RNA polymerase II carboxyl-terminal domain (RNAPII CTD) phosphatases are responsible for the dephosphorylation of the C-terminal domain of the small subunit of RNAPII in eukaryotes. Recently, we demonstrated the identification of several interacting partners with human small CTD phosphatase1 (hSCP1) and the substrate specificity to delineate an appearance of the dephosphorylation catalyzed by SCP1. In this study, using the established cells for inducibly expressing hSCP1 proteins, we monitored the modification of β-O-linked N-acetylglucosamine (O-GlcNAc). O-GlcNAcylation is one of the most common post-translational modifications (PTMs). To gain insight into the PTM of hSCP1, we used the Western blot, immunoprecipitation, succinylated wheat germ agglutinin precipitation, liquid chromatography-mass spectrometry analyses, and site-directed mutagenesis and identified the Ser residue of hSCP1 as the O-GlcNAc modification site. These results suggest that hSCP1 may act as a O-GlcNAcylated protein in vivo, and its N-terminus may function as a scaffold for binding the protein(s). [BMB Reports 2014; 47(10): 593-598]

INTRODUCTION

RNA polymerase II (RNAPII) is a key complex multisubunit enzymatic component in the transcription apparatus for the synthesis of mRNAs and many noncoding RNAs and for its regulation (1, 2). Eukaryotic RNAPII has several (up to 52 in mammals) conserved tandem heptad repeats (Y1S2P3T4S5P6S7, named C-terminal domain (CTD)) at its C-terminus that is not present in other RNAPIs (3). The phosphorylation of S2 and S5 of this repeat along with proline cis/trans isomerization and glycosylation creates a code that seemingly can be read to mediate a number of processes (4, 5) and is thought to act as a scaffold to coordinate the binding of proteins involved in the different phases of transcription and couples the transcription with other nuclear process. Indeed, the phosphorylation status of this repeat is a hallmark for different transcriptional shifts of the RNAPII complexes (6). Thus, the enzymes responsible for the phosphorylation status of these serine (Sr) residues play pivotal roles in the regulation of transcription and related processes (7). These events are largely mediated by the participation of the proline-directed kinases such as cyclin-dependent kinases. Moreover, several phosphatases have been implicated for the removal of phosphates from the CTD, thereby mediating transitions in the transcription cycle (6, 9). A systematic approach investigating the genome-wide distribution of the CTD modifications indicated a considerable crosstalk between the CTD kinases and phosphatases, suggesting that the transcription operates in a uniform mode at virtually all the genes (10).

In higher eukaryotes, small CTD phosphatases (SCPs) with activities preferential for phosphoryl-Ser(S5(5)) were identified, containing a catalytic domain (FCPH domain) with Mg2+-binding DXDX(T/V) signature motif but lack a breast cancer protein related C-terminal domain (11, 12). The SCPs are related to the catalytic subunit of FCP1, the first discovered CTD phosphatase, which is highly conserved, essential enzyme for dephosphorylating the CTD of RNAPII and preferential for phosphorylating(S5). These are also transcriptional regulators for gene silencing activities in neuronal genes and phase regulation in the cell cycle (13, 14). The catalytic mechanisms of the SCPs and the structural basis for their CTD specificity are well understood (15, 16); however, the identity of physiological regulatory mechanisms explaining the biological activities of the SCPs has remained mainly elusive.

The β-O-linked N-acetylglucosamine (O-GlcNAc) modification (O-GlcNAcylation) has been proposed to act as a nutrient sensor and changes in response to many signals, including the morphogens, cell cycle, development, extracellular glucose levels, and numerous forms of cellular stress (17, 18). This modification of Ser and threonine (Thr) residues of various nuclear, mitochondrial, and cytoplasmic proteins is one of a number of post-translational modifications (PTMs) thought to mediate cellular function, and a variety of proteins can be O-GlcNAcylated (19). The O-GlcNAcylation is highly dynamic and interestingly...
regulates protein function in a manner analogous to the phospho- 
ylation of protein by changing the localization of proteins, 
regulating protein-protein or protein-DNA interactions, altering 
the half-life of proteins, and regulating the activity of proteins
(20). Taken together, it is well established that the O-GlcNAcy-
lation is a key PTM employed by the cells and animals to rapidly
respond for the survival against stress.

