**O-GlcNAcylation/Phosphorylation Cycling at Ser\(^{10}\) Controls Both Transcriptional Activity and Stability of Δ-Lactoferrin**

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Δ-Lactoferrin (ΔLf) is a transcription factor that up-regulates *DepS*, *Skp1*, and *Bax* genes, provoking cell cycle arrest and apoptosis. It is post-translationally modified either by O-GlcNAc or phosphate, but the effects of the O-GlcNAc/phosphorylation interplay on ΔLf function are not yet understood. Here, using a series of glycosylation mutants, we showed that Ser\(^{10}\) is O-GlcNAcylated and that this modification is associated with increased ΔLf stability, achieved by blocking ubiquitin-dependent proteolysis, demonstrating that O-GlcNAcylation protects against polyubiquitination. We highlighted the KSQSSDDPNCVD\(^{301}\) sequence as a functional PEST motif responsible for ΔLf degradation and defined Lys\(^{379}\) as the main polyubiquitin acceptor site. We next investigated the control of ΔLf transcriptional activity by the O-GlcNAc/phosphorylation interplay. Reporter gene analyses using the *Skp1* promoter fragment containing a ΔLf response element showed that O-GlcNAcylation at Ser\(^{10}\) negatively regulates ΔLf transcriptional activity, whereas phosphorylation activates it. Using a chromatin immunoprecipitation assay, we showed that O-GlcNAcylation inhibits DNA binding. Deglycosylation leads to DNA binding and transactivation of the *Skp1* promoter at a basal level. Basal transactivation was markedly enhanced by 2–3-fold when phosphorylation was mimicked at Ser\(^{10}\) by aspartate. Moreover, using double chromatin immunoprecipitation assays, we showed that the ΔLf transcriptional complex binds to the ΔLf response element and is phosphorylated and/or ubiquitinated, suggesting that ΔLf transcriptional activity and degradation are concomitant events. Collectively, our results indicate that reciprocal occupancy of Ser\(^{10}\) by either O-phosphate or O-GlcNAc coordinately regulates ΔLf stability and transcriptional activity.

O-GlcNAcylation is a ubiquitous post-translational modification consisting of a single N-acetylglucosamine moiety linked to Ser or Thr residues (1). It is a dynamic and reversible process mediated by the combined actions of O-GlcNAc transferase (OGT)\(^{2}\) and O-GlcNAcase (OGA). Disruption of β-O-linked N-acetylgalcosamine (O-GlcNAc) cycling through inhibitors or gene manipulations results in cellular defects (2, 3), and alterations of the O-GlcNAc status are associated with type-2 diabetes, neurological disorders, and cancer (4).

Because numerous proteins, such as transcription factors, signaling components, and metabolic enzymes are modified, O-GlcNAcylation is critical to normal cell homeostasis and gene regulation (5). It notably modulates gene expression, depending on the promoter and its associated transcription initiation complexes. For instance, the C-terminal domain of RNA polymerase II and a subset of general transcription factors are O-GlcNAcylated at transcription initiation (6). Gene silencing may be effected via the recruitment of OGT onto promoters by transcriptional corepressors. It then catalyzes the O-GlcNAcylation of specific transcription actors, modulating their activity. For instance, the association of OGT with the co-repressor mSin3A leads to the recruitment of histone deacetylase, thereby increasing transcriptional down-regulation (7, 8). OGA may favor gene transcription, not only by reducing the level of glycosylation but also via its intrinsic histone acetyltransferase domain (9). O-GlcNAcylation may also modulate the activity of transcription factors via the regulation of their trafficking, binding affinity either to protein partners or DNA, and/or turnover (8, 10–13).

Increasing evidence links O-GlcNAcylation to the proteasome pathway. It has been shown that O-GlcNAcylation is associated with lower proteasomal susceptibility of transcription factors, such as Sp1 (14, 15), p53 (16), and the estrogen receptor β (17). Most of these proteins have high PEST scores, and phosphorylation of their PEST (Pro-Glu-Ser-Thr) domain targets them for polyubiquitination (18) and subsequent degradation by the proteasome, whereas O-GlcNAcylation prolongs their half-lives. The proteosome is itself regulated through O-GlcNAcylation of both its regulatory and catalytic subunits (19, 20) as well as the ubiquitin (Ub)-activating enzyme E1 (21). Reduced degradation of O-GlcNAcylated proteins might also be due to their specific interaction with chaperones, such as Hsp70 family members that display lectin activity toward the O-GlcNAc motif, protecting them from proteolysis (22).

In many O-GlcNAcylated proteins, a phosphate group can alternatively occupy the same or adjacent sites (16, 17, 23, 24). This O-GlcNAc/P interplay, which leads to a rapid response element; OA, okadaic acid; GlcNH\(_2\), glucosamine; O-GlcNAc, β-O-linked N-acetylglucosamine; O-GlcNAc/P, O-GlcNAc/phosphorylation; ChIP, chromatin immunoprecipitation; re-ChIP, double ChIP; WT, wild type; qPCR, quantitative PCR; HA, hemagglutinin.

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\(^{2}\) The abbreviations used are: OGT, O-GlcNAc transferase; ΔLf, Δ-lactoferrin; Lf, lactoferrin; OGA, O-GlcNAc hydrolase; Ub, ubiquitin; ΔLfRE, ΔLf response element; OA, okadaic acid; GlcNH\(_2\), glucosamine; O-GlcNAc, β-O-linked N-acetylglucosamine; O-GlcNAc/P, O-GlcNAc/phosphorylation; ChIP, chromatin immunoprecipitation; re-ChIP, double ChIP; WT, wild type; qPCR, quantitative PCR; HA, hemagglutinin.
O-GlcNAc/P Interplay Regulates \( \Delta Lf \) Stability and Activity

mechanism and high molecular diversity and fine tunes protein interactions and functions, may also target \( \Delta \)-lactoferrin (\( \Delta Lf \)) and regulate its transcriptional activity and stability. \( \Delta Lf \) is a transcription factor that was first discovered as a transcript, the expression of which was observed only in normal cells and tissues (25). Its absence from cancer cells (25, 26) is due to genetic and epigenetic alterations (27, 28). \( \Delta Lf \) messenger is therefore a healthy tissue marker, and we previously showed that its expression level is correlated with a good prognosis in human breast cancer, high concentrations being associated with longer overall survival (26). \( \Delta Lf \) is transcribed from the alternative promoter P2 in the lactoferrin (\( Lf \)) gene (29), and the use of the first available AUG codon in frame produces an alternative N terminus. Thus, compared with \( Lf \), its secretory counterpart, \( \Delta Lf \) is a 73-kDa cytoplasmic protein able to enter the nucleus (30). Potential DNA-binding domains have been suggested for \( Lf \), implicating the strong concentration of positive charges at the C-terminal end of the first helix, which is truncated in \( \Delta Lf \), and at the interlobe region (31, 32).

