Role of *O*-Linked β-*N*-Acetylglucosamine Modification in the Subcellular Distribution of Alpha4 Phosphoprotein and Sp1 in Rat Lymphoma Cells

Shauna M. Dauphinee,[1] Marlene Ma,[1] and Catherine K.L. Too[1,2]*

[1]Department of Biochemistry and Molecular Biology, Faculty of Medicine,
Dalhousie University, Halifax, Nova Scotia B3H 1X5, Canada
[2]Department of Obstetrics and Gynecology, Faculty of Medicine,
Dalhousie University, Halifax, Nova Scotia B3H 1X5, Canada

Abstract   The mTOR alpha4 phosphoprotein is a prolactin (PRL)-downregulated gene product that is found in the nucleus of PRL-dependent rat Nb2 lymphoma cells. Alpha4 lacks a nuclear localization signal (NLS) and the mechanism of its nuclear targeting is unknown. Post-translational modification by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) moieties has been implicated in the nuclear transport of some proteins, including transcription factor Sp1. The nucleocytoplasmic enzymes *O*-β-*N*-acetylglucosaminyltransferase (OGT) and *O*-β-*N*-acetylglucosaminidase (*O*-GlcNAcase) adds or remove *O*-GlcNAc moieties, respectively. If *O*-GlcNac moieties contribute to the nuclear targeting of alpha4, a decrease in *O*-GlcNAcylation (e.g., by inhibition of OGT) may redistribute alpha4 to the cytosol. The present study showed that alpha4 and Sp1 were both *O*-GlcNAcylated in quiescent and PRL-treated Nb2 cells. PRL alone or PRL + streptozotocin (STZ; an *O*-GlcNAcase inhibitor) significantly ($P \leq 0.05$) increased the *O*-GlcNAc/alpha4 ratio above that in control quiescent cells. However, PRL + alloxan (ALX; an OGT inhibitor) or ALX alone did not decrease *O*-GlcNAcylation of alpha4 below that of controls and alpha4 remained nuclear. In comparison, PRL ($\pm$ALX/STZ) greatly increased Sp1 protein levels, caused a significant decrease in the GlcNAc/Sp1 ratio ($P \leq 0.05$, n = 3) as compared to controls and partially redistributed Sp1 to the cytosol. Finally, a 50% downregulation of OGT gene expression by small interfering RNA (i.e., siOGT) partially redistributed both alpha4 and Sp1 to the cytosol. The alpha4 protein partner PP2Ac had no detectable *O*-GlcNAc moieties and its nuclear distribution was not affected by siOGT. In summary, alpha4 and Sp1 contained *O*-GlcNAc moieties, which contributed to their nuclear targeting in Nb2 cells. J. Cell. Biochem. 96: 579–588, 2005.   © 2005 Wiley-Liss, Inc.

Key words: prolactin; *O*-GlcNAc; Sp1; alpha4 phosphoprotein; lymphoma cells

A prolactin (PRL)-downregulated cDNA encoding alpha4 phosphoprotein was previously identified by differential display and screening of a rat Nb2-Sp T-lymphoma cDNA library [Too, 1997a; Boudreau et al., 2002]. Alpha4 is the mammalian homolog of yeast Tap42, an essential component of the target-of-rapamycin (TOR) kinase pathway that responds to nutrients to regulate translation initiation in yeast cells [Di Como and Arndt, 1996]. Similarly, the mammalian TOR (mTOR) kinase pathway responds to mitogens and/or nutrients to stimulate ribogenesis, translation initiation, and cell growth [Cutler et al., 1999]. Alpha4, like yeast TAP42, interacts with serine/threonine phosphatase PP2A and the alpha4-PP2A interaction is thought to stimulate translation initiation through effects on p70S6K and/or the translation repressor protein 4E-BP1 [Nanahoshi et al., 1998; Peterson et al., 1999].

Our full-length rat alpha4 cDNA encoded a 39-kDa protein when expressed as a GST-fusion protein in bacterial cells but a glycosylated, 45-kDa alpha4 was detected in the rat PRL-dependent Nb2 lymphoma cells [Boudreau et al., 2002]. Surprisingly, subcellular fractionation and western analysis showed alpha4 and its partner, the catalytic subunit of PP2A

(PP2Ac), predominantly in the nucleus of Nb2 cells. The mechanism by which alpha4 is targeted to the nucleus is not immediately evident since the primary sequence of alpha4 lacks any classical or annotated nuclear localization signal (NLS) [Cokol et al., 2000]. However, nonpeptidic nuclear-targeting signals may be provided by post-translational modifications, such as sumoylation [Pichler and Melchior, 2002], O-phosphorylation [Hood and Silver, 1999] and O-glycosylation [Duverger et al., 1996]. Nuclear and cytoplasmic proteins may also be covalently modified by the linkage of single O-linked β-N-acetylglucosamine (O-GlcNAc) moieties to specific serine and threonine residues [Comer and Hart, 1999]. O-GlcNAc moieties have been implicated in the nuclear targeting of signal-free neoglycoproteins [Duverger et al., 1993, 1995, 1996]. Nuclear transport of the transcriptional regulator c-Myc was impeded by point mutations of dominant sites of O-GlcNAc modification [Kamemura and Hart, 2003] and a reduction of O-GlcNAc incorporation into the microtubule-associated Tau proteins correlated with a decrease in the nuclear transport of Tau [Lefebvre et al., 2003].

