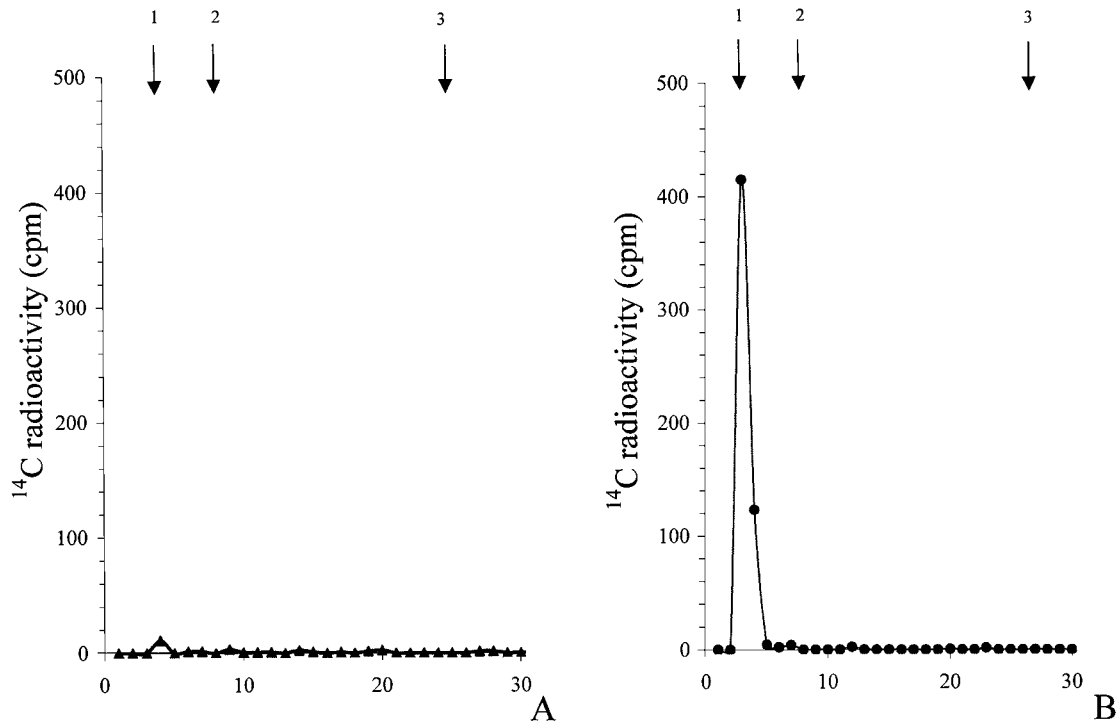

Fig. 4. Determination of the nature of the glycosylation. To determine the nature of the glycans on Pax-6 products, the [^{14}C]glucosamine-labeled Pax-6 products were subjected to reductive alkaline treatment in the presence of excess sodium borohydride in order to release *O*-linked sugars from the proteins. The released saccharides were analyzed by HPAEC. (1, glucosaminitol; 2, lactosaminitol; 3, glucosamine). **A**: Pre-immune serum 13 and (**B**), immune serum 11.

residues of the QNR Pax-6 products peptidic backbone. No larger oligosaccharides were observed.

WGA-Selected Pax-6 Proteins Bind DNA Efficiently In Vitro

Binding of p53 transcription factor to DNA has been reported to be modulated by *O*-glycosylation occurring at the carboxy-terminus [Shaw et al., 1996]. In the same idea, we decided to investigate the DNA binding capacity of WGA-selected Pax-6 isoforms in vitro. Reticulocyte lysates programmed with Pax-6 RNA encoding each a distinct isoform (Fig. 5A) were incubated with the WGA-sepharose in order to recover the glycosylated proteins (Fig. 5B). Three percent of the paired-containing proteins were recovered after incubation with the WGA beads and only 1% of the paired-less proteins. In contrast to the previous experiments, and especially the results presented in Figure 1B (in which the p43 and p32/33 bands were easily detectable), all the QNR Pax isoforms are glycosylated when expressed in the reticulocyte. If p48 and p46 are effectively glycosylated in the original cell (QNR), all of the isoforms appear as being glycosylated in the reticulocyte. This observation could reflect a difference in the *N*-acetylglucosaminyltransferase activity of the different cells, but more probably may be related to a difference in the protein conformation in both cell types.

The WGA-selected p46 proteins or, as control, reticulocytes programmed with an empty pSG5, were incubated with ^{32}P-labeled P6CON, a specific DNA sequence known to be recognized by p46 [Epstein et al., 1994]. As shown in Figure 5C, the WGA-selected p46 protein is able to bind the P6CON DNA probe. The same experiment was repeated with a non-glycosylated GST-p46 fusion protein reexpressed in *Escherichia coli* (GST was used as control). Both the glycosylated p46 form, and the unglycosylated p46 form bind to the DNA sequence.