\( \Delta Lf \) expression provokes anti-proliferative effects and induces cell cycle arrest in S phase (33). It is a transcription factor interacting via a \( \Delta Lf \) response element (\( \Delta Lf \)RE) found in the \( Skp1 \) and \( Dep5 \) promoters (30, 34). \( \Delta Lf \) is also at the crossroads between cell survival and cell death because we recently linked \( \Delta Lf \) overexpression to up-regulation of the \( Bax \) promoter and apoptosis (35). Because \( \Delta Lf \) has several crucial target genes and may act as a tumor suppressor, modifications in its activity or concentration may have marked effects on cell survival, and its transcriptional activity should be strongly controlled. Results of screening \( \Delta Lf \) for O-GlcNAcylation and phosphorylation sites showed that the protein potentially undergoes both post-translational modifications. Four putative O-GlcNAc/phosphorylation sites were found at Ser\(^{10} \), Ser\(^{227} \), Ser\(^{472} \), and Thr\(^{559} \), and their mutation led to a marked effect on cell survival, and its transcriptional activity should be strongly controlled. Results of screening \( \Delta Lf \) for O-GlcNAcylation and phosphorylation sites showed that the protein potentially undergoes both post-translational modifications. Four putative O-GlcNAc/phosphorylation sites were found at Ser\(^{10} \), Ser\(^{227} \), Ser\(^{472} \), and Thr\(^{559} \), and their mutation led to a constitutively active \( \Delta Lf \) mutant (34). Here, we map the major O-GlcNAc/P site to Ser\(^{10} \), the PEST sequence (amino acids 391–404), and the main poly-Ub site to Lys\(^{379} \). We also report that O-GlcNAcylation at Ser\(^{10} \) down-regulates \( \Delta Lf \) transcriptional activity and up-regulates its stability by abrogating Ub-mediated proteolysis, whereas phosphorylation activates both transcription and degradation.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Transfection—HEK 293 cells (ATCC CRL-1573) were grown in monolayers and transfected as described (2 µg of DNA for 2 × 10\(^6\) cells) (30) using DreamFect\(^\text{TM} \) (OZ Biosciences). The amounts of \( \Delta Lf \) expression vectors were adjusted to maintain \( \Delta Lf \) amounts similar to those found in normal NBEC cells (26). Transfections were done in triplicate (n ≥ 4). Cell viability was assessed by counting using trypan blue 0.4% (v/v). Cell culture reagents were from Dutscher, Cambrex Corp., and Invitrogen. Other reagents were from Sigma. Antibodies against the 3xFLAG epitope (mouse monoclonal anti-FLAG M2 antibody, Sigma), HA epitope (goat HA tag polyclonal antibodies, BD Biosciences; mouse monoclonal HA11 antibody 16B12, Covance Research Products), O-GlcNAc motif (mouse monoclonal RL2 antibody, Affinity Bioreagents; mouse monoclonal CTD110.6, Covance Research Products), Ser(P) motif (rabbit polyclonal antibodies, Millipore), and actin (goat polyclonal antibodies I-19, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)) were used for immunofluorescence, immunoprecipitation, and/or immunoblotting.

Immunofluorescence and Microscopy—HEK 293 cells were transfected by \( \Delta Lf \) C-terminal fused GFP expression vector 24 h prior the 4′,6-diamidino-2-phenylindole (Sigma) staining. The p\( \Delta Lf \)-N-EGF vector was kindly provided by Dr. C. Teng (National Institutes of Health, Research Triangle Park, NC). Immunofluorescence and microscopy were performed as described (30). Fluorescent microscopy images were obtained at room temperature with a Zeiss Axioplan 2 imaging system (Carl Zeiss S.A.S., Le Pecq, France) equipped with appropriate filter cubes using a ×40 objective lens.

Purification of DNA, RNA, and Poly(A\(^+\)) RNA—Genomic DNA was purified using the QIAprep Spin Miniprep Kit (Qiapgen), total RNA using the RNaseasy mini kit (Qiagen), and poly(A\(^+\)) RNA using the polyATrack\(^\text{R} \) RNA isolation system (Promega). The purity and integrity of each extract were checked using the nanodrop ND-1000 spectrophotometer (Labtech International) and the Bioanalyzer 2100 (Agilent Technologies).

qPCR Conditions—qPCR analyses were performed as described (30). The primer pairs used for the detection of \( \Delta Lf \) (forward, 5′-AAGCCAGTGCAAGTGCTCA-3′; reverse, 5′-GCTTTGTGGATTTGATGT-3′; annealing temperature, 55 °C), ribosomal protein, large, P0 (34), and hypoxanthine-guanine phosphoribosyltransferase (forward, 5′-GACCAGTCAACAGGGGACAT-3′; reverse, 5′-AACACTTCTGTTGGGTCCTTTT-3′; annealing temperature, 55 °C) were purchased from Eurogentec.

Plasmid Construction and Site-directed Mutagenesis—pGL3-S1\( ^{skp1} \)-Luc, pcDNA-\( \Delta Lf \), and p3xFLAG-CMV10-\( \Delta Lf \) were constructed as described (30). p3xFLAG-CMV10 (Sigma) and pcDNA3.1 (Invitrogen) were used as null vectors. The Ub-HA expression vector was a gift from Dr. C. Couturier (IBL, Lille, France). The pcDNA-OGT expression vector was constructed using OGT cDNA isolated from the pShuttle-OGT vector (36) (kind gift of Dr. J. Hart, The Johns Hopkins University School of Medicine (Baltimore, MD)) and further cloned into the pcDNA3.1 vector. Mutants were generated using the QuikChange\(^\text{R} \) site-directed mutagenesis kit (Stratagene) with pcDNA-\( \Delta Lf \) as template and primer pairs listed in Table 1. The constructs in which several sites were mutated were done sequentially. Following sequencing, the HindIII-NotI digests were cloned either into pcDNA3.1 for reporter gene assays or into p3xFLAG-CMV10 for protein experiments.

Reporter Gene Assay—Reporter gene assays were performed using the pGL3-S1\( ^{skp1} \)-Luc reporter vector and the different pcDNA-\( \Delta Lf \) mutant constructs or a null vector as described (34). Cell lysates were assayed using a luciferase assay kit (Promega) in a Tristar multimode microplate reader LB 941 (Berthold Technologies). Relative luciferase activities were normalized to basal luciferase expression and protein content as described (30) and expressed as a percentage; 100% corresponds to the relative luciferase activity of \( \Delta Lf \) WT. Basal luciferase expression was assayed using a null vector and was determined for each condition (OGT, okadaic acid (OA), and glucosamine (GlcNH\(_2\)) at each concentration.
Each experiment represents at least three sets of independent triplicates.

In Vivo DNA Binding Assays—Chromatin immunoprecipitation (ChIP) and double ChIP (re-ChIP) assays were performed as described (34, 37) with some modifications introduced for re-ChIP. Briefly, ChIP complexes (8 × 10⁶ cells) were immunoprecipitated with M2, RL2, HA tag, or anti-Ser(P) antibodies all used at 1:250 and twice eluted with 100 μM dithiothreitol for 30 min at 37 °C. After centrifugation, pooled eluted fractions were diluted 40 times to reduce the dithiothreitol concentration to 25 μM with ChIP dilution buffer and then immunoprecipitated with either M2 or rabbit anti-IgG (GE Healthcare) or without antibodies. Amplification conditions of Skp1 and albumin promoters were as described (34). ChIP or re-ChIP results presented in Fig. 5 correspond to one representative experiment among three. qPCR was performed only for the ChIP assay. Amplification was carried out in triplicate (n = 3) in the presence of 2 μl of purified DNA, primer pairs used to amplify the Skp1 promoter fragment (34), and Brilliant SYBER Green QPCR Master Mix (Stratagene) according to the manufacturer’s instructions. Samples were then submitted to 40 cycles of amplification (denaturation, 30 s at 90 °C; hybridation, 30 s at 55 °C; elongation, 30 s at 72 °C) in a thermocycler Mx4000 (Stratagene). Data presented in Fig. 5D are expressed as a percentage of input.

