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Human alpha 1-acid glycoprotein as a model protein for glycoanalysis in baculovirus expression vector system

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ABSTRACT

Glycosylation is an important post-translational modification that confers various biological activities, structural stability, and inter-molecular interactions to proteins. Baculovirus expression vector system (BEVS) is widely used to produce recombinant glycoproteins, which may not be suitable for clinical use due to differences in the *N*-linked glycan structure between insects and mammals. It is necessary to develop an appropriate model protein-base platform for glycoanalysis to engineer the insect-type *N*-glycosylation pathway into human type efficiently. In this study, we employed human plasma protein alpha 1-acid glycoprotein (α1AGP). It was highly secreted from cultured silkworm cells and larvae when using the BEVS and glycosylated with insect type *N*-linked glycans. Interestingly, when separated on SDS-PAGE, the purified recombinant α1AGP secreted into silkworm haemolymph generated six distinct products from three alternative translates, suggesting that α1AGP has variations for the recognition or choice of glycosylation sites.

Introduction

The *N*-linked glycan is known to enhance protein stability (Wormald and Dwek, 1999) and regulate protein–protein or cell–cell interactions (Dwek, 1995). *N*-glycosylations with precise carbon structures are involved in the biological activity of a certain protein; thus, abnormal glycosylations often result in disease, including developmental deficiency, neurodegenerative disorders or serious tumors (Cazet et al., 2010; Pochechueva et al., 2012). Therefore, it is essential to obtain the elaborate linkage of a glycoprotein for functional analyses and medical treatment of a particular symptom. Sometimes, in vitro expressions of recombinant glycoproteins with desired constructs are convenient for analyzing their molecular and structural basis, antibody synthesis and medical applications.

To this end, the mammalian expression system is often applied for the production of glycoproteins (Varki, 1993), although it has several disadvantages, such as relatively lower productivity and higher cost. Recently, the baculovirus expression vector system (BEVS) using lepidopteran insects and cells has been reported to be suitable for mass-production of glycoproteins with mammalian-like post-translational modifications and safety profiles (Summers, 2006; Kato et al., 2010). However, it is known that there are some differences in *N*-linked glycosylation pathways between mammalians and insects. Proteins secreted from insect cells are paucimannosidic and generally not terminally galactosylated or sialylated (Marchal et al., 2001) due to the existence of b-N-acetylglucosaminidase (FDL) removing the terminal GlcNAc of the hybrid-type structure (Kim et al., 2009). Then, when tested in mammals, the recombinant glycoproteins from BEVS are less stable in blood than native glycoproteins. Therefore, it has been, for a long time, a problem to use recombinant glycoproteins produced from BEVS in practical medicine research. To date, various approaches have been reported to change the *N*-linked glycan structure of insect proteins into terminally sialylated complex-type *N*-glycans by transgenic expression of mammalian glycosyltransferases in insect cells (Jarvis, 2003; Harrison and Jarvis, 2006; Toth et al., 2014). However, these studies need the recombinant glycoprotein as model to monitor and analyze the *N*-glycosylation linkages from insect-BEVS. For example, human erythropoietin (hEPO) (accession no. BC093628) is often used as a model glycoprotein for research of glycoengineering in BEVS (Mabashi-Asazuma et al., 2013). hEPO is a good model glycoprotein because it has three potential *N*-glycosylation sites in relatively short polypeptide, and has been researched and applied for a long time (Lin et al., 1985; Sytkowski et al., 1991; Rahbek-Nielsen et al., 1997). However, except for hEPO, there are rare model proteins highly glycosylated and easily handled. Therefore, it is required to investigate new candidates to develop an excellent glycoprotein reporter platform.

Alpha 1-acid glycoprotein (α1AGP) is a secretary protein from human plasma containing 5 potential *N*-glycosylation sites, on asparagine (Asn) residues at the consensus sequences of Asn-X-Ser or Asn-X-Thr tripeptides, in a relatively short peptide of 183 amino acid residues (Fournier et al., 2000). In addition, α1AGP has been reported to

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be a potential biomarker of several kinds of cancer where glycosylation linkage was found to be altered (Ceciliani and Pocacqua, 2007). Thus, α1AGP is expected to be an excellent research candidate for the glycoprotein analysis expressed by BEVS.

In this study, we expressed and purified in a high yield, for the first time, recombinant human α1AGP in silkworm larva- and cell-based BEVS. Then, we observed the linkage changed α1AGP product when expressed in FDL-depleted silkworm cells. Furthermore, we analyzed the *N*-linked glycan structures of the recombinant α1AGP from silkworms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). We confirmed that the protein was highly glycosylated with insect-specific paucimannosidic-type glycans.

Materials and methods

Cell lines and silkworms

The NIAS-Bm-oyanagi2 (kindly provided from Dr. Imanishi), BmN4 (a kind gift from Dr. Chisa Aoki), BmN4-SID1 (Mon et al., 2012) and Bme21 cells (Lee et al., 2012) were maintained in IPL41 insect medium (Sigma, USA) with 10% fetal bovine serum (Gibco, USA) at 27 °C. The silkworm strains used in this study were supplied by the silkworm stock center in Kyushu University. The larvae were reared on mulberry leaves at 25–27 °C.

