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Production of O-GlcNAc Modified Recombinant Proteins in *Escherichia coli*

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Abstract O-linked *N*-acetylglucosamine (O-GlcNAc) is an abundant posttranslationally modified compound in eukaryotic cells. Human O-GlcNAc transferase (OGT) was produced as a maltose binding protein (MBP) fusion protein, which showed significant catalytic activity to modify recombinant Sp1, transcription factor. To facilitate the production of O-GlcNAc modified proteins, instead of using the tedious *in vitro* glycosylation reaction or expression in eukaryotic cells, a MBP-fusion OGT expression vector (pACYC184-MBPOGT) was constructed using pACYC184 plasmid, which could coexist with general prokaryotic expression vectors containing ColE1 origin. By cotransforming pACYC184-MBPOGT and pGEX-2T vectors into *Escherichia coli* BL21, intracellular O-GlcNAcylated proteins could be obtained by a simple purification procedure. It is expected that this may be a useful tool for production of O-GlcNAc modified proteins.

Key words: O-linked *N*-acetylglucosamine, O-GlcNAc transferase, cotransformation

O-GlcNAc modification (O-GlcNAcylation) is a reaction of the O-linked attachment of monomeric *N*-acetylglucosamine on serine or threonine residues of various eukaryotic cellular and viral proteins, including transcription factors, nuclear pore complex, and cytoskeletal proteins [14]. O-GlcNAc is found in all high eukaryotes and is distinct from classical glycosylation for its strict intracellular localization [12]. This modification has also been shown to be dynamic and regulatory in the nucleus and cytosol [16]. O-GlcNAcylation sites are similar or even identical to known phosphorylation sites, which implies that O-GlcNAc and phosphate may compete with each other [7, 8]. O-GlcNAcylation of Sp1 inhibits its proteolytic degradation by proteasome and interactions with TAFII110 [25, 28], suggesting that O-GlcNAc may be involved in transcriptional regulation. Recently, heat shock protein 70 (HSP70) has been identified as an endogenous O-GlcNAc selective lectin [20]. Because HSP70 plays various roles [5, 15, 23], the finding of HSP70 as a lectin provides new insights in the field of HSP70 functions. It has been implied that abnormal O-GlcNAcylation may be involved in some diseases, including diabetes and neurodegenerative diseases [1, 2, 3, 10, 18, 27, 30]. Glucose-stimulated accumulation of O-GlcNAc may be linked to apoptosis of pancreatic β-cell [1], and streptozotocin (STZ), a widely used β-cell toxin, is an inhibitor of O-GlcNAc removing enzyme (O-GlcNAcase) [18]. Therefore, O-GlcNAcylation may be an underlying mechanism of β-cell death by high glucose and streptozotocin.

O-GlcNAc transferase (OGT) has been cloned in several organisms [19, 21]. OGT is highly conserved among eukaryotes ranging from *C. elegance* to human, and contains tetratricopeptide motifs, which are considered to mediate protein-protein interaction [21]. Furthermore, OGT is tyrosine-phosphorylated and O-GlcNAcylated, suggesting that cell signaling may regulate OGT activity [19].

Recently, studies on O-GlcNAcylation and its relevance to cellular physiology have been expanded using O-GlcNAcylated proteins obtained by infection of animal or insect cells with recombinant viral vectors [6, 29]. The present report demonstrated that O-GlcNAcylated proteins could easily be obtained by coexpression of OGT and target proteins in *E. coli*, as an alternative of *in vitro* glycosylation or expression in animal cells.