Previously, we demonstrated the identification of several in-
teracting partners with hSCP1 and the substrate specificity to
delineate an appearance of the dephosphorylation reaction cata-
lized by SCP1 phosphatase using a specific inducible express-
sing system of the human SCP1 (hSCP1) (21). SCP1 protein sub-
jected to diverse PTMs in spite of the known O-GlcNAcylation of 
RNAPII (5), particularly CTD, has not been reported. In this
study, we interrogated the PTMs into this SCP1 as a CTD phos-
phatase and report hSCP1 as a target for the O-GlcNAcylation.

The O-GlcNAcylated hSCP1 peptides were analyzed by mass
spectrometry using the established NIH/3T3 cells expressing
hSCP1 proteins under the tight control by doxycycline.

RESULTS AND DISCUSSION

Establishment of NIH/3T3 cell lines for hSCP1

The active site of the CTD-phosphatases was characterized by 
the signature motif ψψψDXDX(T/V)ψψ, where ψ is a hydro-
phobic residue. The D96 in the hSCP1 has been shown to be 
catalytically pivotal. The hSCP1 was tagged either with the Flag
sequence at its N-terminus (5'-region) or V5 epitope sequence
at its C-terminus (3'-region) and then subcloned into
pTRE-ires-EGFP mammalian inducible plasmid (21-23). After 
the transfections of wild type (Wt) and D96N mutant hSCP1 in

![Fig. 1. Microscopic assays for the EGFP expression in hSCP1 inducible cells. Images of hSCP1(Wt and D96N)-induced NIH/3T3 cells in the absence (A) or presence (B) (Phase contrast image) and (C) of 2 μg/ml doxycycline for 72 h were taken using a Leika M205FA stereo-

microscope (Leika Microsystems, Wetzler, Germany) equipped with the LAS software for fluorescence imaging.]

![Fig. 2. Establishment of hSCP1-expressing inducible NIH/3T3 cells (A) NIH/3T3/hSCP1-V5 and (B) NIH/3T3/M2-hSCP1). The expression levels of hSCP1 (Wt and D96N) treated with or without the inducer for the indicated periods of time were monitored by Western blot analyses with the appropriate antibodies. For reverted cells, the cells were treated with the inducer for the indicated periods, and after the replacement with fresh medium, the cells were allowed to grow for the indicated periods without the inducer. (C) For the comparison of molecular sizes of hSCP1 (Wt and D96N) from NIH/3T3/hSCP1-V5 and NIH/3T3/M2-hSCP1 cells, equal amounts (30 μg) of total cell lysates from the abovementioned cell lines were analyzed by immunoblotting with a mixture of α-DYKDDDDK and α-V5 antibodies and detected with HRP-conjugated secondary antibodies.]

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pTRE-IREs-EGFP vector placing together pEF1α-Tet, several candidates were primarily chosen based on their antibiotic selection (puromycin, 10 μg/ml) and microscopic appearance for the EGFP monitored into the IRES-driven EGFP expression in order for the expression to be tracked by fluorescence (Fig. 1) and finally selected using the Western blot analysis with α-Flag and α-DYKDDDDK antibodies or α-V5 epitope antibodies in all the NIH/3T3 cells (Fig. 2). The selected and used expression clones in this study were shown to be >99% expression of the EGFP whenever they were induced by 2 μg/ml doxycycline as an inducer (Fig. 1C). An increase in the hSCP1 expression was only shown in response to doxycycline depending on the induction time (Fig. 2). However, after the treatment of doxycycline for the indicated periods of time, when the cells were replated on new culture dishes with fresh medium without the inducer, the hSCP1 finally reverted to no expression. Moreover, the immunoprecipitation analyses with 2 mg of the total cell lysates with appropriate antibodies did not show any positive signal from the noninduced cell lines. Thus, its expression was under the tight control of doxycycline and is a suitable, unique, and useful system for characterizing hSCP1. The established inducer, the hSCP1 finally reverted to no expression. Moreover, the usefulness of our inducible protein expressing cell systems for validating the proteome profile changes, dissect the signaling network, and monitor the PTM, particularly phosphorylation has been previously (22, 23, 29, 30) considered. In this study, we first addressed the question whether the hSCP1 is a target substrate of O-GlcNAcase (OGT). For this, immunologically confirmed epitope tagged hSCP1s (Wt and D96N) at different induction time points (0, 24, 48, 72 h) were used in the presence of Thi amet-G as an inhibitor of O-GlcNAcase, immunoprecipitated with either the α-Flag or α-V5 epitope antibody-conjugated agarose beads and analyzed for the O-GlcNAcylation by Western blot analysis using α-O-GlcNAc antibody (CDT110.6) at each induction time point. As shown in Figs. 3A and 3B, hSCP1 except the truncated hSCP1(D96N) from NIH/3T3/hSCP1-V5, lacking the N-terminal region, was modified by the O-GlcNAc. Most proteins undergo PTMs, which might be important for their function. Moreover, in many proteins, some regions are partially unstructured or even native folded under the physiological conditions. Furthermore, the cell lysates from each hSCP1-expressed cells were incubated with sWGA Agarose, a lectin that specifically interacts with terminal GlcNAc moieties. The sWGA precipitates were resolved by SDS-PAGE and Western blotting using either α-Flag or α-V5 epitope antibody. Fig. 3C shows that also hSCP1 except the truncated hSCP1(D96N) from NIH/3T3/hSCP1-V5 was detected in the sWGA precipitates, indicating that they carry O-GlcNAc moiety in their N-terminus and is in a very good agreement with the previous immunoprecipitation result.