Construction of recombinant baculovirus

The coding region human α1AGP was amplified by PCR using the specific primers Hsα1AGP-5 (5′-GCGCTGTCCTGGGTTCTTACAGTCCTGAGC-3′) and Hsα1AGP *Xho*I-3 (5′-GTCC*CTCGAG*GATTCCCCCTCCTCCTGTTTC-3′). The PCR product was digested with *Xho*I, and inserted into an *EcoRV-Xho*I site of the pENTR11L21TEVH8STREP vector (Mitsudome et al., 2014) containing a lobster L21 sequence at the N-terminal, and the tobacco etch virus (TEV) protease cleavage site, the 8-histidine (H8) tag, and the STREP-tag at C-terminal. Baculovirus transfer plasmid was generated by Gateway LR reaction between pDEST8 vector (Invitrogen, USA) and the α1AGP entry plasmid according to the manufacturer's protocol. The transfer plasmid (pDEST8-*polh*-α1AGP-TEVH8STREP) was used for the BmNPV baculovirus generation according to the protocols described previously (Ono et al., 2007).

Expression of recombinant α1AGP

The expression levels of the recombinant α1AGP protein from viral infection in BmN4 cells and Bme21 cells were determined as

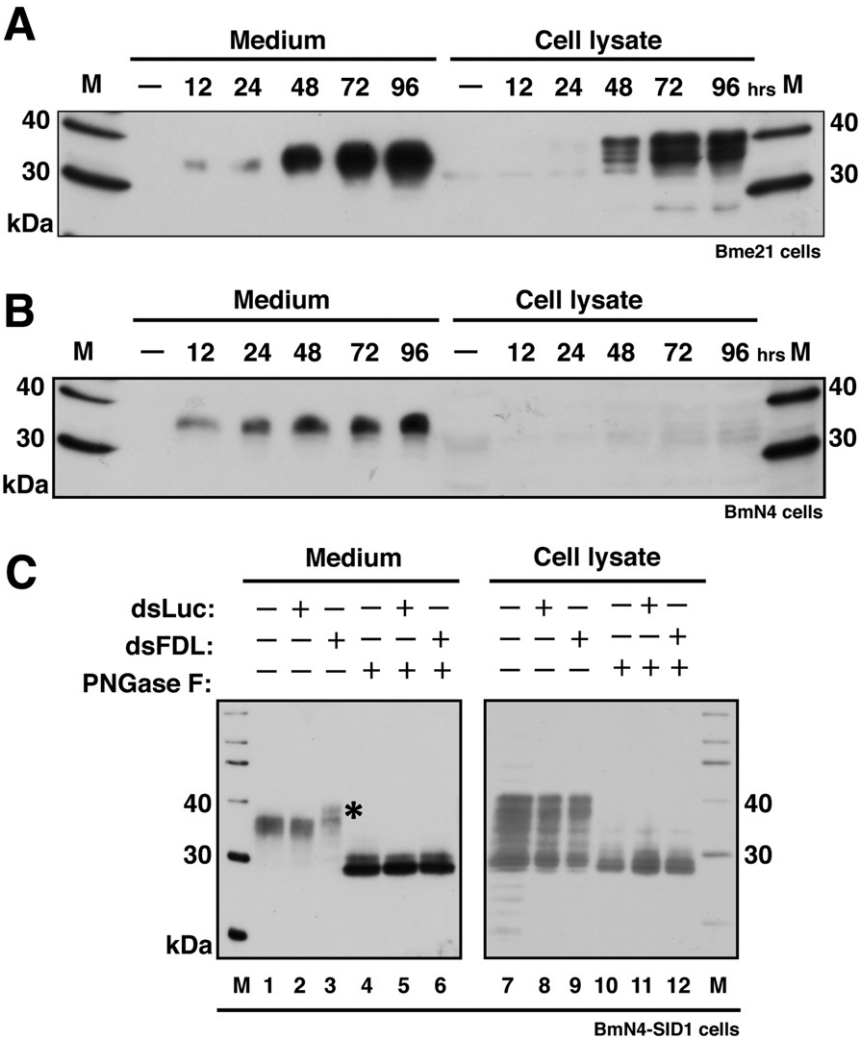

Fig. 1. Expression of human α1AGP in cultured silkworm cells. Time courses of the expression of α1AGP protein in Bme21 cells (A) and BmN4 cells (B). The cells and culture medium were collected at 12, 24, 48, 72, and 96 hpi. The recombinant α1AGP was detected by Western blotting using His-Probe. (C) Effect of FDL depletion on *N*-glycan structures of α1AGP in BEVS. Soaking RNAi-sensitive BmN4-SID1 cells were incubated without (lanes 1, 4, 7, 10) or with 100 ng/ml dsBmLuc (lanes 2, 5, 8, 11) or 100 ng/ml dsBmFDL (lanes 3, 6, 9, 12) and further infected with recombinant BmNPV. The medium (lanes 1–6) and the cell lysate (lanes 7–12) were incubated with PNGaseF (lanes 4–6, 10–12) or without (lanes 1–3, 7–9) were analyzed by Western blotting. Minus (—) represents mock infection. M: molecular mass markers.