DISCUSSION

The limited amount of Pax-6 products, the poor efficiency of tritium, and ^{14}C labeling, makes the detection of *O*-linked glycans on Pax-6 very difficult. In this study, we clearly

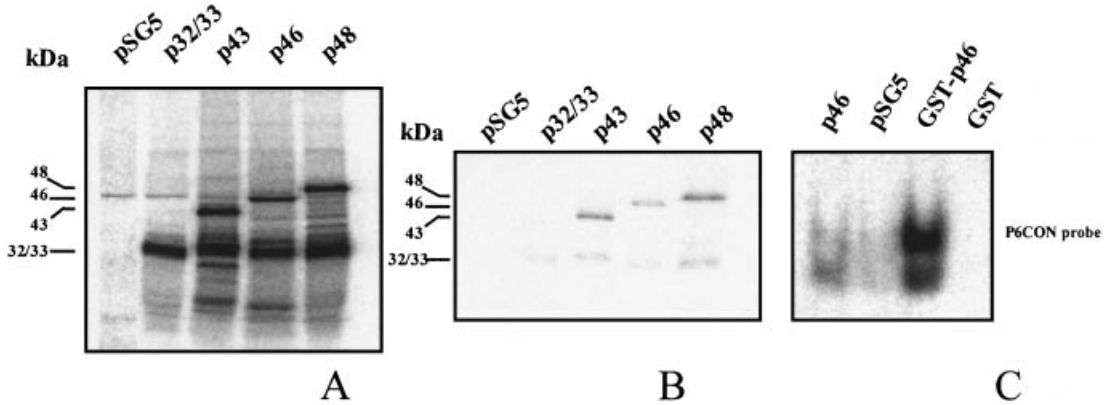

Fig. 5. Pax-6 products recognized by WGA exhibit a DNA-binding activity in vitro. **A**: The Pax-6 cDNAs encoding the p48, p46, p43, and p32/33 proteins were translated in rabbit reticulocyte lysates in the presence of L-[^{35}S]methionine. **B**: Equal amounts of translated proteins were incubated with WGA-agarose beads. The percentage of bound proteins was calculated using a PhosphorImager. **C**: The DNA–protein complexes formed between the labeled p6CON probe, and the WGA-bound p46 protein were recovered. The probe was extracted and subjected to electrophoresis. GST-p46 represents p46 product in *E. coli*.

demonstrate using different experimental approaches that the Pax-6 products are modified with *O*-GlcNAc. *O*-GlcNAc is the most abundant glycosylation type found within the cytosolic and the nuclear compartments. Different potential functions have been speculated for *O*-linked GlcNAc, including a role in the assembly of multimeric protein complexes, a role as phosphorylation antagonist, its involvement in nuclear transport of cytosolic proteins with involvement of the nuclear pore complex *O*-GlcNAc proteins. According to previous reports of peptide requirements for *O*-GlcNAc transferase, and using the YinOYang 1.2 Prediction results (http://www.cbs.dtu.dk/services/YinOYang/), we attempted to predict the potential *O*-GlcNAc attachment sites for the p46 isoform as indicated in Table I.

The peptidic sequence analysis indicates at least 12 potential regions for *O*-*N*-acetylglucosaminylation (bold type). These regions include Ser/Thr-rich sequences or sequences containing a serine or a threonine residue near (2 or 3 residues) a proline residue. Potential sites for *O*-GlcNAc are located in the two DNA-binding domains (Paired box; Black box, Homeodomain; Stirred box and the L-domain is between these two DNA-binding domains). The highest concentration of putative sites is found in the carboxyterminal portion that contains the transactivator domain. Nevertheless, the WGA-selected proteins of nuclear Pax-6 products suggest that the major *O*-GlcNAc sites are rather localized in the paired box domain than in the homeo and carboxyterminal domains. The differences observed between the presumed sites from the primary sequence and the effective sites may be explained by the 3-D structure of the different Pax-6 products domains.

In QNR, *O*-GlcNAc glycosylation was demonstrated to preferentially occurring on the p48 and p46 isoforms, which are strictly localized in the nucleus, whereas we did not detect *O*-GlcNAc on the p43 and p32/33 isoforms. These two last isoforms are distributed both in the cytosolic and in the nuclear compartments. In this idea, a possible role for *O*-GlcNAc in the nucleo-cytoplasmic transport has been recently advanced by Duverger et al. [1993, 1995, 1996]. These authors have demonstrated that serum albumin substituted with β-di-*N*-acetylchitobioside (GlcNAc β-1, 4 GlcNAc) or α-glucosyl is transported in the nucleus in a time-and ATP-dependent manner. In view of this finding, we suggest that *O*-GlcNAc could be a resident signal for nuclear proteins.