Reversible O-linked N-acetylglucosaminylation (O-GlcNAcylation) is catalyzed by the nucleocytoplasmic enzymes O-β-N-acetylglucosaminyltransferase (OGT) and O-β-N-acetylglucosaminidase (O-GlcNAcase), which adds or removes O-GlcNAc moieties, respectively [Haltiwanger et al., 1992; Dong and Hart, 1994]. The pancreatic β-cell toxin alloxan (ALX) and the diabetogenic antibiotic streptozotocin (STZ) have been used to inhibit OGT and O-GlcNAcase activities, respectively, to modify protein O-GlcNAcylation [Roos et al., 1998; Konrad et al., 2002]. ALX, an analog of uracil, inhibits OGT via SH-group oxidation [Miller et al., 1999; Konrad et al., 2002] but may also inhibit many cellular reactions involving uridine nucleotides. STZ, a potent alkylating agent of DNA, stimulates protein O-GlcNAcylation [Konrad et al., 2000; Liu et al., 2000]. Used together, ALX blocked STZ-induced glycosylation of pancreatic β-cell proteins and a known OGT substrate, nucleoporin p62 [Konrad et al., 2002]. The sites of O-GlcNAcylation are often the same or adjacent to sites of serine/threonine phosphorylation, suggesting that the dynamic interplay between O-GlcNAcylation and O-phosphorylation regulates protein–protein

interactions [Roos et al., 1997], protein stability [Han and Kudlow, 1997] and subcellular localization [Kamemura and Hart, 2003].

Transcription factor Sp1 is known to undergo O-GlcNAcylation, which blocks Sp1-protein interactions [Roos et al., 1997] and inhibits Sp1 transcriptional capability on some promoters [Yang et al., 2001]. Recently, O-GlcNAcylation of Sp1 by insulin was shown to facilitate cytosol to nuclear movement of Sp1 [Majumdar et al., 2003]. Sp1 is involved in the transcriptional activation of many genes, including the PRL-regulated immediate early gene interferon-regulatory-1 (IRF-1) in Nb2 lymphoma cells [Book McAlexander and Yu-Lee, 2001]. Unlike PRL downregulation of alpha4 [Boudreau et al., 2002], PRL rapidly increases de novo biosynthesis of Sp1 and Sp1-DNA-binding in the Nb2 cells [Too, 1997b].

The present study examined the role of O-GlcNAc moieties in the nuclear targeting of alpha4 and Sp1 in Nb2 cells. We showed that nuclear alpha4 and Sp1 were O-GlcNAcylated in quiescent cells. PRL alone or PRL + ALX/STZ elevated Sp1 protein levels above that in quiescent cells, significantly decreased the O-GlcNAc/Sp1 ratio and resulted in a partial redistribution of Sp1 from the nucleus to the cytosol. PRL (±ALX/STZ) did not decrease O-GlcNAcylation of alpha4 below that in quiescent cells and alpha4 remained nuclear. However, downregulation of OGT gene expression (to decrease O-GlcNAcylation) by small interfering RNA (siRNA) caused a partial redistribution of alpha4 and Sp1 to the cytosol.

MATERIALS AND METHODS

Antibodies and Reagents

Rabbit anti-alpha4 antibody was purified on Protein A-Sepharose and desalted as previously described [Boudreau et al., 2002]. The commercial sources of antibodies used were as follows: mouse anti-O-GlcNAc (CTD110.6) (Covance Research Products, Inc., Denver, PA); rabbit anti-Sp1 (sc-59), anti-TFIIB (SI-1), anti-PRLr, and horse radish peroxidase (HRP)-conjugated-goat-anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); donkey anti-rabbit IgG-HRP conjugate (Amersham Pharmacia Biotechnology, Baie d'Urfe, Quebec, Canada); and mouse anti-PP2Ac (Transduction Laboratories, Lexington, KY). STZ, ALX, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine

were from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Human PRL was a generous gift from Dr. Robert P.C. Shiu (Department of Physiology, University of Manitoba).

Cell Culture

Suspension cultures of PRL-dependent rat Nb2-11C (Nb2) lymphoma cell line were maintained at 37°C in Fischer's medium containing 10% fetal bovine serum (FBS) as a source of lactogens and 10% lactogen-free horse serum (HS) as previously described [Too et al., 1987]. Confluent Nb2 cells ($\sim 1.0 \times 10^6$ cells/ml) were made quiescent in medium containing HS alone (18–24 h). Quiescent cells were treated for 1 h with 5 mM STZ or 5 mM ALX or left untreated (control) before addition of PRL (10 ng/ml). Cell viability after STZ or ALX treatment for 24 h were more than 95% and 70%, respectively, as determined by the Celltiter 96 AQueous nonradioactive cell proliferation assay (Promega, Madison, WI).

Immunofluorescent Confocal Microscopy

Alpha4 is abundantly expressed in quiescent Nb2 cells, therefore, quiescent cells were fixed, detergent-solubilized and cytospun onto microscope slides for immunofluorescent microscopy as previously described [Too et al., 2001]. Cells were double-labeled by sequential incubation with the first primary antibody (1:10 of 4 mg/ml anti-alpha4 IgG), the first secondary antibody (1:50 of AlexaFluor 488 goat anti-rabbit IgG conjugate), the second primary antibody (1:10 of monoclonal anti-PP2Ac) and, finally, the second secondary antibody (1:50 of AlexaFluor 594 goat anti-mouse IgG conjugate). Each antibody incubation was performed in the dark for 1 h at room temperature and separated by $3 \times$ PBS washes. After the final PBS wash, the slides were mounted in Citifluor-glycerol/PBS AF1 solution (Marivac Halifax Ltd., Halifax, Nova Scotia, Canada). Confocal immunofluorescence microscopy was performed at $100\times$ magnification. To validate the quiescent Nb2 cell morphology, quiescent cells ($\pm$PRL for 24 h) were prepared. Cell nuclei were visualized by propidium iodide staining (Annexin-V-Fluos Staining Kit, Boehringer Mannheim) whereas the cytoplasm was double-labeled with anti-Cdc5 antibodies followed by Alexa-Fluor 488 goat anti-rabbit IgG. Cdc5 is a transcription factor whose biosynthesis is upregulated by PRL [Johnson and Too, 2001].

Subcellular Fractionation

Nb2 cell pellets ($\sim 10 \times 10^6$ cells) were resuspended in 100 µl cold lysis buffer containing 150 mM NaCl, 50 mM Tris, 1 mM EGTA, 50 mM sodium pyrophosphate, and freshly added protease inhibitors (1 mM phenylmethylsulphonyl fluoride; 10 µg/ml each of antipain, leupeptin, and pepstatin). Cell lysates were processed for nuclear (800 × g pellet, 5 min) and cytosolic (800 × g supernatant) fractions as described [Dodd et al., 2000]. The nuclear pellet was resuspended in 100 µl of lysis buffer and BioRAD protein assays were performed for all fractions. Proteins (10–20 µg/lane), representing about 20% of total protein from each fraction, were electrophoresed on 10% SDS–PAGE gels and used for Western analysis.