TABLE 1
Names of mutants, amino acid modification, location, and oligonucleotides used for mutagenesis

| Name of mutants | Mutated amino acid(s)* | Site-directed mutagenesis oligonucleotides |
|-----------------|------------------------|------------------------------------------|
| ΔLefS10A       | S10A                   | F: 5’-CGTGGCCCTCTCTGTCCTGCAAAGAGAGA-3’ R: 5’-CTCTCTCTCTATGCAGCAGGAGGGCAG-3’ |
| ΔLefS227A      | S227A                  | F: 5’-CCCGGGCCTCCCTGTCGTGCAAGAGGAGGGACG-3’ R: 5’-CAACCGCATGAGCAGGAGGAGGGCAG-3’ |
| ΔLefS472A      | S472A                  | F: 5’-GTCGCCCTCTGCGCTGCAGCAGGAGGGCAG-3’ R: 5’-ATCCTGGCATGAGGAGGGCAGG-3’ |
| ΔLefS559A      | T559A                  | F: 5’-ACGGAGACCTGTCGTGAGGAGGATAGGAGC-3’ R: 5’-GCTCTTATGCTCAGGAGAGGAGGACG-3’ |
| ΔLefS10D       | S10D                   | F: 5’-GTCGATACAGAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS16D       | S16D                   | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |
| ΔLefS10A, S227A, S472A, T559A | S227A, S472A, T559A | F: 5’-CTGGATACAGAGAGAGAGAGAGAGAGGAC-3’ R: 5’-GATACTGGGTGGGGGTGTCTCTTATAGGAG-3’ |

* Single-letter amino acid codes are used.
O-GlcNAc/P Interplay Regulates ΔLf Stability and Activity

(A) Graph showing the effect of different treatments on living cells.

(B) Table showing the IP : M2 interactions:
- ΔLf-3xFLAG: + + + + +
- GlcNH₂: - + - - -
- OA: - - + - -
- OGT: - - - + +

(IB : CTD110.6, IB : RL2, IB : M2 images with 75 kD markers)

(C) Graph showing the effect of GlcNH₂ and OA on ΔLf, RPLP0, and HPRT.

(D) Graph showing the relative luciferase activity.

(E) Graph showing the relative protein activity.

(F) Graph showing the relative luciferase activity.

(G) Graph showing the RPF for different treatments.

(H) Table showing the IP: anti-HA interactions:
- ΔLf-3xFLAG: - + + + + +
- Ub-HA: + + + + + +
- GlcNH₂ (mM): 0 0 0 5 10 0 0
- OGT: - - - - - -

(IB : M2, polyub-ΔLf, IB : HA.11 images with 75 kD markers)

(I) Images showing GFP, DAPI, and MERGE for pEGFP, untreated, GlcNH₂, and OA conditions.

(IB : M2, IB : HA.11 images with 75 kD markers)
Proteasomal Degradation Assay—Proteasomal activity assay was performed according to the assay instructions (Chemicon International) on HEK 293 cell lysates. Lactacystin was used as a 20 S proteasome inhibitor. Fluorescence data were collected using a Tristar multimode microplate reader LB 941 (Berthold Technologies) using 380-nm excitation and 460-nm emission filters.

Immunoblotting and Immunoprecipitation—Proteins were extracted from frozen cell pellets in radioimmune precipitation buffer as described (30). For direct immunoblotting, samples mixed with 4× Laemmli buffer were boiled for 5 min. 20 μg of protein from each sample were submitted to 7.5% SDS-PAGE and immunoblotted. For immunoprecipitation experiments, 1 mg of total protein was preabsorbed with protein G-Sepharose 4 Fast Flow (GE Healthcare). RL2 (1:250), M2 (1:500), or anti-HA polyclonal (1:100) antibodies were mixed with Protein G-Sepharose beads for 1 h prior to an overnight incubation with the preabsorbed lysate supernatant at 4 °C. The beads were then washed five times with lysis buffer. Proteins bound to the beads were eluted with 4× Laemmli buffer and analyzed by immunoblotting as above. Blots were probed at room temperature with primary antibodies (M2, 1:2000; CTD110.6, 1:3000; HA.11, 1:4000; anti-Ser(P), 1:500; RL2, 1:2000; and anti-actin, 1:10,000) for 2 h and with either secondary anti-IgG antibodies conjugated to horseradish peroxidase (GE Healthcare) or secondary anti-IgM antibodies conjugated to horseradish peroxidase (Chemicon International) for 1 h before detection by chemiluminescence (ECL Advance, GE Healthcare). Each result in which immunoblots are presented corresponds to one representative experiment among at least three.

Densitometric and Statistical Analyses—The densitometric analyses were performed using Quantity One version 4.1 software (Bio-Rad), and acquisition was carried out with a GS710-calibrated densitometer (Bio-Rad). M2 densitometric values were normalized to actin and expressed as R = Dn/M2/Dact. The -fold stability (X) is expressed as this ratio related to the wild type ratio and to the t0 value as follows for ΔLf

\[ X = \frac{R_{ΔLf}}{R_{WT}} \]

All of the statistical analyses were done using Origin® 7 software (OriginLab Corp.). Means were statistically analyzed using the t test or analysis of variance, and differences were assessed at p < 0.05 (*) or p < 0.01 (**).

RESULTS

Impact of the O-GlcNAc/P Interplay on ΔLf Transcriptional Activity and Stability—Investigation of the O-GlcNAc function has mainly relied on the manipulation of the hexosamine biosynthesis pathway via an increased production of UDP-GlcNAc, the substrate for OGT (38). Thus, cells exposed to increased concentrations of GlcNH2 or overexpressing OGT exhibit enhanced levels of protein O-GlcNAcylation (39). On the other hand, the use of OA, an inhibitor of PP2A and PP1 phosphatases, is a valuable tool for inducing protein hyperphosphorylation (40, 41).

Prior to investigating whether ΔLf transcriptional activity is regulated via O-GlcNAc/P interplay, we first established that HEK 293 cells possess rapid, inducible O-GlcNAc/P mechanisms at the OGT, GlcNH2, and OA concentrations employed (42). First of all, we verified that cell viability was not perturbed (Fig. 1A). At the concentrations usually used in the literature, such as 40 mM GlcNH2 and 50 nM OA, cell viability was markedly decreased in HEK 293 cells. For this reason, we used lower concentrations, such as 10 mM GlcNH2 and 10 nM OA, that did not affect cell viability but at which modulation of the O-GlcNAc/P status was visible (Fig. 1B). Co-transfection of ΔLf (1 μg DNA/10⁶ cells) and OGT (2.5 μg DNA/10⁶ cells) expression vectors did not significantly affect cell viability (Fig. 1A).

Fig. 1B shows that ΔLf is indeed sensitive to OA and GlcNH2 or OGT but with opposite effects. Treatment with OA led to decreased ΔLf glycosylation, whereas treatment with GlcNH2 or co-transfection with OGT increased it. The same GlcNAcylation pattern was observed using either the RL2 or the CDT110.6 antibody. This result demonstrates clearly that ΔLf possesses O-GlcNAc site(s). OA treatment, which favors phosphorylation, decreases the ΔLf glycosylation level, suggesting that glycosylation site(s) may exist in balance with phosphorylation site(s). Because RNA polymerase II activity is also controlled by this interplay, we next verified that transcription of ΔLf, ribosomal protein, large, P0; or hypoxanthine-guanine phosphoribosyltransferase was indeed not altered under OGT, GlcNH2, or OA treatment (Fig. 1C). Our control experiments showed that modulation of the O-GlcNAc content does not impair cell functions at the concentration of OA, GlcNH2, or OGT we used.

