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Abstract

Objective To synthesize complex type *N*-glycans in silkworms, shRNAs against the fused lobe from *Bombyx mori* (BmFDL), which codes *N*-acetylglucosaminidase (GlcNAcase) in the Golgi, was expressed by recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) in silkworm larvae.

Results Expression was under the control of the actin promoter of *B. mori* or the U6-2 and ie-2 promoters from *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV). The reduction of specific GlcNAcase activity was observed in Bm5 cells and silkworm larvae using the U6-2 promoter. In silkworm larvae, the partial suppression of BmFDL gene expression was observed. When shRNA against BmFDL was expressed under the control of U6-2 promoter, the $Man_3GlcNAc(Fuc)GlcNAc$ structure appeared in a main *N*-glycans of recombinant human IgG. These results suggested that the control of BmFDL expression by its shRNA in silkworms caused the modification of its *N*-glycan synthetic pathway, which may lead to the alteration of *N*-glycans in the expressed recombinant proteins.

Conclusions Suppression of BmFDL gene expression by shRNA is not sufficient to synthesize complex *N*-glycans in silkworm larvae but can modify the *N*-glycan synthetic pathway.

Keywords RNAi · BmNPV · Silkworm · *N*-Glycan · IgG · MALDI-TOF-MS

Introduction

Insect cells have been utilized for recombinant protein production because post-translational modifications, including phosphorylation, processing and *N*-glycosylation, of expressed recombinant proteins are conducted as well as in mammalian cells. Some therapeutic products produced in insect cells have been commercialized, for example, Cervarix®, a human papillomavirus vaccine provided by GlaxoSmithKline, and Flublok®, an influenza A virus vaccine provided by Protein Science Corporation (Felberbaum 2015). However, regarding *N*-glycosylation in insect cells, most *N*-glycans produced in insect cells are of the pauci-mannose type, rather than the complex types of glycans in mammalian cells (Altmann et al., 1999). To overcome this problem, the *N*-glycan synthetic pathway in insect cells has been engineered by the transient or constitutive expression of mammalian glycosyltransferases. For transient expression, a baculovirus was used, in addition to the expression of recombinant proteins (Jarvis and Finn, 1996; Wolff et al., 1999). In this case, both a recombinant protein and mammalian glycosyltransferases must be expressed by recombinant baculoviruses, and the number of glycosyltransferases to be expressed is limited. Therefore, transformed insect cells constitutively expressing certain mammalian glycosyltransferases were developed (Aumiller et al., 2012; Tomiya et al., 2003). Moreover, to modify sialic acid to *N*-glycans efficiently in insect cells, enzymes in the cytidine-5'-monophosphate (CMP) -sialic acid synthetic pathway and CMP-sialic acid transporter were expressed in insect cells in addition to mammalian glycosyltransferases (Geisler and Jarvis, 2012a; Mabashi-Asazuma et al., 2013; Toth et al., 2014). These transformed insect cells that express only mammalian glycosyltransferases can produce larger amounts of sialylated *N*-glycan on recombinant proteins than normal insect cells.

A constitutive promoter, for example, the ie-2 promoter from *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV), was used for the expression of mammalian glycosyltransferases in insect cells even when mammalian glycosyltransferases were expressed transiently in insect cells (Jarvis and Finn, 1996, Wolff et al., 2003). In using baculoviruses to express mammalian glycosyltransferases in insects, several recombinant baculoviruses must be infected to produce *N*-glycan-engineered recombinant proteins. In general, multiple infections with several recombinant baculoviruses lead to decreased expression levels of recombinant proteins. Therefore, to overcome this problem, insect cells stably expressing several mammalian glycosyltransferases have been developed (Aumiller et al., 2012; Tomiya et al., 2003). Recently, SweetBac containing bovine β1,4-galactosyltransferase I (βGalTI) and *Caenorhabditis elegans N*-acetylglucosaminyltransferase II (cGnTII) was constructed based on the multibac system, and human IgG H and L chains were expressed in addition to these glycosyltransferases in a SweetBac bacmid in insect cells (Bieniossek et al., 2012; Palmberger et al., 2012). These heterologous glycosyltransferases were under the control of the polyhedrin and p10 promoters, and inducible promoters were investigated also for *N*-glycan synthetic pathway engineering in insect cells (Toth et al., 2014).