Immunoprecipitation (IP) and Western Analysis

IP and western analysis were carried out as previously described [Dodd et al., 2000], but with sparing use of purified anti-alpha4 antibodies ($\sim$7 µg/µl for $\sim 50 \times 10^6$ cells) to minimize the 50-kDa IgG band during immunoblotting (IB). IB was performed with primary rabbit anti-α4 (1:300 of 7.34 µg/µl purified IgG in 10% milk-TTBS), monoclonal anti-PP2Ac (1:5,000), rabbit anti-Sp1 (1 µg/ml), or monoclonal anti-*O*-GlcNAc (1:4,000). HRP-conjugated secondary antibodies (1:5,000 donkey anti-rabbit IgG or 1:1,250 goat anti-mouse IgG) were used to detect immunoreactive signals with SuperSignal ULTRA (Pierce, Rockford, IL). For competition experiments, 25 mM *N*-acetylglucosamine or *N*-acetylgalactosamine were included during primary antibody incubation.

siRNA Gene Silencing

Nb2 cells (5×10^6 cells) in 400 µl of siPORT™ siRNA electroporation buffer (Ambion, Inc., Austin, TX) were placed in 4 mm curvettes (BioRAD). Predesigned siRNAs (Ambion, Inc) targeting OGT (ID no. 88214) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or the negative control siRNA (200 nM each) were electroporated into Nb2 cells with a single pulse of a set voltage (250–350 V) at 960 mF capacitance, zero resistance and time constants of $\sim$36 ms, using a BioRAD GenePulser II electroporator. Nb2 cells were difficult to electroporate with cell viability of 80%–90% at 250 V and $\sim$50% at 300–350 V. Electroporated cells were resuspended in 10 ml of 10% FBS-Fischer's

medium. After a 48 h recovery period at 37°C, cells were harvested for protein (nuclear and cytosol fractions) and RNA using Ambion's PARIS™ Kit. Gene expression was analyzed by semi-quantitative RT-PCR with GAPDH primers as previously described [Too et al., 2001]. OGT primers, 5′-gtggcggcagtagaagtc-3′ (forward) and 5′-gccgcaaagcatgtcgat-3′ (reverse), gave a 376-bp PCR product. Proteins (10–20 µg/lane), ~20% of total protein from the nuclear and cytosolic fractions, were used for Western analysis.

Statistical Analysis

Analysis of variance was performed using InStat software program (GraphPad Software, Inc., San Diego, CA).

RESULTS

Alpha4 is Predominantly Nuclear

We previously showed, by subcellular fractionation and western analysis that alpha4 was abundantly expressed in quiescent Nb2 cells and the protein was localized predominantly in the nucleus [Boudreau et al., 2002]. Quiescent Nb2 cells have large nuclei with little cytoplasm (Fig. 1A). Confocal immunofluoresent microscopy confirmed that alpha4 was nuclear in quiescent cells and showed its partial co-distribution with nuclear PP2Ac (Fig. 1B).

Alpha4 Is Modified by *O*-GlcNAc Moieties

The rat Nb2 alpha4 is glycosylated with *N*- and/or *O*-linked polysaccharides [Boudreau et al., 2002]. We now examined if it also contained single *O*-GlcNAc moieties with potential nuclear targeting action. A computer-based prediction server (http://www.cbs.dtu.dk/services) identified seven serine/threonine residues in alpha4 that may potentially undergo *O*-GlcNAcylation (Table I). To test this prediction, IP/IB analysis was performed. The monoclonal anti-*O*-GlcNAc CTD110.6 antibody detected *O*-GlcNAc residues in the Nb2 alpha4 protein (Fig. 2A). The addition of 25 mM glucosamine competed with antibody binding whereas an equivalent concentration of galactosamine had no effect (Fig. 2B), thus confirming antibody specificity.

PP2Ac Has No Detectable *O*-GlcNAc Moieties

The Nb2 alpha4 interacts with PP2Ac [Boudreau et al., 2002]. The prediction server also identified a total of five serine/threonine residues in PP2Ac that may be modified by *O*-GlcNAcylation (Table I). Figure 2A showed that alpha4 co-immunoprecipitated with its protein partner PP2Ac (36 kDa) but no 36-kDa *O*-GlcNAc immunoreactive band was detected, suggesting that the PP2Ac may not have *O*-GlcNAcylated residues. Specific IP of PP2Ac
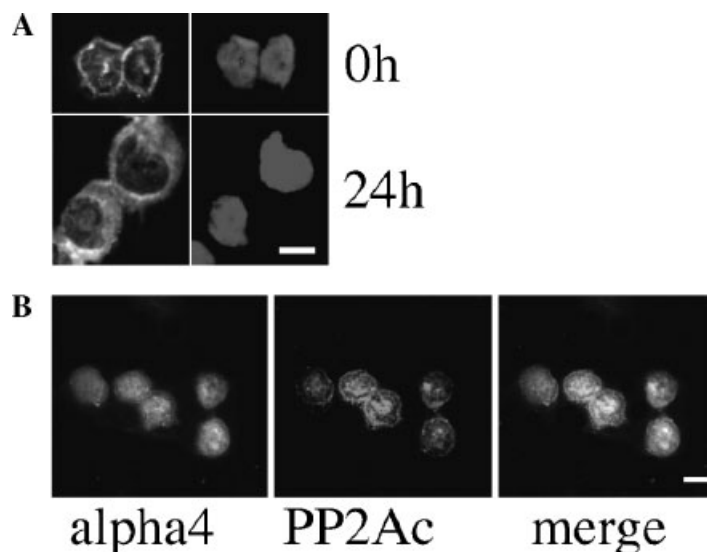

Fig. 1. Confocal microscopy shows nuclear alpha4 and PP2Ac. **A**: The thin layer of cytoplasm in quiescent Nb2 cells was robustly restored within 24 h of PRL treatment. The nuclei were demarcated by propidum iodide staining (**right panels**) and the cytoplasm by PRL-inducible Cdc5 (**left panels**) as described in Materials and Methods. **B**: Quiescent Nb2 cells were double-labeled with anti-alpha4 (**left panel**) or anti-PP2Ac (**middle panel**) antibodies and with the appropriate AlexaFluor secondary antibodies. Merged fluorescence (**right panel**). Bar, 10 µm.