We then investigated ΔLf transcriptional activity using reporter gene assays and a Skp1 promoter fragment containing O-GlcNAc/P Interplay Regulates ΔLf Stability and Activity

FIGURE 1. O-GlcNAc/P interplay regulates ΔLf transcriptional activity. HEK 293 cells were incubated with GlcNH2 or OA, or transfected with an OGT construct (OGT) to assess the impact of the O-GlcNAc/P interplay on ΔLf. A, cell viability. Cell viability of 10⁶ HEK 293 was assayed 24 h after GlcNH2, or OA treatment or after transfection with pcDNA-OGT at 2.5 or 5 μg of DNA/10⁶ cells (n = 9). B, ΔLf O-GlcNAcylation status. Treated and untreated 3xFLAG-ΔLf-expressing HEK 293 cell extracts were M2-immunoprecipitated prior to SDS-PAGE and CTD110.6 or RL2 immunodetection. Input was used as the loading control (n = 3). C, gene expression is not altered under GlcNH2, or OA treatment or OGT overexpression. Poly(A)+ RNA was purified from total RNA of ΔLf-expressing cells treated with OGT, GlcNH2, or OA 24 h after transfection and assay using real-time PCR. RPLPO and hypoxanthine-guanine phosphoribosyltransferase are internal controls (n = 3). D–F, ΔLf transcriptional activity is modulated by OGT, GlcNH2, or OA treatment. Cells were co-transfected with pcDNA-ΔLf and pGL3-S1Skp1-Luc and incubated with GlcNH2, or OA or co-transfected with pcDNA-ΔLf, pcDNA-OGT, and pGL3-S1Skp1-Luc for 24 h prior to lysis. Relative luciferase activities are expressed as described under "Experimental Procedures" (n = 9). G, the endoproteolytic activity of the proteasome is not altered under OGT, GlcNH2, or OA treatment. The histograms represent the proteasomal activity assessed by following the fluorescence emitted during the degradation of a synthetic fluorescent peptide (69). Lactacystin is a proteasome inhibitor (n = 3). H, Ub-dependent degradation of ΔLf is GlcNH2-sensitive. Cells were co-transfected with or without 3xFLAG-tagged ΔLf (3xFLAG ΔLf) and Ub-HA vectors, treated or not with GlcNH2, or transfected or not with pcDNA-OGT. Cells were incubated 2 h with 10 μM MG132 before lysis in order to inhibit proteasomal degradation. Total cell extracts were immunoprecipitated with anti-HA polyclonal antibodies or used as input. Samples were immunoblotted with M2 (top and bottom) or HA.11 (middle) antibodies. I, ΔLf trafficking is not affected by GlcNH2, or OA treatment. Cells were transfected with pEGFP empty or pEGFP-ΔLf vector and incubated with GlcNH2 or OA. Fluorescent microscopy was performed after 4′,6-diamidino-2-phenylindole (DAPi) staining. Error bars, S.D. IB, immunoblot; IP, immunoprecipitation.
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M2.

Development at two exposure times is shown for M2. C and D, mapping of ΔLf-O-GlcNAcylated sites. Cells were transfected by

the above constructs and lysed 24 h later. C, lysates were immunoprecipitated with RL2 and immunoblotted with M2. D, lysates were immunoprecipitated with M2 and immunoblotted with M2. E, relative luciferase activity of ΔLf and its mutants. Cells were co-transfected with pGL3-S1Skp1-Luc reporter vector and pcDNA-ΔLf (ΔLfWT) vector or the O-GlcNac mutant constructs. Relative luciferase activities are expressed as described under “Experimental Procedures” (n = 9, **, p < 0.01). Error bars, S.D. IB, immunoblot; IP, immunoprecipitation.

the ΔLfRE known to be highly transactivated by ΔLf (30). ΔLf transcriptional activity increased in line with OA concentration (Fig. 1D), whereas it decreased in a dose-dependent manner in the presence of GlcNH2 (Fig. 1E). Thus, when phosphorylation was augmented, transactivation was increased 6–7-fold compared with controls, whereas when O-GlcNAcylation was increased in ΔLf-expressing cells, transactivation of the Skp1 promoter was strongly reduced. ΔLf transcriptional activity also decreased when cells overexpressed OGT but at a lower level (Fig. 1F).

Because, as for many transcription factors, ΔLf is rapidly degraded, we next investigated whether its turnover is depen-

dent on both the Ub-proteasome pathway and O-GlcNAc/P interplay. We first verified that treatment with OA, GlcNH2, or OGT did not disturb the proteasome pathway. As shown in Fig. 1G, these treatments did not alter or exacerbate proteasomal degradation compared with the untreated and lactacystin-treated conditions. Fig. 1H shows that a ladder of polyubiqui-

nated ΔLf forms is visible (top, lanes 3 and 6). We next evaluated whether O-GlcNAcylation regulates ΔLf degradation, and the intensity of polyubiquitination was indeed decreased in a dose-

dependent manner after GlcNH2 treatment (Fig. 1H, top, lanes 4 and 5) and after OGT overexpression (Fig. 1H, top, lane 7). Equivalent loadings of Ub-HA protein (Fig. 1H, middle) and ΔLf (Fig. 1H, bottom) were confirmed by immunoblotting. These data demonstrated that ΔLf is more stable in an environment favoring O-GlcNAcylation.

ΔLfM4 (34) and ΔLfWT were used as controls. ΔLf and its glyco-

sylated mutants were then expressed in HEK 293 cells, and their levels of expression were compared. Fig. 2B shows that the ΔLfM4+ mutant was expressed at the same level as ΔLfWT (short exposure time) in contrast to the other mutants (long exposure time). ΔLfS227+ and ΔLfS472+ were slightly more expressed than were ΔLfM4+ and ΔLfT559+, which were both feebly expressed. These data suggest that the post-translational modifications present on Ser10 may participate in ΔLf stability and that its absence from the other mutants leads to their rapid turnover.

O-GlcNAcylation was then investigated on the ΔLf isoforms. Because ΔLf mutants are feebly produced, we first immunopre-

cipitated ΔLf-expressing cell lysates with RL2 in order to accum-

ulate enough O-GlcNAcylated material (Fig. 2C). A reverse immunoprecipitation was then performed using the M2 anti-

body in order to specifically immunoprecipitate ΔLf or its gly-

covariants (Fig. 2D). Fig. 2C shows that ΔLf was effectively glyco-

sylated, whereas ΔLfM4+ was not, confirming that no other O-GlcNAc sites are present on the protein. ΔLfS10+, ΔLfS227+, and ΔLfS472+ mutants were glycosylated, whereas ΔLfT559+ was not (Fig. 2C). The reverse immunoprecipitation of the cell lysates with M2 antibody followed by O-GlcNac immunode-

tection with the CTD 110.6 antibody (Fig. 2D) confirmed that ΔLf and its ΔLfS10+ mutant were glycosylated, whereas ΔLfM4+ and ΔLfT559+ were not. The O-GlcNAcylated signals corre-

sponding to ΔLfS227+ and ΔLfS472+ mutants that were effec-

We further investigated whether ΔLf traffic might be altered, leading to an exclusive nuclear targeting of the phosphoform. As previously described (29, 30), a ΔLf-GFP fused protein localizes predominantly to the cytoplasm but also to the nucleus (Fig. 1, panel 2). Here, we showed that the subcellular localization of ΔLf-GFP was not modi-

fied with either GlcNH2 or OA (Fig. 1I, panels 3 and 4, respectively), sug-

gesting that ΔLf traffic is not regu-

lated by the O-GlcNAc/P interplay.