To synthesize complex *N*-glycans, *N*-acetylglucosamine (GlcNAc) addition to the Manα (1,3)-branch of the core structure by *N*-acetylglucosaminyltransferase I (GnTI) is an important step. However, the activities of *N*-acetylglucosaminidase (GlcNAcase) and α-mannosidase, which removes GlcNAc residue from synthesized *N*-glycans and α1,3-mannose residues from the core structure of *N*-glycan, respectively, are high in insect cells. Therefore, most of the *N*-glycans synthesized in insect cells are of the pauci-mannose type even when indigenous GlcNAcase is expressed (Geisler and Jarvis 2012b;

Tomiya et al., 2003). Three GlcNAcase genes (GlcNAcase I, II and fused lobe (FDL)) were already cloned in *Spodoptera frugiperda*, *Trichoplusia ni*, *Bombyx mori* and *Drosophila melanogaster* (Geisler et al., 2008; Geisler and Jarvis 2010; Kokuho et al., 2010; Léonard et al., 2006; Nomura et al., 2010; Okada et al., 2007; Tomiya et al., 2006). Among these three GlcNAcases, FDL that is localized in the Golgi apparatus, is involved in *N*-glycan processing in insect cells. To engineer the *N*-glycan synthesis pathway to execute complex *N*-glycan synthesis in insect cells, the FDL gene was suppressed by RNAi in insect cells and silkworm larvae (Kim et al., 2011; Nomura et al., 2015). This FDL inhibition led to complex *N*-glycan synthesis in insect cells.

To express *B. mori* FDL (BmFDL) gene-specific shRNA in silkworm, various kinds of promoters, the actin promoter from *B. mori*, the ie-2 promoter from OpMNPV, and the U6-2 promoter from *B. mori,* were used and evaluated. Actin promoter from *B. mori* and ie-2 promoter from OpMNPV are an RNA polymerase II (Pol II) promoter, which works for the synthesis of mRNA precursors, most of snRNA and miRNA (Egloff et al., 2008; Schanen and Li, 2011). U6-2 promoter from *B. mori* is an RNA polymerase III (Pol III) promoter (Tanaka et al., 2009), which works for the synthesis of structural RNAs for translation (tRNA, 5S rRNA, 7SL RNA), RNA processing (RPR1) and splicing (U6). Normally, the Pol III promoter has been used for the expression of shRNA, but the Pol II promoter has also sometimes worked well for shRNA expression (Giering et al., 2008; Su et al., 2008).

In this study, short hairpin RNA (shRNA) was expressed in silkworm larvae through the use of recombinant *B. mori* nucleopolyhedrovirus (BmNPV) to suppress the BmFDL gene for the engineering of the *N*-glycosylation pathway. The expression of shRNA against the BmFDL gene by recombinant BmNPVs in silkworm larvae was evaluated,

and the *N*-glycan structure attached to recombinant human IgG expressed in silkworm larvae under the suppression of BmFDL expression was investigated.

Materials and methods

Construction of recombinant BmNPV bacmids for shRNA expression

Usually, recombinant proteins have been expressed in insect cells under the control of the polyhedrin promoter. In this study, shRNA against the BmFDL gene was expressed using recombinant BmNPV under the control of the constitutive Pol II or Pol III promoters. The actin and U6-2 promoter sequences from *B. mori* were amplified by PCR using the primer sets Actin-F, Actin-R and U6-F, U6-R, respectively (Table 1). The ie-2 promoter from OpMNPV was amplified by PCR using ie-F and ie-R (Table 1). Each amplified DNA fragment was replaced with the polyhedrin promoter sequence in pFastBac1 (Life Technologies Japan, Tokyo, Japan) by InFusion technology (TAKARA Bio, Shiga, Japan). For this InFusion reaction, pFast-F and pFast-R primers (Table 1) were used to amplify pFastbac1 vector lacking polyhedrin promoter sequence. The resulting plasmids were designated as pFast-Actin, pFast-U6, and pFast-ie, respectively. Each pFast vector containing the shRNA sequence was amplified by PCR using sh-FDL primer, and each reverse primer (Actin-R, U6-R, ie-R, Table 1) and its DNA fragment were self-ligated. Actin-R, U6-R and ie-R primers have shRNA sequence, respectively, and this shRNA sequence was placed at the downstream of each promoter sequence after its self-ligation. Each resulting plasmid was transformed into *Escherichia coli* BmDH10BacCP^{-}Chi^{-} (Park et al., 2008), and a white colony was picked up to extract the recombinant BmNPV

bacmid. Constructed recombinant BmNPV bacmids were designated as BmNPVCP$^-$Chi$^-$/Actin/sh-FDL, BmNPVCP$^-$Chi$^-$/U6/sh-FDL and BmNPVCP$^-$Chi$^-$/ie/sh-FDL bacmids, respectively. For a negative control, BmNPVCP$^-$Chi$^-$ bacmids containing the expression cassette of shRNA to the EGFP gene (BmNPVCP$^-$Chi$^-$/Actin/sh-EGFP, BmNPVCP$^-$Chi$^-$/U6/sh-EGFP and BmNPVCP$^-$Chi$^-$/ie/sh-EGFP bacmids) using the EGFP-F primer (Table 1) were constructed. A recombinant BmNPV bacmid containing human IgG H chain and L chain genes was constructed previously (Park et al., 2007) and used in this study.