TABLE I. Predicted *O*-GlcNAc Sites on Alpha4 and PP2Ac

Sequence name	Residue		*O*-GlcNAc	Yin-Yang
Alpha4	34	EPTGS	++	
	90	ALTMK	+	a
	130	PQTKN	+++	
	139	NNTAR	++	
	142	ARSSM	+	
	229	EESAC	+	a
	269	TVSDW	+	
PP2Ac	24	QLSES	+	a
	53	PVTVC	+	
	238	LVSRA	+	
	258	VVTIF	+	
	301	PHVTRR	+	a

[a]Yin-Yang indicates the potential for reciprocal *O*-glycosylation and *O*-phosphorylation.
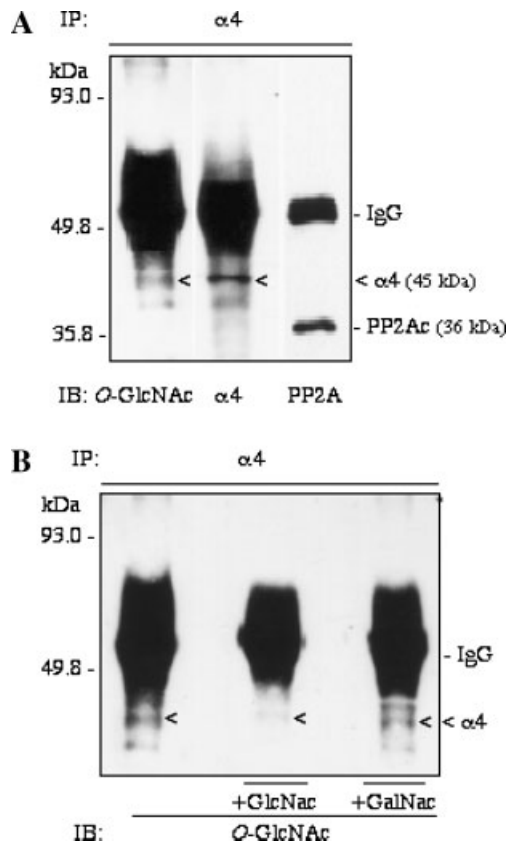

Fig. 2. Nb2 alpha4 contains *O*-GlcNAc moieties. Total Nb2 cell lysates were prepared from log-phase growing cells (50×10^6 cells per treatment). Cell lysates were used for IP of alpha4 as described in Materials and Methods. Immunocomplexes were resolved by 10% SDS–PAGE and transferred to nitrocellulose membranes for (**A**) IB for *O*-GlcNAc moieties, alpha4 or PP2Ac. Blots were stripped in between. (**B**) IB of alpha4 was performed in the presence of 25 mM glucosamine (GlcNac) or 25 mM galactosamine (GalNac) during incubation with primary antibody. Molecular weight markers are shown. Representative of three experiments.
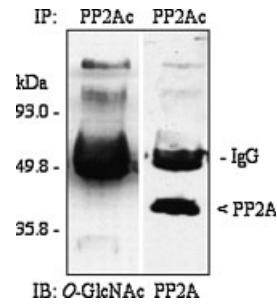

Fig. 3. Nb2 PP2Ac lacks *O*-GlcNAc moieties. Total Nb2 cell lysates were prepared as in Figure 2. IP of PP2Ac was followed by IB for *O*-GlcNAc moieties or for PP2Ac. Molecular weight markers are shown on the left. Representative of two experiments.

also showed no detectable *O*-GlcNAcylation of this protein (Fig. 3). However, it is possible that these moieties, if present on PP2Ac, were not readily detectable by the CTD110.6 antibody.

Sp1 Has *O*-GlcNAc Moieties

Sp1 is known to undergo *O*-GlcNAcylation [Roos et al., 1997] and would be a good positive control to test the CTD110.6 antibody. In Nb2 cells, PRL rapidly induces Sp1 biosynthesis within 30 min and this is abrogated by cycloheximide [Too, 1997b]. Elevated levels of the Sp1 protein (95-kDa) was preserved for at least 24 h after PRL treatment [Too, 1997b] and were consistently higher in the nucleus than cytosol (see Fig. 4). The CTD110.6 antibody detected a number of immunoreactive bands in the Sp1 immunocomplex but there was a distinct 95-kDa band indicating *O*-GlcNAcylation of nuclear Sp1 (Fig. 4).

Effects of PRL, STZ, and ALX on *O*-GlcNAc Modification of Alpha4

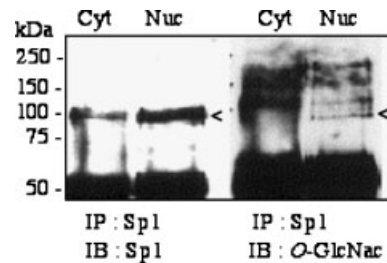PRL regulation of alpha4 *O*-GlcNAcylation was examined with the β-cell toxins, ALX, and

Fig. 4. Nuclear Sp1 is *O*-GlcNAc modified. Quiescent Nb2 cells were given PRL for 24h. Nuclear (Nuc) and cytosolic (Cyt) fractions were prepared for IP/IB analysis as indicated. IB with anti-*O*-GlcNAc was performed, the blots stripped before IB with anti-Sp1 antibodies.

STZ, which have been used successfully to modify protein *O*-glycosylation [Konrad et al., 2000, 2002; Liu et al., 2000]. STZ increases protein *O*-glycosylation by blocking *O*-GlcNAcase activity [Roos et al., 1998] whereas ALX decreases protein *O*-glycosylation by inhibiting OGT [Konrad et al., 2002].