Mapping the Key O-GlcNac Site to Ser10—The low abundance of ΔLf, the necessity for producing a 3xFLAG-tagged protein in order to detect it, and the inherent limitation of the sensitivity of tritium labeling render the detection of carbohydrate moieties on ΔLf and the sub-

sequent mapping of its glycosylated sites extremely difficult. Therefore, in order to confirm the presence of the O-GlcNac sites and character-

ize their roles, we made a series of glycosylation mutants in which only one O-GlcNac site is preserved, named ΔLfS10+, ΔLfS227+, ΔLfS472+, and ΔLfT559+, respectively (Fig. 2A).
O-GlcNAc/P Interplay Regulates ΔLf Stability and Activity

ΔLf Turnover Is Driven through a PEST Motif (Amino Acids 384–404) and Lys379—Short intracellular half-life proteins frequently have a short hydrophilic stretch of amino acids termed a PEST motif. Phosphorylation of the Ser and/or Thr residues and ubiquitination, often of the flanking Lys residues, trigger degradation. Analysis of the ΔLf sequence did not allow identification of a PEST motif in the Ser10 environment but indicated one at the C terminus with three nearly contiguous Ser (Ser392, Ser395, and Ser396) and two flanking Lys (Lys379 and Lys391) residues as potential targets either for kinase/OGT or Ub ligase, respectively. Alignment of Lf sequences from other species to this PEST motif shows that the locus is conserved (Table 2).

We evaluated the functionality of the PEST sequence using a ΔLfPEST mutant in which the three Ser residues were replaced by Ala and showed that this mutation leads to a slight increase in ΔLf content of about 40% compared with WT (Fig. 3, A and B). To measure the ΔLf turnover rate indirectly, we performed incubations (0–150 min) with cycloheximide, a potent inhibitor of de novo protein synthesis (45, 46). The ΔLf content of HEK cells transfected with either ΔLfWT or ΔLfPEST constructs was analyzed following the addition of cycloheximide (Fig. 3C). Differences in the steady state levels of ΔLf were readily apparent after 30 min, which may correspond to the delay necessary for observing the first effects of cycloheximide treatment (Fig. 3C, panel 1). Mutation of the Ser residues in the PEST sequence conferred stability on ΔLf (Fig. 3C, panel 5). GlcNH2 treatment of HEK cells transfected with either ΔLfWT or ΔLfPEST constructs was also performed (Fig. 3C, panels 3 and 7, respectively), and OGT was coexpressed with ΔLfPEST (Fig. 3C, panel 9). Actin, which is stable under the same experimental conditions, was used as an internal control (Fig. 3C, panels 2, 4, 6, 8, and 10). Densitometric data are expressed as -fold stability as described under “Experimental Procedures” (Fig. 3D). Inhibition of the PEST sequence led to a 5–6-fold gain in stability and confirmed that this sequence is determinant for ΔLf degradation.
O-GlcNAc/P Interplay Regulates ΔLf Stability and Activity

A

IB : M2
IB : actin

B

Relative expression values

C

Time (min)

D

Fold stability

E

IB : M2
IB : actin

F

IB : M2
IB : actin

G

IP : anti-HA

Ub-HA + - + + + +

Input

Ub-HA + - + + + +

IB : M2
O-GlcNAc/P Interplay Regulates ΔLf Stability and Activity

We next studied the invalidation of the PEST sequence on the ΔLf<sup>44</sup> mutant. This mutant is detected at low levels in transfected cells, indicating that it is either feebly expressed or rapidly degraded (Fig. 2B). Fig. 3E shows that this invalidation increased ΔLf<sup>44</sup> stability and rendered this mutant more resistant to proteasomal proteolysis. We further investigated whether a particular Ser within the PEST motif was involved in this process using a series of single Ser mutants (Table 2). Whatever the Ser mutated, ΔLf expression was identical, suggesting that the three Ser residues were equivalent phosphorylation targets due to their proximity (Fig. 3E). Moreover, prediction results for putative phosphorylation sites using the NetPhos 2.0 server (CBS.DTU; available on the World Wide Web) also emphasized these three Ser residues as kinase targets, albeit with a higher score for Ser<sup>396</sup> (Ser<sup>392</sup>, 0.766; Ser<sup>395</sup>, 0.789; Ser<sup>396</sup>, 0.977).

We then studied whether ΔLf-mediated ubiquitination occurs predominantly through Lys<sup>379</sup>- or Lys<sup>391</sup>-linked chains by constructing a series of mutants in which residue 379 or 391 was mutated to Ala either in ΔLf or ΔLf<sup>44</sup>. The K379A mutation led to a slightly increased expression level of ΔLf and completely restored stability to ΔLf<sup>44</sup> compared with controls, whereas the K391A mutation had no effect on the ΔLf expression level and only slightly increased expression of ΔLf<sup>44</sup> (Fig. 3F). This result confirms that the flanking Lys<sup>379</sup>, which is highly conserved among species (Table 2), is involved in ΔLf turnover and suggests that it is the major poly-Ub acceptor site. We next verified that ubiquitination of ΔLf is indeed Lys<sup>379</sup>-linked. Fig. 3G shows that polyubiquitination was strongly visible on ΔLf<sup>WT</sup> (top, lane 3), was lower on ΔLf<sup>PEST</sup> (lane 4) and ΔLf<sup>K391A</sup> mutants (lane 6), was poorly visible on ΔLf<sup>L379A</sup> (lane 5), and was not at all visible on the double mutant ΔLf<sup>L379K</sup> (lane 7) in which both Lys residues were mutated. Control levels of ubiquitination and ΔLf expression are shown in the middle and lower panels, respectively (Fig. 3G). These data confirmed that the Lys<sup>379</sup> residue corresponds to the main Ub ligase target and that Lys<sup>391</sup> corresponds to a minor site.

We next investigated which type of relationship may exist between the functional PEST sequence at the C terminus and the O-GlcNAc/P site at the N terminus.

O-GlcNAcylation of Ser<sup>10</sup> Protects ΔLf from Polyubiquitination—To determine whether ΔLf protein stability was controlled via an O-GlcNAc/P switch on Ser<sup>10</sup>, other mutants were constructed (Fig. 4A), such as the ΔLf<sup>S10A</sup> mutant that has only the Ser<sup>10</sup> residue mutated and the ΔLf<sup>S10D</sup> mutant in which an Asp residue was introduced in place of Ser in order to mimic constitutive phosphorylation as described previously (47, 48). Immunoblotting of the different mutants with M2 antibodies is presented in Fig. 4B. ΔLf<sup>S10A</sup> and ΔLf<sup>S10</sup> had expression levels similar to that of WT, whereas ΔLf<sup>S10D</sup> had an extremely short half-life (Fig. 4C), suggesting that this mutant is an interesting tool for studying degradation of the ΔLf phosphoform. Due to the absence of Ser<sup>10</sup>, ΔLf<sup>S10A</sup> was expected, like the ΔLf<sup>L44</sup>, ΔLf<sup>T559+</sup>, ΔLf<sup>T227+</sup>, and ΔLf<sup>S472+</sup> mutants (Fig. 2B), to be less stable than WT. In ΔLf<sup>S10A</sup>, only Ser<sup>10</sup> is mutated. Therefore, the stability of ΔLf<sup>S10A</sup> might be due to the other sites, which could be used as “protecting sites” in the absence of Ser<sup>10</sup>.