Infection of recombinant BmNPV into Bm5 cells or silkworm larvae

Bm5 cells were maintained in Sf-900II (Life Technologies, Japan) supplemented with 1% fetal bovine serum (FBS, Sigma Aldrich, Japan, Tokyo, Japan) and antibiotic-antimycotic solution (Life Technologies, Japan) at 27°C. The hemolymph of silkworm larvae containing recombinant BmNPV was diluted in phosphate-buffered saline (PBS, pH 7.4) and used as a recombinant BmNPV solution. Bm5 cells were infected with each recombinant BmNPV containing shRNA at a multiplication of infection (MOI) of 1.

Each recombinant BmNPV was injected into a silkworm larva at 1×10^7 pfu. In the case of co-injection, two recombinant BmNPVs were mixed at a 1:1 ratio and injected into a silkworm at 1×10^7 pfu per larva. Silkworm larvae were reared on an artificial diet (Silkmate S2, Nosan, Yokohama, Japan). Recombinant BmNPV titers were measured by semi-quantitative real-time PCR with the primer sets Bm ie-1 F and Bm ie-1-R (Table 1) (Kato et al., 2009). In siRNA experiment, 600 pmol of each siRNA was transfected into Bm5 cells in a 25 cm^2 T-flask.

GlcNAcase assay

Bm5 cells and the fat body from silkworm larvae were suspended in 20 mM phosphate buffer (pH 7.4) containing 0.4% Triton X-100 and protease inhibitor cocktail (Roche diagnostics, Mannheim, Germany) and disrupted by sonication (30 s × 3). The homogenate was centrifuged to remove insoluble materials. The supernatant was used as an enzyme solution.

The enzyme solution was added to 20 mM phosphate buffer (pH 7.4) containing 1 mM *p*-nitrophenyl-GlcNAc and incubated at 37°C. The reaction was stopped by the addition of 200 mM Na_2CO_3. One unit of enzyme activity was defined as the amount of enzyme capable of catalyzing the release of 1 μmol of GlcNAc per minute.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from Bm5 cells and the fat body of silkworm larvae using TRIzol reagent (Life Technologies, Japan). The quality of the extracted RNA was checked by both electrophoresis and spectrophotometry. RT-PCR was performed using the Prime Script RT-PCR Kit (TAKARA Bio). In this RT-PCR, the BmFDL-F and BmFDL-R primers were used for the amplification of a part of the BmFDL cDNA; BmGAPDH-F and BmGAPDH-R were used as a control for the amplification of the BmGAPDH gene.

Purification of recombinant human IgG from hemolymph of silkworm larvae

Recombinant human IgG from silkworm larval hemolymph was purified using Protein A

Sepharose gel (GE healthcare science Japan, Tokyo, Japan). Next, 0.1 M citrate buffer (pH 3.0) was used for the elution of recombinant human IgG, and the pH in the eluted fractions was immediately adjusted to approximately pH 7.0 with 1 M Tris-HCl (pH 8.0).

Determination of *N*-glycan structure in recombinant human IgG

All experimental procedures, including the chromatographic conditions and glycosidase treatments, have been described previously (Nakagawa et al, 1995; Takahashi et al., 1995: Tomiya et al., 1987), with slight modifications of the preparation of 2-aminopyridine (PA) -derivatives of the *N*-glycans. The purified recombinant human IgG (~2 mg) was treated with 20 μg pepsin (final concentration 0.5 mg/mL) plus 0.5 mU glycoamidease A (final concentration 125 mU/mL) in 0.1 M citrate-phosphate buffer, pH 4.0, at 37^{o}C overnight. The resultant peptides were further digested by 20 μg pronase (final concentration 0.2 mg/mL) in 1 M Tris-HCl buffer, pH 8.0, at 37^{o}C overnight. After the removal of the peptide materials by using a graphite carbon columns (GL Science, Tokyo, Japan), the reducing ends of the *N*-glycans were derivatized with PA (Wako pure chemical, Osaka, Japan). The mixture of PA-derivatives of the *N*-glycans was individually separated and the components identified sequentially on a Shim-pack HRC-ODS column (Shimadzu, Kyoto, Japan) and on a TSK-gel Amide-80 column (Tosoh, Tokyo, Japan). The identification of *N*-glycan structures was based on their elution positions on two kinds of columns in comparison with PA-glycans in the GALAXY database (Takahashi and Kato, 2003). The *N*-glycans were confirmed by co-chromatography and MALDI-TOF-MS analysis (Yagi et al., 2005).