Materials and Methods

Cloning and Plasmid Construction

Total RNA from mouse liver (for cloning of Sp1), HeLa cells (for serum response factor [SRF]), and HepG2 cells (for OGT) were prepared, and the first strand cDNA was synthesized using oligo $(dT)_{15}$ and M-MLV reverse transcriptase (Promega, Madison, U.S.A.). Mouse Sp1, human SRF, and human OGT cDNA were amplified by polymerase chain reaction (PCR) with synthesized first cDNA, Pfx proof-reading thermal DNA polymerase (Gibco-BRL, Rockville,

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U.S.A.), and the following primers: Sp1 F 5'-CGGAATTC-ACCATGAGCGACCAAGATCACTCC-3', R 5'-CGGAA-TTCTTGGAC- CCATGCTACCTTGCATCC-3', SRF F 5'-GCGGGATCCCTG CCCGTCCGCCCTCCTGCATCG-AGC-3', R 5'-GCGGGATCCAGTCCTGTGGCTGCC CG-TTCCTCCTCCCCG-3', OGT F 5'-GGGATCCATGAAC-ATGGTGGTTTGCACTT GGGTTCTGGTGGCG-3', R 5'-GGAAGCTTCTTTATTTATGCTCACTCAGTGACTTCA ACAGGC-3'. To produce GST-fusion Sp1 (GST-Sp1) and SRF (GST-SRF), the amplified Sp1 and SRF DNA were cloned into *Eco*RI-cut or *Bam*HI-cut pGEX-2T vectors (Amersham Pharmacia, Uppsala, Sweden), respectively, designated pGEX-Sp1 and pGEX-SRF. The amplified OGT cDNA was doubly digested with *Bam*HI and *Hin*dIII and cloned into *Bam*HI and *Hin*dIII-cut pMALc2 vectors (New England Biolabs, Bevenly, U.S.A.) to produce MBP-fusion OGT (MBP-OGT), designated pMALc2-OGT. To produce the GST-fusion C-terminal domain (CTD) of eukaryotic RNA polymerase II, the mouse exon28 DNA was obtained by PCR with chromosomal DNA of Balb/c cells and the following primers: F 5'-TCCTTCCTCCAT-GTTTGTATGCCG-3', R 5'-CGAAGGACTACCATCCT-AAATCCG-3'. The amplified DNA was cloned into pT7 Blue T vector (Takara Shuzo Co., Shiga, Japan). The CTD DNA insert was obtained again by digestion with *Hpa*II and *Hae*III, whose ends were successively flushed by mung bean nuclease, and inserted into *Sma*I-cut pGEX-2T vector, designated pGEX-CTD.

Expression and Purification of Recombinant Proteins

All recombinant proteins were expressed in *E. coli* BL21. *E. coli* BL21 carrying expression vectors was induced to express recombinant proteins by cultivation at 25°C for 3 h after the addition of 0.5 mM IPTG. GST-fusion proteins were purified using glutathione agarose (Sigma Co., St. Louis, U.S.A.) as described previously [11] and O-GlcNAcylated GST-fusion proteins were further purified using WGA-agarose (Sigma Co., St. Louis, U.S.A.) [4]. MBP-OGT was purified by amylose resin (New England Biolabs, Beverly, U.S.A.) according to the manufacturer's manual. The purity and concentration of proteins were estimated by SDS polyacrylamide gel electrophoresis and Bradford protein assay, respectively.

In Vitro Glycosylation Reaction

The reaction mixture for *in vitro* glycosylation contained 50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, 1–5 μg of purified MBP-OGT, 1–5 μg of purified GST-fusion proteins, and 1 mM uridine diphospho-N-acetylglucosamine (UDP-GlcNAc). The reaction was performed at 25°C for 1 h and the reaction mixture was directly subjected to Western blot analysis.

Coexpression of MBP-OGT and GST Fusion Proteins

The region from the Ptac promoter to the OGT DNA of pMALc2-OGT was reamplified by PCR with primers, F 5'-GCCCAAGCTTGCAATTAATGTGAGTTAGCTCAC-TC-3', R 5'-ACGTT GTAAAACGACGGCCAGTGCC-3'. The amplified DNA was digested with *Hin*dIII and cloned into *Hin*dIII-cut pACYC184 vector (a kind gift from Dr. Gerhard Sandmann, Goethe Universitat, Germany) [26], designated pACYC184-MBPOGT. *E. coli* BL21 carrying pGEX-Sp1, -SRF, or -CTD was cotransformed with pACYC184-MBPOGT. The cotransformed *E. coli* BL21 was grown in Luria-Bertani medium containing 100 μg/ml ampicillin and 20 μg/ml chloramphenicol.