Immunoprecipitated alpha4, whether in control or PRL-treated cells, contained *O*-GlcNAc moieties (Fig. 5A). In the presence of PRL (±STZ), the *O*-GlcNAc/alpha4 ratio was significantly ($P \leq 0.05$) increased above controls whereas all other treatments had no significant effect (Fig. 5B). If *O*-GlcNAc moieties contributed to the nuclear targeting of alpha4, a decrease in *O*-GlcNAcylation might redistribute alpha4 to the cytosol. Since the *O*-GlcNAcylated alpha4 was already nuclear in control quiescent cells, a reduction of alpha4 *O*-GlcNAcylation would have to be achieved by other means (see below). Elevation of protein *O*-GlcNAcylation, for example, by using a more potent *O*-GlcNAcase inhibitor such *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc), was not further investigated.

Fig. 6. PRL (±STZ/ALX) decreases *O*-GlcNAc/Sp1 ratio. Quiescent Nb2 cells were treated with PRL, STZ (S), and/or ALX (Al) for 24 h. Control cells (Con) were left untreated. **A:** IP/IB of Sp1 or *O*-GlcNAc moieties were as indicated. **B:** Immunoreactive bands were scanned and the ratio of *O*-GlcNAc/Sp1 plotted. Mean ± SD of three independent experiments, absence of bars was due to small SD between experiments. Significantly decreased below (*) or increased above (+) controls, $P \leq 0.05$ (n = 3).

PRL (±STZ or ALX) Decreases *O*-GlcNAc Modification of Sp1

The effects of STZ and ALX on Sp1 *O*-GlcNAcylation were next examined. Immunoprecipitated Sp1 in quiescent Nb2 cells (controls) or cells treated with PRL showed positive *O*-GlcNAc immunoreactivity (Fig. 6A). In all treatment groups with PRL for 24 h, the Sp1 protein level was substantially increased above controls (Fig. 6A, upper panel) and densitometric analysis revealed a significant decrease in the *O*-GlcNAc/Sp1 ratio ($P \leq 0.05$, n = 3) as compared to controls (Fig. 6B). In the absence of PRL, STZ alone increased the *O*-GlcNAc modification of Sp1 above controls but ALX had no significant effect (Fig. 6B). Since the PRL + ALX treatment decreased the *O*-GlcNAc/Sp1 ratio it might be expected to redistribute Sp1 to the cytosol, and this was further investigated.

Effects of *O*-GlcNAc Modification on the Subcellular Distribution of Alpha4 and Sp1

Nb2 cells were treated with PRL and/or ALX to inhibit OGT activity. Figure 7 showed nuclear localization of Sp1 in control Nb2 cells or cells given PRL ± ALX for 3 h. At 24 h, however, PRL or PRL + ALX caused a partial redistribution of Sp1 from the nucleus to the
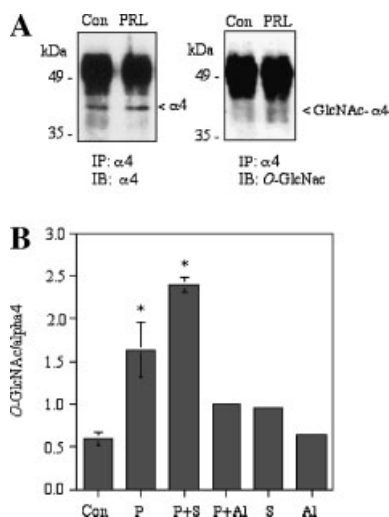

Fig. 5. Effects of PRL, STZ, and ALX on alpha4 *O*-GlcNAcylation. Quiescent Nb2 cells were treated with PRL (P; 10 ng/ml), STZ (S; 5 mM), and/or ALX (Al; 5 mM) for 24 h or left untreated (Con). In (**A**), IP/IB was performed as indicated. In (**B**), immunoreactive bands were densitometrically scanned and the ratio of *O*-GlcNAc/alpha4 plotted. Mean ± standard deviation (SD) of three independent experiments, absence of bar is due to small SD between experiments. *, significantly increased above controls, $P \leq 0.05$ (n = 3).
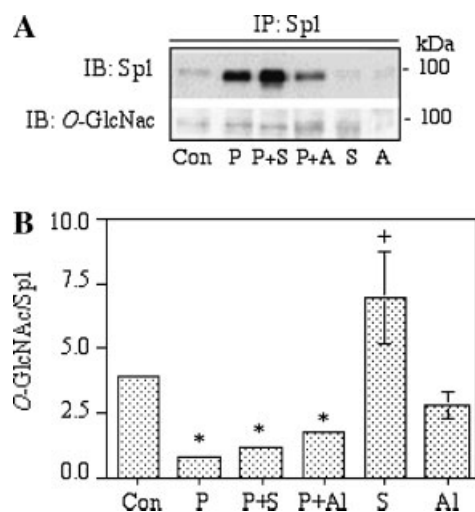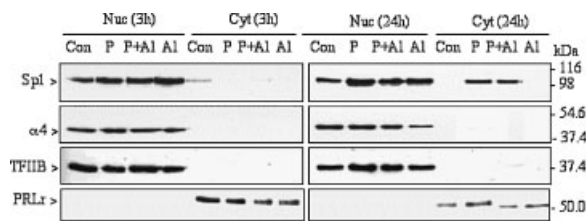
Fig. 7. Subcellular distribution of alpha4 and Sp1. Quiescent Nb2 cells were treated with PRL (P; 10 ng/ml) and/or ALX (Al; 5 mM) for 3 or 24 h. Cell lysates were subcellular fractionated as described in Materials and Methods. For each nuclear fraction, a fixed protein concentration of 20 μg/lane was used for SDS–PAGE and its percentage of the total nuclear protein was noted. The same percentage of the total cytosol protein was used for each corresponding cytosolic fraction. Western analyses for Sp1 and alpha4 were performed. Fraction integrity was confirmed by TFIIB (nuclear) and PRLr (non-nuclear).

cytosol. This effect might be due to the overall decrease in the *O*-GlcNAc/Sp1 ratio (Fig. 6), suggesting that a decrease in *O*-GlcNAcylation impeded Sp1 nuclear transport at 24 h. In contrast, there was no detectable nuclear-to-cytosol redistribution of alpha4 at 3 or 24 h, which was expected as PRL $\pm$ ALX did not decrease *O*-GlcNAcylation of alpha4 below control levels (Fig. 5).