Conformational disorder is proposed to be important for binding diversity in the protein-protein interactions (26-28). In many in vitro studies of the recombinant SCP1, the inability to produce an active full-length version of the SCP1 under native conditions necessitated the use of a truncated form, but the results regarding its protein-protein interaction were inconclusive (15, 16). In this study, an active full-length hSCP1 in mammalian system was successfully produced and shown to possess in vivo O-GlcNAcylation, thus demonstrating a pivotal role of its O-GlcNAcylation of CTD phosphatase SCP1

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N-terminus in events such as PTMs and proteolysis, which might provide a specific location for the protein-protein interaction platform.

**Ser** residue in hSCP1 as an O-GlcNAcylation site

To determine the O-GlcNAcylation site of hSCP1, 20 mg of total cell lysate from NIH/3T3/M2-hSCP1 for Flag-tagged hSCP1(Wt) with the lysis buffer A, described in "Materials and Methods" section, was immunoprecipitated with the α-Flag antibody-conjugated Agarose beads. The immunoprecipitates were resolved onto 6-15% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie Brilliant Blue G250 (Fig. 4A). After confirming the recombinant hSCP1 of the immunoprecipitate (1/30 volume) with α-DYKDDDDK antibody (Fig. 4B), the excised hSCP1 band was digested with Glu-C. The extracted peptides were analyzed using Q-TOF MS, and the O-GlcNAc-modified peptide was identified by calculating the difference of 203.2. Only one O-GlcNAc-modified peptide of hSCP1 corresponding to residue R**36GILHS**A was identified, and the Q-TOF spectrum and sequencing results of the GlcNAc-modified peptide corresponding to residues 36-50 are shown in Fig. 4C. The expected increase in mass by the O-GlcNAc modification was 203.2 Da. To assess the O-GlcNAcylation site at Ser**41**, a full length hSCP1 (S**41A**) tagged with V5-epitope at its C-terminal region was created. Either hSCP1 (S**41A**) or hSCP1 (Wt) in pcDNA3 vector was then transfected into NIH/3T3 cells in the presence of 10 μM Thiamet-G. Alterations in the level of O-GlcNAcylation were monitored by immunoprecipitation with V5-Agarose. Alterations in the level of O-GlcNAcylation were monitored by immunoprecipitation with V5-Agarose.
beads and Western blot analysis. The O-GlcNAcylation level of hSCP1 (S\textsuperscript{14}) proteins was significantly reduced relative to the hSCP1 (Wt) transfected cells (Fig. 4D). Because of the presence of an internal Ser residue in this peptide, we concluded that S\textsuperscript{14} was modified by the O-GlcNAc.

Despite its importance in coordinating phosphor-ylation and dephosphorylation of the CTD in the transcription and mRNA processing, the mechanism of regulation of SCP1 phosphatase remains largely undefined. It is not clear whether the PTMs of the SCP1 phosphatases by enzymatic reactions are essential for their pathway. The PTMs create a highly dynamic relay system, responding to signaling events without requiring de novo protein synthesis and offer a platform for signaling pathway components involved in the regulation of the signaling system, hence providing alternative opportunities for targeting the specific signaling pathway.