The turnover of these different Ser<sup>10</sup> mutants compared with WT and actin (internal control) is shown in Fig. 4C. Differences in the steady state levels of ΔLf and ΔLf<sup>S10+</sup> mutant were readily apparent around 30–60 min and strongly visible after 90 min (Fig. 4C, panels 1 and 5). Immunoblotting of the different ΔLf constructs and the Ub-HA-expressing vector for 24 h and then incubated with a 10 μM concentration of the proteasomal inhibitor MG132 for 2 h prior to lysis. Total cell extracts were immunoprecipitated with anti-HA polyclonal antibodies or used as input. Samples were immunoblotted with M2 (top and bottom) or with HA.11 (middle) antibodies. Error bars, S.D. (B, D, F).

**Figure 3.** Ub-dependent ΔLf degradation is mediated through a PEST sequence at the C terminus and Lys<sup>379</sup> and is inhibited by O-GlcNAcylation. A and B, deletion of the PEST sequence slightly increases ΔLf stability. HEK 293 cells were transfected with either ΔLf<sup>WT</sup> or ΔLf<sup>PEST</sup> constructs for 24 h. Total protein extracts were immunoblotted with M2. C, modulation of ΔLf half-life by O-GlcNAcylation. Cells were transfected with either ΔLf<sup>S10</sup> or ΔLf<sup>PEST</sup> and GlcH<sub>2</sub>O-treated or cotransfected with the OGT-construct or not and then incubated with fresh medium supplemented by 10 μg/ml cycloheximide for the indicated time 24 h after transfection. Total protein extracts were immunoblotted with either M2 or anti-actin antibodies. D, data are expressed as fold-stability as described under "Experimental Procedures." *p < 0.05, C, the three Ser residues of the PEST sequence are equivalent. Mutation of Ser residues was done on the 3xFLAG-ΔLf<sup>WT</sup> construct as template. Cells were transfected by the different constructs, and 24 h after transfection, total protein extracts were immunoblotted with either M2 or anti-actin antibodies. E, mutation of Lys<sup>379</sup> rather than of Lys<sup>391</sup> inhibits degradation. 3xFLAG-ΔLf and 3xFLAG-ΔLf<sup>WT</sup> constructs were used as template to obtain Lys<sup>379</sup> and Lys<sup>391</sup> mutants. Cells were transfected by the different constructs, and 24 h after transfection, total protein extracts were immunoblotted with either M2 or anti-actin antibodies. G, Lys<sup>379</sup> is the main Ub-ligase target. HEK 293 cells were co-transfected with or without the 3xFLAG-ΔLf constructs and the Ub-HA-expressing vector for 24 h and then incubated with a 10 μM concentration of the proteasomal inhibitor MG132 for 2 h prior to lysis. Total cell extracts were immunoprecipitated with anti-HA polyclonal antibodies or used as input. Samples were immunoblotted with M2 (top and bottom) or with HA.11 (middle) antibodies. Error bars, S.D. (B, D, F).
Ser10 mutants exist as phosphoforms. The decreased phosphor-
tivity (gated the phosphorylation signal, confirming antibody specific-ation with the M2 antibody and probing the resulting blot with
be responsible for gene transactivation. Using immunoprecipi-
tactivity, we next questioned whether the phosphoform might
thestated that the degradation process is regulated via the
ubiquitination occurring mainly at Lys379. We also demon-
through a PEST sequence located at the C terminus with poly-
extracts were immunoprecipitated with anti-HA polyclonal antibodies or used as input. Samples were immunoblotted with M2 (top and bottom) or with HA.11 antibodies (middle). Error bars, S.D. IB, immunoblot; IP, immunoprecipitation.

\( \Delta L_f^{S10D} \) mutant or of \( \Delta L_f^{WT} \) under OA treatment precluded
the observation of a polyubiquitination signal (data not shown).

In conclusion, our data showed that \( \Delta L_f \) turnover is driven
through a PEST sequence located at the C terminus with poly-
ubiquitination occurring mainly at Lys379. We also demon-
strated that the degradation process is regulated via the O-
GlcNAc/P interplay, which targets Ser10. As a glycoform, \( \Delta L_f \) is
stable, whereas as a phosphoform, it is sensitive to degradation.
Since proteasomal degradation is triggered by phosphorylation,
we suggest that phosphorylation of Ser10 favors phosphoryla-
tion at Ser10 rendered \( \Delta L_f \) sensitive to degradation. Since
phosphorylation at Ser10 Controls \( \Delta L_f \) Transcriptional Activity—Because OA treatment increases \( \Delta L_f \) transcriptional activity, we next questioned whether the phosphoform might be responsible for gene transactivation. Using immunoprecipitation with the M2 antibody and probing the resulting blot with an anti-Ser(P) antibody, we studied the phosphorylation status of \( \Delta L_f \) (Fig. 5A, left). Phosphatase treatment markedly abrogated the phosphorylation signal, confirming antibody specificity (right). Immunoblotting (Fig. 5A) showed that \( \Delta L_f \) and its Ser10 mutants exist as phosphoforms. The decreased phosphorylation signals observed under GlcNH2 treatment confirm that phosphorylation and O-GlcNAcylation may alternate on some of the sites. Therefore, the weaker phosphorylation signal observed with the hyperglycosylated \( \Delta L_f^{S10D} \) isoform (lane 9) compared with control (lane 5) strongly suggests that the O-GlcNAc/phosphorylation interplay targets the Ser10 site. However, because \( \Delta L_f^{P44} \) is phosphorylated, \( \Delta L_f \) is also phosphorylated on sites different from the O-GlcNAc/P interplay sites.

We next performed gene reporter analyses as described
above and investigated whether phosphorylation at Ser10 controls \( \Delta L_f \) transcriptional activity. Fig. 5B shows that, compared with \( \Delta L_f^{WR} \), \( \Delta L_f^{S10D} \) transcriptional activity was inhibited 2-fold as in Fig. 2E, whereas the transcriptional activity of \( \Delta L_f^{S10A} \) was increased 1.5–2-fold, and that of \( \Delta L_f^{S10D} \) was increased 4.5–5-fold. The prevention of glycosylation of Ser10 favored transcription, suggesting that O-GlcNAcylation at this site inhibits \( \Delta L_f \) transcriptional activity. Mimicking phosphorylation at Ser10 rendered \( \Delta L_f \) more active than \( \Delta L_f^{P44} \) (Fig. 2E) and strongly suggests that the presence of a phosphate group on this site favors transactivation (Fig. 5B). This result reinforces the status of \( \Delta L_f^{S10D} \) as a constitutive phosphorylated mutant. Because Ser10 is present in a basic environment (‘MKRVGRPPVSCKR4’) within a putative truncated DNA-binding domain, we constructed a \( \Delta L_f^{P11-14} \) mutant in which

\[ \text{O-GlcNAc/P Interplay Regulates } \Delta L_f \text{ Stability and Activity} \]
the first 14 amino acid residues were deleted. Surprisingly, this deletion did not affect ΔLf transcriptional activity (Fig. 5B), suggesting that the ΔLf DNA-binding domain must be located at the hinge region (31, 32). Because O-GlcNAcylation and phosphorylation might occurr on neighboring sites, we screened the vicinity of Ser10 and identified Ser16 that might be used as a replacement target by kinases. We therefore constructed a ΔLfS16D mutant in order to mimic phosphorylation at this site. Expression of this mutant led to a basal expression level of the reporter gene (Fig. 5B), showing that constitutive phosphorylation at this locus does not lead to increased transactivation as for ΔLfS10D and does not take over when the major acceptor site is invalidated, confirming the key role of Ser10.