Downregulation of OGT Partially Redistributes Alpha4 and Sp1 to the Cytoplasm

RNA interference (RNAi) of OGT gene expression could potentially decrease protein *O*-GlcNAcylation and redistribute glycosylated nuclear proteins, including alpha4, to the cytosol. In the positive control, siRNAs targeting GAPDH (siGDH), electroporated at 300 V, decreased GAPDH mRNA levels by ~70% as compared to the negative siRNA control (Fig. 8A). siOGT were similarly electroporated into Nb2 cells but at varying voltages to achieve a significant gene knock-down without cell death. Semi-quantitative RT-PCR was performed to measure levels of the OGT and GAPDH transcripts, the latter serving as a control. A decrease in OGT mRNA levels by only 30%–50% was achieved when electroporation was performed at 300 and 350 V (Fig. 8A, lanes 2 and 3). siOGT was not electroporated at higher voltages since Nb2 cell viability decreased to less than 50% above 350 V (data not shown). When OGT mRNA was decreased by 50% (350 V), there was a partial redistribution of alpha4 and Sp1 into the cytosol (Fig. 8B), suggesting that *O*-GlcNAc moieties contributed to the nuclear distribution of both proteins. In
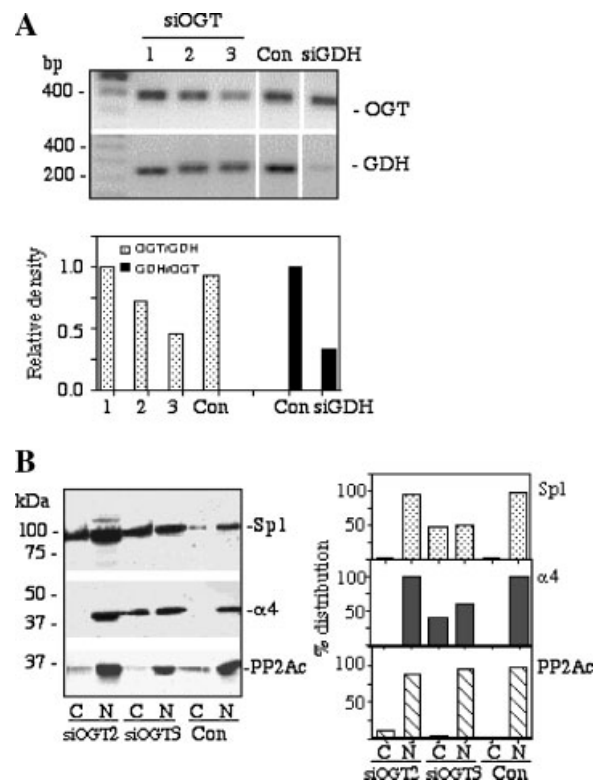

Fig. 8. Downregulation of OGT partially redistributes alpha4 and Sp1 to the cytosol. Nb2 cells were electroporated with 200 nM siOGT at (1) 250, (2) 300, or (3) 350 V, or 200 nM GAPDH siRNA (siGDH) or 200 nM negative siRNA control (Con) at 300 V (see Materials and Methods). (**A**) RT-PCR of OGT and GDH expression (**upper panel**). Plots of OGT/GDH and GDH/OGT ratios from densitometric scans (**lower panel**). (**B**) Western analysis of nuclear (N) and cytosolic (**C**) fractions after electroporation of siOGT at 300 (siOGT2) or 350 (siOGT3) V or the negative siRNA control at 300 V. Representative of 2 or 3 experiments, each in triplicates.

contrast, the predominantly nuclear distribution of PP2Ac was unchanged in controls and siOGT-treated cells (Fig. 8B).

DISCUSSION

Alpha4 and Sp1 localize predominantly in the nucleus of rat Nb2 lymphoma cells but neither protein has a classical NLS or any identifiable non-classical nuclear-targeting motifs. *O*-GlcNAcylation may serve as a nonpeptidic nuclear-targeting signal [Duverger et al., 1996]. The present study demonstrated that alpha4 was post-translationally modified by *O*-GlcNAc moieties in quiescent and PRL-stimulated Nb2 cells. If *O*-GlcNAc moieties played a role in the nuclear targeting of alpha4, then a reduction in *O*-GlcNAc modification might be expected to inhibit nuclear transport of alpha4.

ALX, the OGT inhibitor, was not effective in decreasing the O-GlcNAc/alpha4 ratio below that of controls and alpha4 remained nuclear. However, siRNA silencing of OGT gene expression (by ~50%) was able to partially redistribute alpha4 to the cytosol, suggesting that the O-GlcNAc moieties contributed to the nuclear targeting of alpha4. There is also the possibility that the nuclear transport of alpha4 arises from its interaction with a nuclear-targeted protein partner. Others and we have shown that alpha4 interacts with PP2Ac [Chen et al., 1998; Boudreau et al., 2002]. Nuclear and/or cytoplasmic forms of PP2Ac have been observed at different phases of the cell cycle [Turowski et al., 1995], clearly demonstrating that PP2Ac can shuttle between the nucleus and cytosol. The present study showed no detectable O-GlcNAc moieties on PP2Ac and there was no obvious effect of siOGT on the subcellular distribution of PP2Ac, indicating that nuclear PP2Ac itself was not directly due to these moieties.

O-GlcNAcylation may occur reciprocally with O-phosphorylation to regulate nuclear transport of proteins. For example, Tau proteins that were hyperphosphorylated but with a reciprocal reduction in O-GlcNAcylation showed reduced nuclear transport [Lefebvre et al., 2003]. There may not be a reciprocal relationship between O-GlcNAcylation and O-phosphorylation for alpha4. Table I shows that these two distinct modifications involve different serine/threonine residues on alpha4, with the exception of two weak O-GlcNAcylation sites on T90 and S229.