Western Blot Analysis

The *in vitro* glycosylation reaction mixtures or GST-fusion proteins purified from cotransformants were electrophoresed

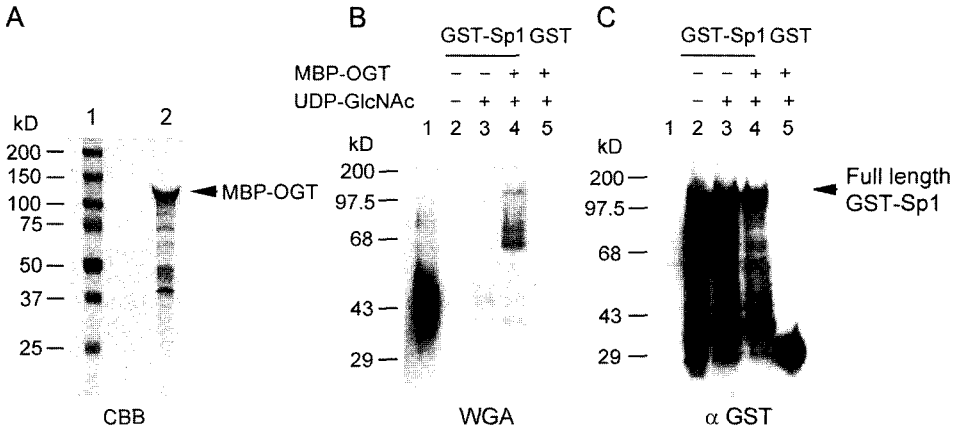

Fig. 1. The recombinant MBP-OGT catalyzes O-GlcNAcylation of Sp1 *in vitro*.
(A) Recombinant MBP-OGT purified from *E. coli* was electrophoresed on 10% SDS-polyacrylamide gel and stained with Coomasie Brilliant Blue (CBB). (B) GST-Sp1 or GST alone were glycosylated *in vitro* by MBP-OGT. The reaction mixture was subjected to Western blotting using anti-GST antibody or WGA. Ovalbumin, a glycoprotein containing terminal GlcNAc, was used as a WGA-reactive marker (Lane 1).

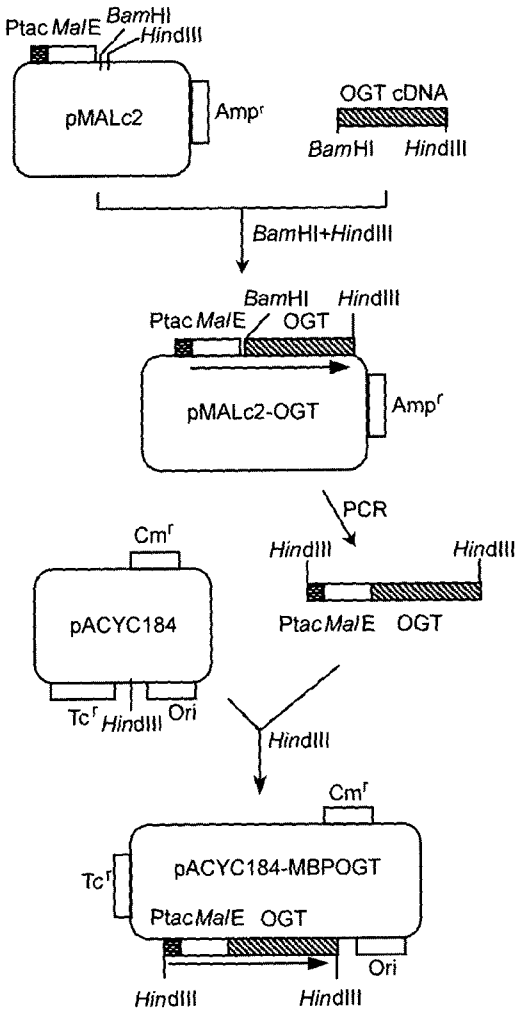

Fig. 2. Construction of pACYC184-MBPOGT.
The region from Ptac to OGT cDNA in pMALc2-OGT was inserted into pACYC184 vector. Ptac, Tac promoter; MalE, maltose binding protein; Amp^r, Ampicillin resistant gene; Cm^r, chloramphenicol resistant gene; Tc^r, tetracycline resistant gene; Ori, replication origin of plasmid p15A.