Results and discussion

Target sequence in BmFDL gene for siRNA-mediated suppression of its expression

Among the three GlcNAcases in *B. mori*, BmFDL, which resides in the Golgi, participates in the *N*-glycan synthetic pathway. First, to find the target sequence of the BmFDL gene for the suppression of BmFDL gene expression by shRNA, RNA interference (RNAi) by siRNA was performed in Bm5 cells. Three sequences in the BmFDL gene were screened by siRNA Target Finder (http://www.genscript.com/ssl-bin/app/rnai) and were selected as target sequences: FDL420; from 420 to 442 bp, FDL830; from 830 to 852 bp, FDL1159; from 1159 to 1181 bp (Fig. 1A). The siRNA to the EGFP gene (from 480 to 502 bp) was used as a negative control. GlcNAcase activity was measured at 2 days after transfection with each siRNA. The addition of siRNA to the BmFDL gene from 1159 to 1181 bp was the most efficient at reducing specific GlcNAcase activity among the three siRNAs (Fig. 1B). The sequence was selected as a target sequence in the BmFDL gene for shRNA-mediated suppression of its expression.

Expression of shRNA against BmFDL gene in Bm5 cells and silkworm larvae

To produce recombinant proteins whose *N*-glycan synthetic pathway has been modified for producing complex *N*-glycans in silkworms, siRNA injection into silkworm larvae is not practical because of its cost. Therefore, shRNA expression by recombinant BmNPV in Bm5 cells and silkworm larvae was conducted to suppress BmFDL gene expression. First, shRNA against the BmFDL gene was expressed in Bm5 cells and silkworm larvae

using recombinant BmNPVs to suppress the activity of GlcNAcase, which is involved in the *N*-glycan synthetic pathway in insect cells. The suppression of BmFDL gene expression by shRNA expression was performed in Bm5 cells. The constructed shRNA to the BmFDL gene is shown in Fig. 2A. After infection with BmNPVCP$^-$Chi$^-$/U6/sh-FDL, the specific GlcNAcase activity of Bm5 cells was significantly reduced by 62% compared with Bm5 cells infected with BmNPVCP$^-$Chi$^-$/U6/sh-EGFP (Fig. 2B). However, the specific GlcNAcase activity of Bm5 cells was not significantly reduced in the case of infection with BmNPVCP$^-$Chi$^-$/Actin/sh-FDL or BmNPVCP$^-$Chi$^-$/ie/sh-FDL. This indicates that the Pol III promoter, which has been used for shRNA expression, works well for shRNA expression in Bm5 cells. It was previously reported that the expression of shRNA by the U6-2 promoter from *B. mori* led to specific gene knockdown in Bm cells (Tanaka et al., 2009). At 3 days after infection with BmNPVCP$^-$Chi$^-$/U6/sh-FDL, the specific GlcNAcase activity of Bm5 cells was significantly reduced by 34% compared to Bm5 cells infected with BmNPVCP$^-$Chi$^-$/U6/sh-EGFP (Fig. 2C). The infection of BmNPVCP$^-$Chi$^-$/ie/sh-FDL decreased specific GlcNAcase activity by 25% at 3 days after infection.

In the case of silkworm larvae, the specific GlcNAcase activity of the fat body was significantly reduced by infection with BmNPVCP$^-$Chi$^-$/U6/sh-FDL and BmNPVCP$^-$Chi$^-$/ie/sh-FDL at 3 days after infection but was not altered by infection with BmNPVCP$^-$Chi$^-$/Actin/sh-FDL (Fig. 3A). The mRNA level of BmFDL during each shRNA-expressing recombinant BmNPV was investigated by RT-PCR. The agarose gel electrophoresis in the RT-PCR experiment was shown in Fig. 3B and the rate of its mRNA reduction was calculated. The level of BmFDL mRNA was reduced to 71, 63, and 80% by the infection with each BmFDL gene-specific shRNA expressing recombinant BmNPVCP$^-$Chi$^-$

/Actin/sh-FDL, /U6/sh-FDL and /ie/sh-FDL, respectively, compared with BmNPVCP^{-} Chi^{-}/U6/sh-EGFP (Fig. 3B). *B. mori* has three GlcNAcase genes, and BmFDL and BmGlcNAcase2 are expressed in the fat body (Nomura et al., 2015). Multiple expression of GlcNAcase genes in the fat body seems to complicate the evaluation of specific GlcNAcase activity. However, the suppression of BmFDL gene expression in the fat body by recombinant BmNPV was not efficient compared with the results of a previous paper using RNAi transgenic silkworms (Nomura et al., 2015).

N-glycan structure of recombinant human IgG expressed in silkworm larvae

From the results of BmFDL gene suppression using Bm5 cells and silkworm larvae, the U6-2 promoter was used to express BmFDL gene-specific shRNA for recombinant protein *N*-glycan analysis. To investigate the *N*-glycan structure in recombinant human IgG expressed in silkworms with the co-expression of shRNA against the BmFDL gene, recombinant human IgG was purified from the hemolymph of silkworm larvae with co-expression of shRNA against the BmFDL gene. *N*-glycans were released from recombinant human IgG and animated with PA. PA-labeled oligosaccharides were assigned by HPLC mapping (Fig. 4). When shRNA against the BmFDL gene was expressed under the control of the actin or ie-2 promoter, Man_2GlcNAc(Fuc)GlcNAc was predominantly observed (actin: 65%, ie-2: 61.5%). When the BmFLD gene was not suppressed, the ratio of Man_2GlcNAc(Fuc)GlcNAc was 77.5%, which is higher ratio than the results of Fig. 4, indicating that the suppression of BmBDL gene shows a limited effect. All *N*-glycans observed in recombinant human IgG co-expressed with shRNA under the control of the actin or ie-2 promoter were of the high-mannose or pauci-