Because ΔLf transcriptional activity is altered by O-GlcNAcylation at Ser10 and an OGT-OGA complex has been described in the vicinity of transcription factors bound to their response elements (8, 9), we next considered whether glycosylated ΔLf binds DNA. Using a ChIP assay we investigated the binding of the different Ser10 mutants compared with WT. As shown in Fig. 5C, specific ChIP PCR products were detected for each mutant. It is interesting to note that the PCR product signals for ΔLfWT and ΔLfS10D were equivalent, whereas treatment with GlcNH2 led to a weaker signal for both, suggesting that fewer promoter sites were occupied. Because ΔLfWT and ΔLfS10D were equivalently expressed (Fig. 4B) even under GlcNH2 treatment (Fig. 1, B and C, respectively), we suggest that glycosylation inhibits binding to DNA and that among the ΔLf intracellular pool, only the Ser10 phosphoforms bind ΔLfRE. These results were confirmed by the detection of a PCR product signal comparable with that of WT for ΔLfS10D, which was poorly expressed but extremely active (Fig. 4B), suggesting that a large proportion of ΔLfS10D binds ΔLfRE (Fig. 5C). The detection of a weaker signal for ΔLfA, which was expressed similarly to WT, shows that without phosphorylation and glycosylation at Ser10, ΔLf still binds DNA, but its capacity to occupy promoter sites is reduced.

Real time PCR was next performed to quantify promoter site occupancy (Fig. 5D). The qPCR data confirmed the PCR results except that promoter site occupancy for ΔLfS10D and ΔLfS10A was twice as high as that of WT. Treatment with GlcNH2 led to a 0.5-fold promoter site occupancy compared with WT, confirming that favoring GlcNAcylation prevents DNA binding.

FIGURE 5. O-GlcNAcylation at Ser10 negatively controls DNA binding and ΔLf transcriptional activity. A, ΔLf is a phosphorylated protein. HEK 293 cells were transfected by different ΔLf constructs in the presence or not of GlcNH2 for 24 h prior to lysis. M2 immunoprecipitates were immunoblotted with anti-Ser(P), and as a loading control, input was immunoblotted with M2 (left). Phosphatase treatment (1 unit/IP) confirms anti-phosphate antibody specificity (AP, right). B, relative luciferase activity of ΔLf and its O-GlcNAc mutants. HEK 293 cells were co-transfected with pGL3-S1Skp1-Luc vector and pcDNA3.1-Skp1 or pcDNA3.1-LfWT or Ser10 mutant constructs. Relative luciferase activities are expressed as described under “Experimental Procedures” (n = 9; **, p < 0.01). C and D, O-GlcNAcylation inhibits DNA binding. The in vivo binding of ΔLf and its Ser10 mutants to the Skp1 promoter fragment was examined in HEK 293 cells treated or not with GlcNH2 (n = 3). Cross-linked DNA-ΔLf complexes were immunoprecipitated, and precipitated DNA fragments were PCR-amplified (D) with specific primers covering the ΔLfRE present in the Skp1 promoter. The PCR-amplified DNA purified from the sonicated chromatin was used as input and loading control. ChIP assays were performed using M2, anti-rabbit IgG antibodies as a nonspecific control (irrelevant; IR) and without antibody (NIP). Amplification of the albumin promoter region was used as a negative control. E, ΔLf transactivation complex is not O-GlcNAcylated. Re-ChIP was performed as above for the ChIP assay with some modifications. The first immunoprecipitation was performed using M2, RL2, anti-Ser(P) or anti-HA antibodies. Then, prior to reversal of protein-DNA cross-linking, the chromatin fragments were subjected to reprecipitation using M2, irrelevant antibody, or no antibodies (n = 3). Error bars, S.D. IB, immunoblot; IP, immunoprecipitation.

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In addition, we performed a re-ChIP assay to investigate whether ΔLf or a ΔLf-associated transcriptional complex binds to the endogenous human Skp1 promoter in vivo as a phosphoform. Moreover, since the half-life of ΔLf is short as a phosphoform, we studied the possibility that ΔLf also exists as a ubiquitinated isoform on DNA. Using a re-ChIP assay, we showed that phosphorylated and ubiquitinated but not O-GlcNAc ΔLf complexes were specifically co-localized on the Skp1 promoter fragment (Fig. 5E). The slight amplification observed in panel 1 (NIP and IR) might be due to the fact that the two immunoprecipitations were performed with the same antibody, increasing the background level. Our results clearly demonstrate that phosphorylated and/or ubiquitinated ΔLf or ΔLf associated with phosphorylated and/or ubiquitinated proteins specifically binds the Skp1 promoter segment with close proximity in vivo, whereas glycosylated ΔLf or ΔLf associated with glycosylated proteins does not. Because ΔLf is ubiquitinated at Lys379 and phosphorylated at Ser10, we suggest that these two post-translational modifications might be concomitantly present on ΔLf bound to DNA and may both be determinant in its activity. Further work will have to be done to demonstrate such a partnership, and for that, specific antibodies against the phosphorylated or the Ub-Lys379 or poly-Ub-Lys379 will be obtained.

DISCUSSION

O-GlcNAc/P modification of transcription factors modulates their transcriptional activity by regulating their turnover, traffic, binding to DNA, or cofactor recruitment. ΔLf is a transcription factor controlling the expression of key molecular actors and as such should be highly regulated. In this study, we demonstrated that it is alternatively O-GlcNAcylated or O-phosphorylated at Ser10 and that these two alternative modifications play distinct roles in modulating its turnover and transcriptional activity.

The concentration of transcription activators and the rate of their degradation are under the control of the proteasome, and there is direct evidence that a switch between O-GlcNAcylation and phosphorylation regulates the process. Phosphorylation drives proteins to degradation via the capping of PEST hydroxyl groups, whereas O-GlcNAcylation hinders it mainly by competing for and masking these hydroxyl groups from kinases. Numerous proteins, such as the transcription factor Sp1 (14), the estrogen receptor (49), the eukaryotic initiation factor elF2α-p67 (50), or p53 (16), are protected from proteasomal degradation by O-GlcNAcylation. Here, we show that ΔLf has a short half-life compatible with its function and is stabilized when Ser10 is O-GlcNAcylated. Moreover, we showed that Ser10 is not present within a phosphodegron, which is a recognition signal for Ub ligases. The ΔLf degradation motif (KASQEQSDDPDPCVD) is conserved in Lf from different species, and the mutation of all three Ser residues led to increased stability of the protein, clearly confirming the functionality of this motif. Mutation of each Ser separately indicated that they behave similarly, suggesting that they are equivalent targets of kinases due to their proximity, but we do not know whether they are also substituted with GlcNAc moieties. YinOYang l.2 server predictions indicated Ser292 and Ser395 as OGT targets but with low scores. The ΔLfS10D isoform is not glycosylated, suggesting that no further glycosylation sites are present, but we cannot exclude glycosylation of the PEST motif only when Ser10 is glycosylated or the possibility that the ΔLfpp4 isoform, which is extremely unstable, exists only as a phosphorylated PEST isoform.