Sp1 is a well-known nuclear protein that is post-translationally modified by O-GlcNAc moieties [Roos et al., 1997; Haltiwanger et al., 1998]. The C-terminal region of Sp1 is required to target it to the nucleus of HeLa cells [Kuwahara et al., 1999]. This region contains a zinc-finger DNA-binding domain [Kuwahara and Coleman, 1990], purported to be responsible for the nuclear transport of Sp1 [Kuwahara et al., 2000]. The C-terminus of Sp1 also contains a dominant O-GlcNAcylation site [Roos et al., 1997] and O-GlcNAcylation of Sp1 by insulin was shown to facilitate cytosol to nuclear movement of Sp1 [Majumdar et al., 2003]. Our studies confirmed O-GlcNAcylation of Sp1. In Nb2 cells, PRL elevation of Sp1 protein levels was accompanied by a reduction in the O-GlcNAc modification of this nuclear protein. PRL ± ALX significantly decreased, but did not abolish, the overall O-GlcNAcylation of Sp1 and caused a partial redistribution of Sp1 in the cytosol at 24 h. Furthermore, siRNA silencing of OGT gene expression (by ~50%) also resulted in an equal distribution of Sp1 in the Nb2 nucleus and cytosol, providing additional evidence that O-GlcNAcylation facilitated Sp1 nuclear tranport. O-GlcNAc modification of Sp1 was reported to inhibit its hydrophobic interactions with other transcription factors, thereby preventing proper assembly on the DNA template and, ultimately, repressing Sp1 transcriptional activity [Roos et al., 1997; Yang et al., 2001]. PRL activation of the immediate early IRF-1 gene requires the constitutive interaction of Sp1 with the IRF-1 promoter [Book McAlexander and Yu-Lee, 2001]. The abundant increase in Sp1 protein levels by PRL and the resulting reduction in the O-GlcNAc/Sp1 ratio may have a dual function, that is, to facilitate Sp1 interaction with other components of the transcriptional machinery at the IRF-1 promoter but also act as a negative regulator of Sp1 action by reducing its nuclear transport.

There is increasing evidence that post-translational modification by O-GlcNAcylation plays a role in the regulation of signal transduction pathways. An increase in O-GlcNAcylation, resulting from exogenous glucosamine or by PUGNac-inhibition of O-GlcNAc removal, was shown to increase the activities of several critical signaling intermediates (i.e., the small GTPase Rac, p42/44, and p38 MAP kinases) involved in the regulation of chemotactic signal transduction and motility in neutrophils [Kneass and Marchase, 2005]. O-GlcNAcylation has also been shown to modulate protein phosphorylation and expression of essential and conserved cell signaling pathways. The X-linked OGT gene is necessary for embryonic stem cell viability but by conditional mutagenesis in vivo, OGT gene mutation in somatic cell types such as thymocytes, neurons, and fibroblasts, was shown to result in the loss of O-GlcNAc and causes T-cell apoptosis, neuronal Tau hyperphosphorylation, and fibroblast growth arrest with altered expression of c-Fos, c-Jun, c-Myc, Sp1, and p27 [O'Donnell et al., 2004]. The mTOR alpha4 and Sp1 are PRL-regulated gene products. PRL regulation of O-GlcNAcylation of alpha4 and Sp1 reveals an additional role for PRL in the post-translational modification of its target proteins in distinct signaling cascades to facilitate PRL action in the Nb2 lymphoma cells.

In summary, both alpha4 and Sp1 are *O*-GlcNAcylated and located in the nucleus of quiescent Nb2 cells. PRL (±ALX) reduced Sp1 *O*-GlcNAcylation below that of quiescent cells and caused a partial redistribution of Sp1 in the cytosol. PRL (±ALX) did not reduce *O*-GlcNA-cylation of alpha4 below that of quiescent cells and alpha4 remained in the nucleus. However, siRNA silencing of OGT gene expression (by ~50%) partially redistributed alpha4 and Sp1 to the cytosol. PP2Ac had no detectable *O*-GlcNAc moieties and its nuclear distribution was not affected by siOGT.

ACKNOWLEDGMENTS

REFERENCES

Book McAlexander M, Yu-Lee LY. 2001. Sp1 is required for prolactin activation of the interferon regulatory factor-1 gene. Mol Cell Endocrinol 184:135–141.

Boudreau RTM, Sangster SM, Johnson LM, Dauphinee S, Li AW, Too CKL. 2002. Implication of a4 phosphoprotein and the rapamycin-sensitive mTOR pathway in prolactin receptor signalling. J Endocrinol 173:493–506.

Chen J, Peterson RT, Schreiber SL. 1998. Alpha4 associates with protein phosphatases 2A, 4, and 6. Biochem Biophys Res Commun 247:827–832.

Cokol M, Nair R, Rost B. 2000. Finding nuclear localization signals. EMBO Rep 1:411–415.

Comer FI, Hart GW. 1999. O-GlcNAc and the control of gene expression. Biochim Biophys Acta 1473:161–171.

Cutler NS, Heitman J, Cardenas ME. 1999. TOR kinase homologs function in a signal transduction pathway that is conserved from yeast to mammals. Mol Cell Endocrinol 155:135–142.

Di Como CJ, Arndt KT. 1996. Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev 10:1904–1916.

Dodd F, Limoges M, Boudreau RTM, Rowden G, Murphy PR, Too CKL. 2000. L-arginine inhibits apoptosis via an NO-dependent mechanism in Nb2 lymphoma cells. J Cell Biochem 77:624–634.

Dong DL, Hart GW. 1994. Purification and characterization of an O-GlcNAc selective *N*-acetyl-beta-D-glucosaminidase from rat spleen cytosol. J Biol Chem 269:19321–19330.