mannose type. In the case of the U6-2 promoter, $Man_3GlcNAc(Fuc)GlcNAc$ (46.8%) was observed in addition to $Man_2GlcNAc(Fuc)GlcNAc$ (40.0%). However, all *N*-glycans observed were still of the high-mannose or pauci-mannose type. Any complex glycans containing GlcNAc residues at the ends were not observed with co-expression of shRNA against to the BmFDL gene, but the co-expression of shRNA under the control of the U6-2 promoter had significant effects on *N*-glycan structure in recombinant human IgG in silkworm larvae. In silkworms, a GlcNAc residue was detected at the Manα(1,3)-branch of the core structure of *N*-glycans attached to recombinant glycoproteins, indicating that *B. mori* exhibits GnTI activity (Nomura et al., 2015). However, in this study, no GlcNAc residues were observed even when shRNA against the BmFDL gene was co-expressed to suppress BmFDL gene expression.

GnTI and mannosidase II or III (ManII or ManIII) operate on $Man_3GlcNAc(Fuc)GlcNAc$ in insect cells, leading to the production of $GlcNAcMan_3GlcNAc(Fuc)GlcNAc$ and $Man_2GlcNAc(Fuc)GlcNAc$, respectively (Altmann et al., 1999; Aoki et al., 2007; Francis et al., 2002). $GlcNAcMan_3GlcNAc(Fuc)GlcNAc$ is converted to $Man_3GlcNAc(Fuc)GlcNAc$ by FDL. Glycosyltransferases in the Golgi are known to form heterodimeric complexes and interact with each other (Hassinen et al., 2010; Schoberer et al., 2013). In HeLa cells, GnTI and ManII interact, and the localization of GnTI influences the localization of ManII (Nilsson et al., 1996). Mouse GnTI inhibitory protein (GnT1IP-L), which is a glycosyltransferase-like protein, inhibits GnTI in Golgi by forming the heteromeric complex with GnTI, leading to the synthesis of high-mannnose type *N*-glycans in glycoproteins (Huang and Stanley, 2010; Huang et al., 2015). It is possible that the suppression and overexpression of Golgi-resident enzymes influence the expression and

localization of other enzymes in the Golgi and can be utilized to re-design the *N*-glycan synthetic pathway to produce complex *N*-glycans. In this study, BmFDL is speculated to form a complex with BmManII or BmManIII and the suppression of BmFDL expression by its shRNA may influence the function of BmManII or BmManIII in Golgi, leading to increase the level of $Man_3GlcNAc(Fuc)GlcNAc$ in *N*-glycans of recombinant IgG expressed in silkworm larvae (Fig. 5).

In conclusions, shRNA against the BmFDL gene was expressed by recombinant BmNPVs in Bm5 cells and silkworm larvae to suppress BmFDL gene expression. Specific GlcNAcase activity was reduced in Bm5 cells and silkworm larvae by the expression of shRNA under the control of the U6-2 promoter. The expression of shRNA under the control of the U6-2 promoter led to increased levels of $Man_3GlcNAc(Fuc)GlcNAc$ in recombinant human IgG compared with the expression of shRNA under the control of the actin promoter or ie-2 promoter. This shRNA expression by recombinant BmNPV under the control of the U6-2 promoter can be utilized for the modification of the *N*-glycan synthetic pathway in silkworms together with the heterologous expression of mammalian glycosyltransferases.

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Table 1. Used primers

Name	Sequence (5’ – 3’)
Actin-F	CCGGAATATTAATAGAGGTACCACCACCCTGCC
Actin-R	CTTCGGACCGGGATCTCGATATCAAGCTTATCGATAC
U6-F	CCGGAATATTAATAGAGGTTATGTAGTACACATTG
U6-R	CTTCGCACCGGGACTTGTAGAGCACGATATT
ie-F	CCGGAATATTAATAGCATGATGATAAACAATGTATGG
ie-R	CTTCGGACCGGGATCAACAGATGCTGTTCAACTGTG
pFast-F	GATCCCGGTCCGAAGCGCGCG
pFast-R	CTATTAATATTCCGGAGTACAC
sh-FDL	TTCTAGTAAATCGTATATGTGTTCAAGAGACATATACGATTTACTAGAACAT TTTTTATCGGGCGCGGATCCCGGTC
sh-EGFP	TTGAAGTTCACCTTGATGCCGTTCAAGAGAGCATCAAGGTGAACTTCAAGAT TTTTTATCGGGCGCGGATCCCGGTC
Bmie-1 F	CCCGTAACGGACCTTGTGCTT
Bmie-1 R	TATCGAGATTTATTTACATACAACAAG
BmGAPDH-F	CGCTGGAATTTCTTTGAATGAC
BmGAPDH-R	CAATGACTCTGCTGGAATAACC