on SDS polyacrylamide gel, and electrotransferred onto PVDF membrane (Bio-Rad Hercules, U.S.A.). The blots were probed with horseradish peroxidase (HRP)-conjugated wheat germ agglutinin (WGA) [9] and the bound WGA was visualized by enhanced chemiluminescence (Supersignal West Pico, Pierce Rockford, U.S.A.). The presence of GST-fusion proteins was confirmed by reprobing the blots with goat anti-GST antibody (Pharmacia Uppsala, Sweden) and alkaline phosphatase-conjugated rabbit anti-goat IgG antibody (Sigma Co., St. Louis, U.S.A.). The signals were detected with chemiluminescence (CSPD, Roche Mannheim, Germany) or by color deposit using a mixture of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP). To detect MBP-OGT, bacterial extracts were subjected to Western blot analysis using rabbit anti-MBP antibody (New England Biolabs, Beverly, U.S.A.) and HRP-conjugated goat anti-rabbit IgG antibody (Sigma Co., St. Louis, U.S.A.).

RESULTS

In Vitro Glycosylation of GST-Sp1 by Recombinant MBP-OGT

The OGT has been functionally expressed in *E. coli* as S-tagged form by cloning into pET32 vector and shown to glycosylate Nup 62, a subunit of nuclear pore complex, and two mammalian kinases, casein kinase II and GSK-3β [22]. Similarly, we cloned human OGT cDNA into pMALc2 expression vector for the production of MBP-OGT. As shown in Fig. 1, soluble MBP-OGT with approximate MW of 125 kD was purified (Fig. 1A) and could glycosylate recombinant GST-Sp1 *in vitro* (Fig. 1B).

Coexpression of MBP-OGT and GST-Sp1 in *E. coli*

Because *E. coli* has no endogenous OGT-like activity [22], it was tested whether or not MBP-OGT could glycosylate

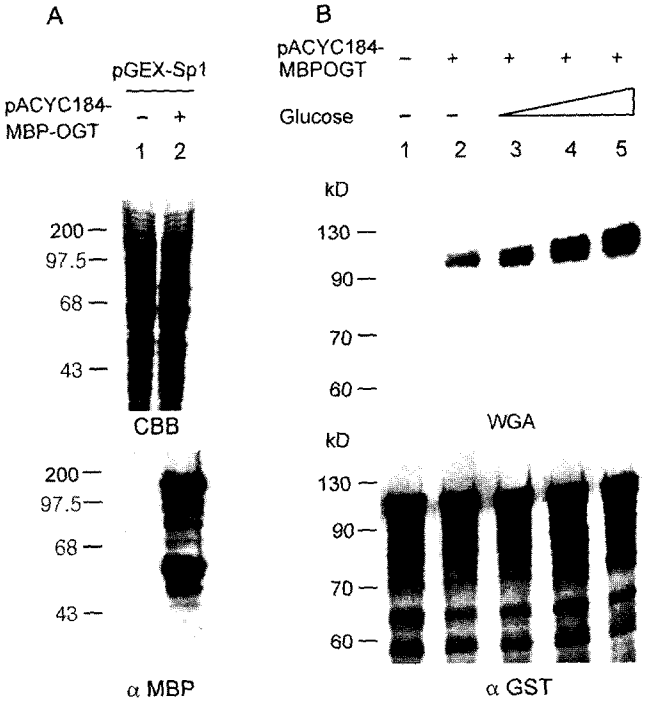

Fig. 3. Coexpression of MBP-OGT and GST-Sp1 produces O-GlcNAcylated Sp1 in *E. coli*.
(A) The extracts of *E. coli* BL21 harboring pGEX-Sp1 only (lane 1) and cotransformed with pACYC184-MBPOGT and pGEX-Sp1 (lane 2) were analyzed by Western blotting using anti-MBP antibody. (B) GST-Sp1 was purified with glutathione agarose from the extracts of the *E. coli* BL21 coexpressing MBP-OGT and GST-Sp1 under various concentration of glucose in growth medium (Lane 2: no addition; Lane 3: 0.1% glucose; Lane 4: 0.5% glucose; Lane 5: 2% glucose). The purified GST-Sp1 proteins were analyzed by Western blotting using anti-GST antibody or WGA.