In this study, we established clonal cell lines in which the expressions of hSCP1(Wt) and dominant negative hSCP1 (D\textsuperscript{96}N) are under the tight control of an antibiotic, doxycycline as an inducer. Moreover, we demonstrate that hSCP1 was modified with O-GlcNAcylation in NIH/3T3 cells and identified an O-GlcNAcylated peptide from the hSCP1, and S\textsuperscript{41} located near the N-terminal region the O-GlcNAcylation site. These experiments may shed some light on not only the successful production of the functional full-length hSCP1 in the mammalian system, but also the future evaluation of the functional role of its N-terminus, which is the protein-protein interaction. Although the functional role of the O-GlcNAcylation on the N-terminal region of SCP1 such as the role of O-GlcNAcylated RNAPII CTD remains speculative, accumulating evidences indicate that the reversible PTMs such as O-GlcNAc is mostly used to modulate the protein stability, allowing catalytic functions of the ordered proteins and is frequently used for the signaling activities of intrinsically disordered proteins. We believe that a novel O-GlcNAcylated protein, hSCP1, identified in this study could function as a valuable resource for a further study of the role of RNAPII CTD phosphatases, a newly emerging family of phosphatases.

**MATERIALS AND METHODS**

**Cell culture and establishment of inducible hSCP1-expressing cell lines**

NIH/3T3 cells were purchased from ATCC (Bethesda, MD, USA) and maintained in high glucose (25 mM) DMEM (Life Technologies, Burlington, ON, USA) supplemented with 10% bovine serum and antibiotics (Life Technologies) in a humidified incubator at 37°C with 5% CO\textsubscript{2} as recommended by the manufacturer’s instruction. Tetracycline-inducible cell lines for the specific hSCP1 protein expression were established as previously described (23, 29, 30). The expressed hSCP1(Wt and D\textsuperscript{96}N mutant) were tagged with V5 epitope and Flag sequence at the C- and N-terminal regions of the hSCP1 cDNA, respectively. For O-GlcNAcase inhibition, the established expressing cells were cultured in the presence of 10 μM Thiamet-G (Cayman Chemical, Ann Arbor, MI, USA) during the induction time.

**Immunoblot analysis, immunoprecipitation assays, and sWGA lectin precipitation**

The cells were washed with ice-cold PBS and lysed using lysis buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 containing protease inhibitor cocktails (Roche, Manhein, Germany) for particularly Tagged hSCP1 as per manufacturer’s recommendation or conventional RIPA buffer containing protease inhibitors. The procedures for Western blot analysis and immunoprecipitation assays were performed as described previously (21, 29). The anti-V5 epitope antibody-conjugated Agarose beads and α-Flag antibody-conjugates were purchased from Sigma-Aldrich. Primary antibodies used are as follows: mouse α-Flag (MI clone, Sigma-Aldrich), rat α-DYKDDDDK (BioLegend, San Diego, CA, USA), mouse α-V5 epitope (Santa Cruz Biotech., Santa Cruz, CA, USA), mouse α-O-GlcNAc (CTD110.6 clone, Covance, Prinston, NJ, USA), and rabbit α-tubulin (AbClon, Seoul, Korea). Succinylated wheat germ agglutinin (sWGA) lectin provides a convenient method for enriching and detecting O-GlcNAc-modified proteins. Precipitation with sWGA Agarose (Vector, Burlingame, CA, USA) was performed as previously described (19). The detection of α-tubulin as a primary antibody was used as an internal control. For reprobing the blots, the blots were washed in 1X TBS to remove the chemiluminescent substrate and incubated with BlotFresh Western Blot Stripping Reagent (SigmaGen Laboratories, Gaithersburg, MD, USA) according to the manufacturer’s instruction.

**Phosphatase assays**

The hSCP1-catalyzed dephosphorylation of the phosphorylated substrate was performed as previously described (21).

**Site-directed mutagenesis and transient expression of SCP1**

Ser\textsuperscript{41} of the hSCP1 cDNA was mutagenized to Ala using a QuikChange™ site-directed mutagenesis kit (Stratagen, La Jolla, CA). The sequence change was verified using an ABI Prism sequencing kit (Applied Biosystems, Foster City, CA, USA). Transfections for the transient expression were performed using FuGENE 6 Reagent (Roche) according to the manufacturer’s instruction. 3 μg of cDNA construct in pcDNA3 was used for all the transfections.

**Mass spectrometric analysis**

The specific protein bands from the CBB-stained gel of the immunoprecipitated complexes (20 mg of total cell lysate) were digested with endoproteinase Glu-C (Promega, Madison, WI, USA) at 37°C according to the manufacturer’s instruction. The resulting peptides were extracted using 50% acetonitrile and dried in a vacuum evaporator for further analysis. Mass spectrometric analysis was performed using a QSTAR Pulsar Q-TOF MS (Applied Biosystems) equipped with a nanoelectrospray ion source (Protana, Odense, Denmark) as described previously (21, 29).

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