Figure legends

Fig. 1 Effect of siRNA on GlcNAcase activity in Bm5 cells. (A) Construction of the prepared siRNAs. FDL420, FDL830 and FDL1159 target the sequences of BmFDL from 420 to 442 bp, from 830 to 852 bp and from 1159 to 1181 bp, respectively. The siRNA to the EGFP gene targets the sequence of the EGFP gene from 480 to 502 bp and was used as a negative control. (B) Specific GlcNAcase activity of Bm5 cells transfected with each siRNA. Each GlcNAcase activity was measured 2 days after siRNA transfection. Specific GlcNAcase activity of Bm5 cells was represented as a value relative to cells transfected with siRNA for the EGFP gene. Bars in B denote standard deviation (n=3).

Fig. 2 Effect of shRNA expression and activity of GlcNAcase in Bm5 cells by recombinant BmNPV . (A) Constructed shRNAs in this study. (B) Specific GlcNAcase activity in Bm5 cells 1 day after infection with BmNPVCP$^-$Chi$^-$/Actin/sh-FDL, BmNPVCP$^-$Chi$^-$/U6/sh-FDL or BmNPVCP$^-$Chi$^-$/ie/sh-FDL. BmNPVCP$^-$Chi$^-$/Actin/sh-EGFP, BmNPVCP$^-$Chi$^-$/U6/sh-EGFP and BmNPVCP$^-$Chi$^-$/ie/sh-EGFP were used as negative controls. (C) Specific GlcNAcase activity of Bm5 cells 3 days after infection with BmNPVCP$^-$Chi$^-$/Actin/sh-FDL, BmNPVCP$^-$Chi$^-$/U6/sh-FDL or BmNPVCP$^-$Chi$^-$/ie/sh-FDL. Specific GlcNAcase activity in B and C was represented as a value relative to the negative control. The collected data in B and C were analyzed with the statistical test of t-test, and were considered statistically significant ($p < 0.05$). Bars in B and C denote standard deviation (n=3).

Fig. 3 Effect of shRNA expression by recombinant BmNPV on the expression and activity of GlcNAcase in silkworm larvae. (A) Specific GlcNAcase activity in the fat

body of silkworm larvae 3 days after infection with BmNPVCP$^-$Chi$^-$/Actin/sh-FDL, /U6/sh-FDL or /ie/sh-FDL. BmNPVCP$^-$Chi$^-$/Actin/sh-EGFP, /U6/sh-EGFP and /ie/sh-EGFP were used as each negative control. Specific GlcNAcase activity was represented in terms of a value relative to the negative control. The collected data were analyzed with the statistical test of t-test, and were considered statistically significant ($p< 0.05$). (B) The mRNA level of BmFDL in the fat body of silkworm larvae 3 days after infection with BmNPVCP$^-$Chi$^-$/Actin/sh-FDL, /U6/sh-FDL or /ie/sh-FDL, compared with BmNPVCP$^-$Chi$^-$/U6/sh-EGFP. The mRNA level of the negative control is shown as 1. Bars in A and B denote standard deviation (n=3). In the electrophoresis of RT-PCR products, 1.5% agarose gel was used.

Fig. 4 *N*-glycan structure of recombinant human IgG. The purified recombinant human IgG treated pepsin and glycoamidease, was further digested by 20 μg pronase. After the removal of the peptide materials by using a graphite carbon, the reducing ends of the *N*-glycans were derivatized with 2-aminopyridine (PA). The mixture of PA-derivatives of the *N*-glycans was individually separated and the components identified sequentially on a Shim-pack HRC-ODS column and on a TSK-gel Amide-80 column. The identification of *N*-glycan structures was based on their elution positions on two kinds of columns in comparison with PA-glycans in the GALAXY database (Takahashi and Kato, 2003). The *N*-glycans were confirmed by co-chromatography and MALDI-TOF-MS analysis (Yagi et al., 2005).