GST-Sp1 in *E. coli* when MBP-OGT and GST-Sp1 were coexpressed. For coexpression in *E. coli*, the region containing Ptac promoter, MBP and OGT of pMALc2-OGT, was inserted into pACYC184 carrying the replication origin of plasmid p15A, which enabled it to coexist in cells with vectors harboring a ColE1 compatible origin (Fig. 2) and the resultant plasmid pACYC184-MBPOGT was introduced by cotransformation into *E. coli* which contained pGEX-Sp1 plasmid. MBP-OGT was expressed in a significant level in the cotransformant (Fig. 3) and the glycosylated GST-Sp1 could be obtained by purification with glutathione agarose (Fig. 3B, Lanes 1, 2). Furthermore, the addition of high concentration of glucose in the culture medium elevated the level of GST-Sp1 glycosylation (Fig. 3B, Lanes 3-5). This might be because the high glucose concentration increased intracellular UDP-GlcNAc, which donated GlcNAc for O-GlcNAcylation as described previously in eukaryotic cells [13].

Coexpression of MBP-OGT and other O-GlcNAcylable Proteins in *E. coli*

Having observed significant glycosylation of Sp1 by coexpression with OGT, a question arose on whether MBP-OGT could also glycosylate other O-GlcNAcylable proteins. C-terminal domain (CTD) of RNA polymerase II and serum response factor (SRF) are typical O-GlcNAcylated proteins [17, 24]. Thus, CTD and SRF gene were cloned

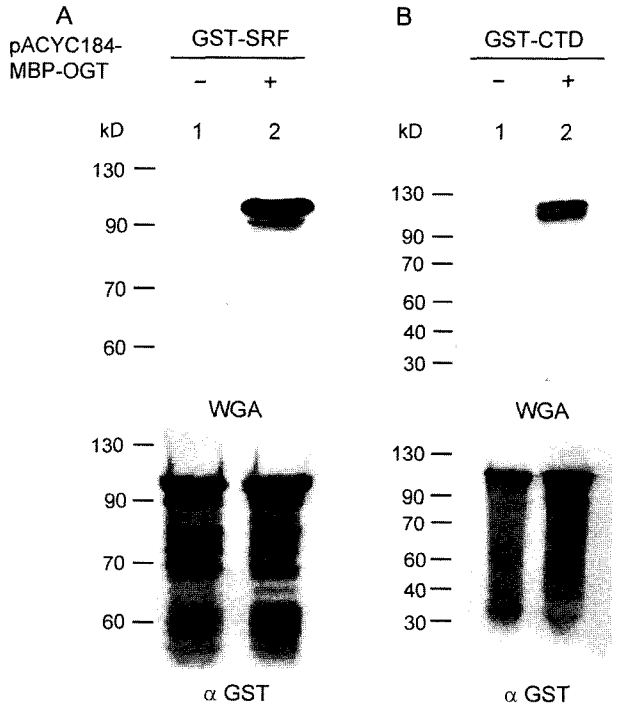

Fig. 4. O-GlcNAc modification of SRF and CTD by coexpression with MBP-OGT.
pACYC184-MBPOGT was introduced into *E. coli* BL21 expressing GST-SRF and GST-CTD. The GST proteins were purified and subjected to Western blotting using anti-GST antibody or WGA.