Duverger E, Carpentier V, Roche AC, Monsigny M. 1993. Sugar-dependent nuclear import of glycoconjugates from the cytosol. Exp Cell Res 207:197–201.

Duverger E, Pellerin-Mendes C, Mayer R, Roche AC, Monsigny M. 1995. Nuclear import of glycoconjugates is distinct from the classical NLS pathway. J Cell Sci 108 (Pt 4):1325–1332.

Duverger E, Roche AC, Monsigny M. 1996. *N*-acetylglucosamine-dependent nuclear import of neoglycoproteins. Glycobiology 6:381–386.

Haltiwanger RS, Blomberg MA, Hart GW. 1992. Glycosylation of nuclear and cytoplasmic proteins. Purification and characterization of a uridine diphospho-*N*-acetylglucosamine: Polypeptide beta-*N*-acetylglucosaminyltransferase. J Biol Chem 267:9005–9013.

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-beta-*N*-acetylglucosaminidase inhibitor O-(2-acetamido-2-deoxy-D-glucopyranosylidene)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.

Hood JK, Silver PA. 1999. In or out? Regulating nuclear transport. Curr Opin Cell Biol 11:241–247.

Johnson LM, Too CKL. 2001. Prolactin, interleukin-2 and FGF-2 stimulate expression, nuclear distribution and DNA-binding of rat homolog of pombe Cdc5 in Nb2 T lymphoma cells. Mol Cell Endocrinol 184:151–161.

Kamemura K, Hart GW. 2003. Dynamic interplay between O-glycosylation and O-phosphorylation of nucleocytoplasmic proteins: A new paradigm for metabolic control of signal transduction and transcription. Prog Nucleic Acid Res Mol Biol 73:107–136.

Kneass ZT, Marchase RB. 2005. Protein O-GlcNAc modulates motility-associated signaling intermediates in neutrophils. J Biol Chem 280:14579–14585.

Konrad RJ, Janowski KM, Kudlow JE. 2000. Glucose and streptozotocin stimulate p135 O-glycosylation in pancreatic islets. Biochem Biophys Res Commun 267:26–32.

Konrad RJ, Zhang F, Hale JE, Knierman MD, Becker GW, Kudlow JE. 2002. Alloxan is an inhibitor of the enzyme O-linked *N*-acetylglucosamine transferase. Biochem Biophys Res Commun 293:207–212.

Kuwahara J, Coleman JE. 1990. Role of the zinc(II) ions in the structure of the three-finger DNA binding domain of the Sp1 transcription factor. Biochemistry 29:8627–8631.

Kuwahara J, Azumano M, Takeda T. 1999. Nuclear localization of transcription factor Sp1. Nucleic Acids Symp Ser 42:293–294.

Kuwahara J, Watanabe Y, Kayasuga T, Itoh K. 2000. Zn finger and nuclear localization of transcription factor Sp1. Nucleic Acids Symp Ser 44:265–266.

Lefebvre T, Ferreira S, Dupont-Wallois L, Bussiere T, Dupire MJ, Delacourte A, Michalski JC, Caillet-Boudin ML. 2003. Evidence of a balance between phosphorylation and O-GlcNAc glycosylation of Tau proteins—a role in nuclear localization. Biochim Biophys Acta 1619:167–176.

Liu K, Paterson AJ, Chin E, Kudlow JE. 2000. Glucose stimulates protein modification by O-linked GlcNAc in pancreatic beta cells: Linkage of O-linked GlcNAc to beta cell death. Proc Natl Acad Sci USA 97:2820–2825.

Majumdar G, Harmon A, Candelaria R, Martinez-Hernandez A, Raghow R, Solomon SS. 2003. O-glycosylation of Sp1 and transcriptional regulation of the calmodulin

gene by insulin and glucagon. Am J Physiol Endocrinol Metab 285:E584–E591.

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

Nanahoshi M, Nishiuma T, Tsujishita Y, Hara K, Inui S, Sakaguchi N, Yonezawa K. 1998. Regulation of protein phosphatase 2A catalytic activity by alpha4 protein and its yeast homolog Tap42. Biochem Biophys Res Commun 251:520–526.

O'Donnell N, Zachara NE, Hart GW, Marth JD. 2004. Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. Mol Cell Biol 24:1680–1690.

Peterson RT, Desai BN, Hardwick JS, Schreiber SL. 1999. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. Proc Natl Acad Sci USA 96:4438–4442.

Pichler A, Melchior F. 2002. Ubiquitin-related modifier SUMO1 and nucleocytoplasmic transport. Traffic 3:381–387.

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

Roos MD, Xie W, Su K, Clark JA, Yang X, Chin E, Paterson AJ, Kudlow JE. 1998. Streptozotocin, an analog of N-acetylglucosamine, blocks the removal of O-GlcNAc from intracellular proteins. Proc Assoc Am Physicians 110:422–432.

Too CKL. 1997a. Differential expression of elongation factor-2, alpha4 phosphoprotein and Cdc5-like protein in prolactin-dependent/independent rat lymphoid cells. Mol Cell Endocrinol 131:221–232.

Too CKL. 1997b. Induction of Sp1 activity by prolactin and interleukin-2 in Nb2 T-cells: Differential association of Sp1-DNA complexes with Stats. Mol Cell Endocrinol 129:7–16.

Too CKL, Walker A, Murphy PR, Cragoe EJJ, Jacobs HK, Friesen HG. 1987. Identification of amiloride-sensitive Na+/H+ exchange in rat Nb2 node lymphoma cells. Stimulation by 12-0-tetradecanoyl-phorbol-13-acetate. Endocrinology 121:1503–1511.

Too CKL, Vickaryous N, Boudreau RT, Sangster SM. 2001. Identification and nuclear localization of a novel prolactin and cytokine-responsive carboxypeptidase D. Endocrinology 142:1357–1367.

Turowski P, Fernandez A, Favre B, Lamb NJ, Hemmings BA. 1995. Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression. J Cell Biol 129:397–410.

Yang X, Su K, Roos MD, Chang Q, Paterson AJ, Kudlow JE. 2001. O-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability. Proc Natl Acad Sci USA 98:6611–6616.