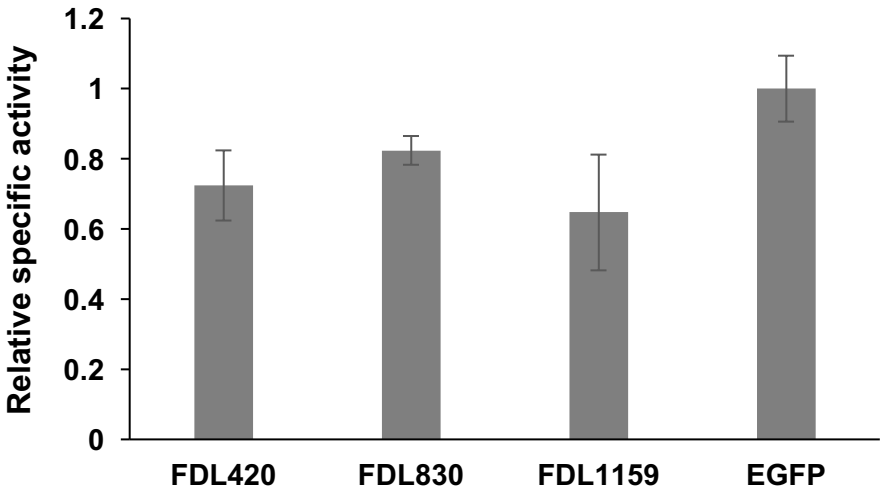
Fig. 5 A proposed pathway of the increase of $Man_3GlcNAc(Fuc)GlcNAc$ in *N*-glycans of recombinant IgG expressed in silkworm larvae by the suppression of the BmFDL

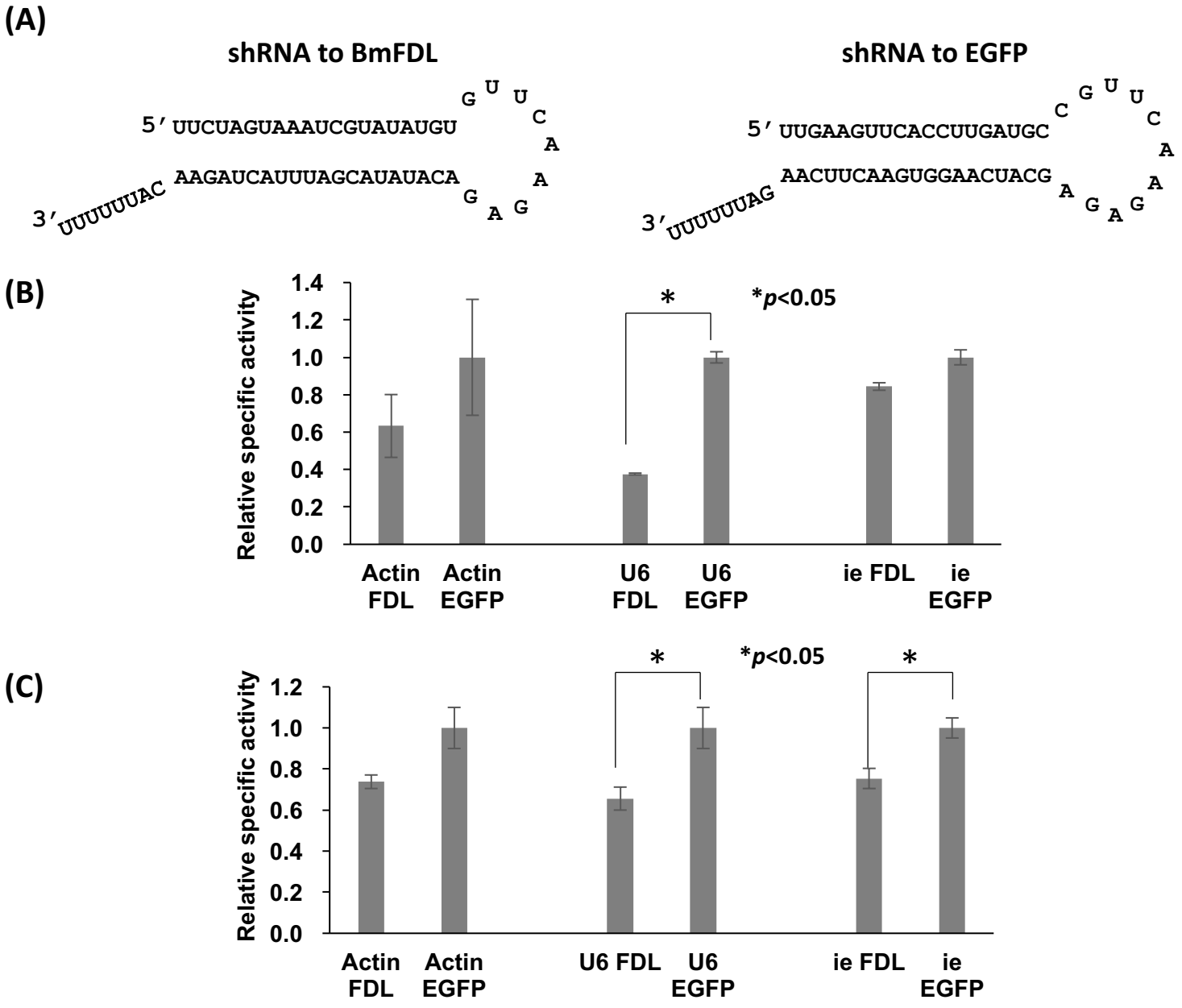
expression. BmFDL speculates to form the complex with BmManII or BmManIII and the suppression of BmFDL expression may influence the function of BmManII or BmManIII.