We next investigated Ub targets by mutating lysine residues neighboring the PEST motif and demonstrated that ΔLf ubiquitination occurs on Lys379 and Lys391 with Lys379 as the main target. The ΔLfK379 double mutant was devoid of Ub, confirming that only these two residues are involved. The formation of Ub ladders observed with ΔLfK379 and ΔLfK391 also revealed that, despite the possibility of its multimonoubiquitination, ΔLf undergoes polyubiquitination. Unexpectedly, the ΔLfPEST mutant was still ubiquitinated, suggesting the existence of other degradation motifs. ΔLf is involved in S phase control and should be ubiquitinated via the SCF complex, but it is possible that another complex, such as anaphase promoting complex/cyclosome, might be involved. Interestingly, ΔLf possesses a R57RSNLCAL sequence, which may behave as a potential RXXLXX(L/I/V/M) D-box motif (ELM D-box entry), the target of anaphase promoting complex/cyclosome (51). The presence of two degradation motifs suggests that ΔLf may be degraded throughout the cell cycle. Nevertheless, further work has to be done in order to prove the functionality of this D-box.

The relationship linking O-GlcNAcylation and the Ub pathway has not yet been elucidated. Although Yang et al. (16) demonstrated that O-GlcNAcylation inhibits ubiquitination of p53, a recent study by Guinez et al. (21) shows that O-GlcNAc and Ub can coexist on the same protein and suggests that the Ub/O-GlcNAc ratio may send proteins either to destruction or repair. Here, we demonstrated that enhancement of the O-GlcNAc status within the cells inhibited ΔLf ubiquitination, and the absence of Ser10 as in the ΔLfS10A mutant was accompanied by a decrease in polyubiquitination, suggesting that this modification of ΔLfS10A only occurs on the phosphoforms. Phosphorylation at Ser10, by acting through the creation of a negatively charged region and/or the triggering of transient conformational changes, may lead to phosphorylation at the PEST locus, conferring a priming site role on Ser10.

The O-GlcNAc/P interplay also modulates transcriptional activity. O-GlcNAcylation directly activates FoxO1 (52), p53 (53, 54), and NF-κB (55) and Sp1 indirectly via cofactors (14, 15), whereas it inhibits c-Myc (24) and mouse estrogen receptor β (49). In this work, we have demonstrated that GlcNAcylation inhibits ΔLf transcriptional activity, whereas phosphorylation activates it, and that Ser10 is central to this regulation. An absence of modification at Ser10 leads to gene transactivation, whereas phosphomimetism increases it, confirming the inhibitory role of glycosylation. Because the expression of ΔLfS10A is much greater than that of ΔLfpp4, we suggest that ΔLf exists normally in the cell as a pool of stable but inactive glycoforms that, under appropriate stimuli, become activated by phosphorylation and sensitive to degradation. However, another explanation is that only the phosphoform is present in the nucleus. Nucleocytoplasmic traffic may be regulated via O-GlcNAcylation because the modification of the O-GlcNAc status leads to a change in the cellular distribution of Tau (56), Alpha4, and Sp1 (57) but does not influence Stat5a traffic (58). Here, we showed that ΔLf-GFP traffic was not affected by
GlcNH₂ or OA treatment. But even if the nucleocytoplasmic traffic is not governed by the O-GlcNAc/P interplay, because the OGT-OGA complex and kinases are present within both compartments (59, 60), nuclear ΔLf might exist only as a phosphoform.

Phosphorylated transcription factors are usually more competent to bind DNA and activate transcription than their non-phosphorylated counterparts, but there is direct evidence for the involvement of O-GlcNAcylation. PDX-1 O-GlcNAcylation increases its ability to bind DNA (61) and enhances p53 DNA binding by hiding an inhibitory domain at the N terminus (54). O-GlcNAcylation of HIC1 does not affect its specific DNA binding (62), and whatever the modifications present on Stat5, it binds its response element similarly (58). Nevertheless, we can infer that transactivation by ΔLf, by down-regulating Skp1 expression, may alter SCF activity, whereas phosphorylation of ΔLf may increase it. Regulation of the transcriptional activity of ΔLf by the O-GlcNAc/P interplay may therefore modulate the Ub-proteasome-mediated degradation of cell cycle regulators. Furthermore, we demonstrated that ΔLf is itself ubiquitinated; thus, its turnover could be regulated by feedback control via overexpression of Skp1. On the other hand, ubiquitination also occurs on the ΔLf DNA complex. Modification by Ub is not only a destruction signal but also determines membrane receptor internalization, sorting at the endosomal compartment, activation of DNA repair, or transcriptional activation of transcription factors, such as c-Myc and SRC-3 (66–68). As an example, SRC-3 is first activated by multi-(mono)ubiquitination and then polyubiquitinated prior to degradation. Therefore, ΔLf might require concomitant preubiquitination and phosphorylation as a transcriptional activation signal before being degraded as a polyubiquitinated isoform.

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REFERENCES

1. Hart, G. W., Haltiwanger, R. S., Holt, G. D., and Kelly, W. G. (1989) Ciba Found. Symp. 145, 102–112, discussion 112–108
2. Hanover, J. A. (2001) FASEB J. 15, 1865–1876
3. O’Donnell, N., Zachara, N. E., Hart, G. W., and Marth, J. D. (2004) Mol. Cell. Biol. 24, 1680–1690
4. Lefebvre, T., Dehennaut, V., Guinez, C., Olivier, S., Drouget, L., Mir, A. M., Mortuaire, M., Vercootter-Edouart, A. S., and Michalski, J. C. (2010) Biochim. Biophys. Acta 1800, 67–79
5. Comer, F. L. and Hart, G. W. (1999) Biochim. Biophys. Acta 1473, 161–171
6. Hart, G. W. (1997) Annu. Rev. Biochem. 66, 315–335
7. Roos, M. D. and Hanover, J. A. (2000) Biochem. Biophys. Res. Commun. 271, 275–280
8. Yang, X., Zhang, F., and Kudlow, J. E. (2002) Cell 110, 69–80
9. Toleman, C., Paterson, A. J., Whisenhunt, T. R., and Kudlow, J. E. (2004) J. Biol. Chem. 279, 53665–53673
10. Wells, L., Kruppel, L. K., Comer, F. I., Wadzinski, B. E., and Hart, G. W. (2004) J. Biol. Chem. 279, 38466–38470
11. Hart, G. W., Housley, M. P., and Slawson, C. (2007) Nature 446, 1017–1022
12. Slawson, C., Housley, M. P., and Hart, G. W. (2006) J. Cell. Biochem. 97, 71–83
13. Zachara, N. E., and Hart, G. W. (2004) Trends Cell Biol. 14, 218–221
14. Han, I. and Kudlow, J. E. (1997) Mol. Cell. Biol. 17, 2550–2558
15. Yang, X., Su, K., Roos, M. D., Chang, Q., Paterson, A. J., and Kudlow, J. E. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 6611–6616
16. Yang, W. H., Kim, J. E., Nam, H. W., Ju, J. W., Kim, H. S., Kim, Y. S., and Cho, J. W. (2006) Nat. Cell Biol. 8, 1074–1083
17. Cheng, X., Cole, R. N., Zaia, J., and Hart, G. W. (2000) Biochemistry 39, 11609–11620