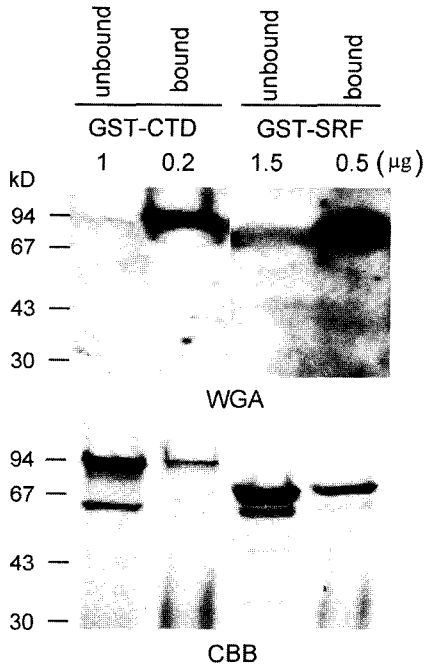

Fig. 5. Isolation of O-GlcNAcylated proteins using WGA chromatography.
GST-CTD and GST-SRF were initially purified using glutathione agarose from cotransformants and the O-GlcNAc-modified forms were further purified using WGA agarose. WGA-bound and unbound proteins were analyzed by Western blotting using WGA or CBB staining on SDS-polyacrylamide gel.

into pGEX-2T vector for expression as GST-fusion proteins, designated pGEX-CTD and pGEX-SRF, and *E. coli* BL21 was cotransformed with pGEX-CTD or pGEX-SRF and pACYC184-MBPOGT. As shown in Fig. 4, MBP-OGT could intracellularly glycosylate the GST-SRF and GST-CTD. To isolate O-GlcNAcylated forms, the GST-CTD and GST-SRF, which had been initially purified from cotransformants by glutathione agarose, were applied to WGA lectin chromatography. Total 200 μg of GST-CTD and 300 μg of GST-SRF were incubated with 20 μl of WGA agarose beads, and 8 μg of O-GlcNAcylated GST-CTD and 20 μg of O-GlcNAcylated GST-SRF, respectively, were obtained. Subsequently, WGA agarose bound and unbound proteins were analyzed by WGA lectin blotting (Fig. 5). It was found that O-GlcNAcylated molecules could be almost completely separated from unglycosylated forms by WGA chromatography.

DISCUSSION

O-GlcNAcylation is now believed to be an important protein modification involved in diverse aspects of cellular physiology [14]. However, since its discovery, there has been only a few studies on the effects of O-GlcNAc on

proteins. This is partly due to the difficulty to obtain O-GlcNAcylated proteins. Most of O-GlcNAcylated eukaryotic proteins are also phosphoproteins [12]. Thus, O-GlcNAcylated proteins purified from eukaryotic cells may be a mixture of proteins with different modifications. Because *E. coli* does not have the usual eukaryotic modification systems, especially glycosyltransferase activities, it can be an ideal expression host to produce pure O-GlcNAcylated proteins. At present, O-GlcNAcylated proteins are obtained by expression of baculovirus vector in insect cells or vaccinia virus vector in mammalian cells [6, 29]. In addition, *in vitro* glycosylation with recombinant OGT purified from *E. coli* may be used [22]. Usually, these methods are time-consuming, highly expensive, and yields are low. Furthermore, careful examinations to confirm that purified proteins do not contain other types of modifications such as phosphorylation are necessary. In the present study, it was shown that coexpression of target proteins with OGT in *E. coli*, which is devoid of eukaryotic protein modification systems, produced O-GlcNAcylated proteins, which could then be simply purified. High glucose concentration or the addition of glucosamine in the growth medium was shown to elevate the intracellular O-GlcNAc level of eukaryotic cells, probably by providing more UDP-GlcNAc needed for O-GlcNAcylation [13]. Similarly, the addition of a large amount of glucose in the growth medium of *E. coli* cotransformant yielded more O-GlcNAcylated proteins, most likely due to the identical reason. Sp1, SRF, and CTD of RNA polymerase II are typical O-GlcNAcylated eukaryotic proteins, and they were all glycosylated efficiently by coexpression with OGT. This system of coexpression with OGT is expected to provide a convenient tool for the production of O-GlcNAcylated eukaryotic proteins.

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