Gene transcription is under the control of *cis*-acting sequences that are recognized by DNA binding proteins, which activate or repress the transcription, via interactions with other components of the transcriptional machinery. *O*-GlcNAc residues expected to be present in the paired box and in the homeodomain could play a role in the DNA binding and/or in protein–protein interaction. WGA-enriched proteins are able to precipitate p46 together with a specific DNA sequence: P6CON [Epstein et al., 1994]. Additionally non-glycosylated p46 expressed by *E. coli* is able to bind the same DNA target. This observation indicates that *O*-GlcNAc residues

TABLE I. Primary Sequence of the QNR Pax-6 p46 Isoform

1 MQNSHSGVNQLGGVFVNGRPL**PDSTR**QKIVELAHSGARPCDISRILQVSN 50

51 GCVSKILGRYYETGSIRPRAIGGSKPRVATPEVVSKIAQYKRECPSIFAW 100

101 EIRDRLLSEGVCTNDNI**PSVSS**INRVLRNLASEKQQMGADGMYDKLRMLN 150

151 GQTGTWGTRPGWY**PGTS**VPGQPAQDGCPQQEGGGEN**TNSISS**NGEDSDEA 200

201 QMRLQLKRKLQRNR**TSFT**QEQIEALEKEFERTHYPDVFARERLAAKIDLP 250

251 EARIQVWFSNRRAKWRREEKLRNQRRQA**SNTPS**HI**PISSSFSTS**VYQPIP 300

301 Q**PTTPGS**MLGRTDTALTNTYSALPPM**PSFT**MANNLPMQPPV**PSQTSSYS**C 350

351 ML**PTSPS**VNGRSYDTYTPPHMQTHMNSQPMG**TSGTTST**GLISPGVSVPVQ 400

401 VPGSEPDMSQYWPRLQ 416

Peptidic sequence analysis indicates at least 12 potential regions for *O*-*N*-acetylglucosaminylation (bold type). Potential sites for *O*-GlcNAc are located in the two DNA-binding domains (Paired box; Black box, Homeodomain; Stirred box, and the L-domain is between these two DNA-binding domains).

in p46 isoform of Pax-6 do not directly interact with DNA. Since these residues remain accessible to WGA after binding with the DNA specific sequence, they could also interact with other nuclear components for activation or repression of the transcriptional machinery. Numerous transcription factors have been demonstrated to be *O*-GlcNAc modified. Jackson and Tjian [1988] have tested eight RNA polymerase II transcription factors: Sp1, AP-1, AP-2, AP-4, CTF, Zeste, GAGA, and ADF-1, that are all *O*-GlcNAc glycosylated. These authors demonstrated that the glycosylated Sp1 isoform was 3–5-fold more efficient in activating transcription than the unglycosylated recombinant protein produced in *E. coli*. The results obtained for Sp1 indicated that WGA specifically represses the transcriptional activity of Sp1, suggesting that the glycosylation directly contribute to the transcriptional activation function of Sp1. Roos et al. [1997] have shown that Sp1 *O*-GlcNAc transactivation domain fails to bind to Sp1 partners, whereas non-glycosylated Sp1 transactivation domain binds efficiently to the same protein (the glycosylation block Sp1 dimerization and association with TAFII110). The role of *O*-GlcNAc glycosylation in other transcription factors is still poorly understood.

As a conclusion, *O*-GlcNAc plays different roles in the regulation of the activity of transcription factors. If the glycosylation does not directly affect the DNA binding of many transcription factors, it may modulate their interaction with other proteins. In this respect, inhibitory interactions involving the paired domain have been described between Pax-6 and Engrailed-1 [Plaza et al., 1997], and the Pax-6 homeodomain is found to interact with both the TATA-box-binding protein TBP and the antioncogene Rb [Cvekl et al., 1999]. Since the Pax-6 transcription factor could act as an activator [Plaza et al., 1993, 1995; Carrière et al., 1995] or a repressor of targets promoters transcription [Duncan et al., 1998], the *O*-GlcNAc modification that could function to inhibit the interactions between proteins [Roos et al., 1997] may be an essential determinant to define the positive or the negative activity of Pax-6. Pax-6 proteins exhibit potential *O*-GlcNAc sites in the carboxyterminal activation domain. *O*-GlcNAc modification has been found to undergo dynamic changes often in a signal-dependent manner [Kearse and Hart, 1991], and this modification has been compared to phosphorylation [Comer and Hart, 2000]. We have already described a balance between *O*-GlcNAc modification and phosphorylation [Lefebvre et al., 1999]; such a balance may modulate the activity of the Pax-6 proteins in a cell-specific manner.

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