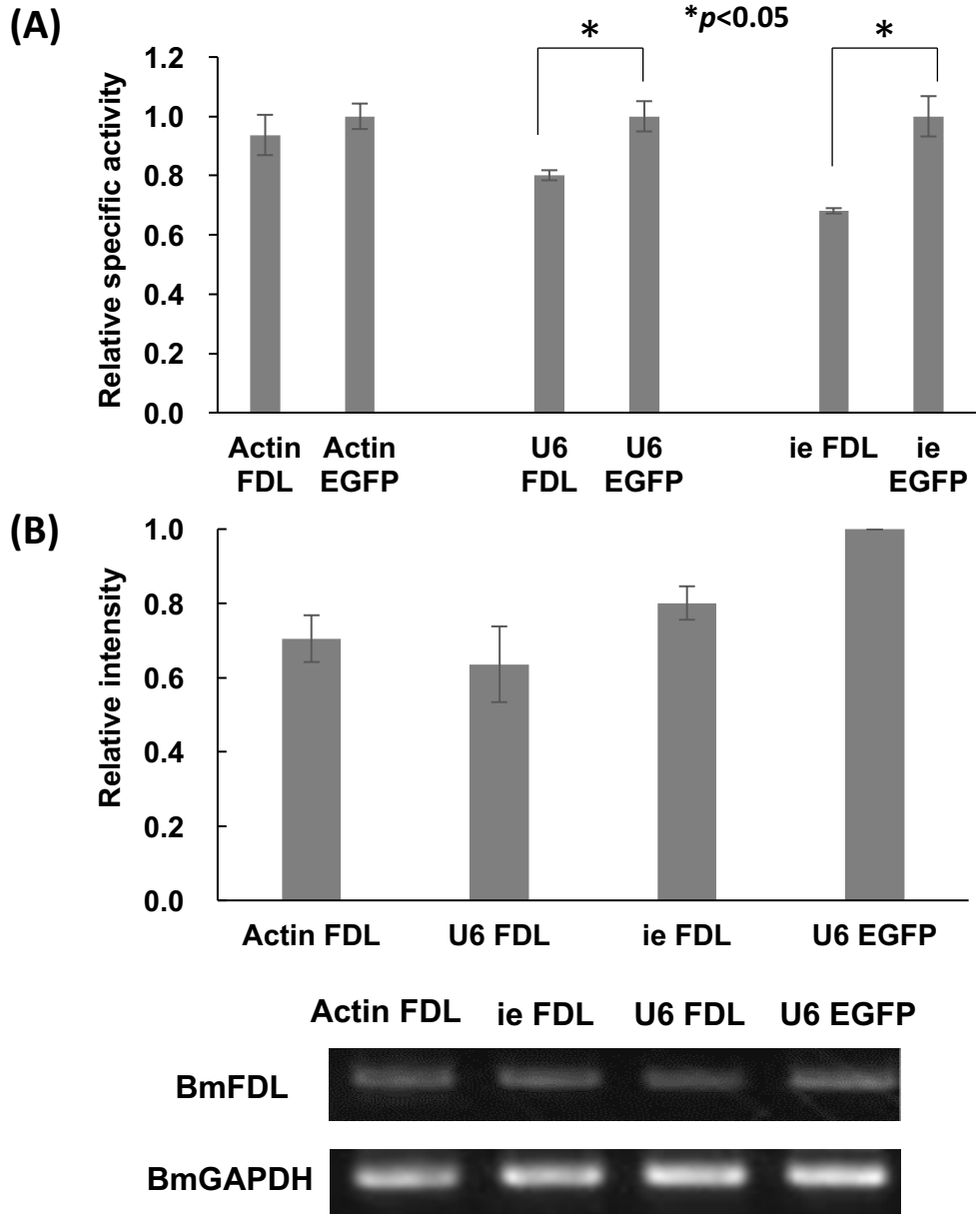
(A)

FDL420	5′ CGUUUAUCGUGAUGCAAAAUC 3′	
	3′ CGCTTTUAGCACUACGUUUU 5′	
FDL830	5′ GCGAACAAACUAAACACGUUC 3′	
	3′ GGCGCUUGUUUGAUUUGUGCA 5′	
FDL1159	5′ CAUAUACGAUUUACUAGAACA 3′	
	3′ GUGUAUAUGCUAAAUGAUCUU 5′	
EGFP	5′ GCAUCAAGGUGAACUUCAAGA 3′	
	3′ GCCGUAGUUCCACUUGAAGUU 5′	

(B)




(A)
shRNA to BmFDL
5′ UUCUAGUAAAUCGUAUAUGU
AAGAUCAUUUAGCAUAUACA
3′ UUUUUUAC
G U U C A A G A G
shRNA to EGFP
5′ UUGAAGUUCACCUUGAUGC
AACUUCAAGUGGAACUACG
3′ UUUUUUAG
C G U U C A A G A G
(B)
Relative specific activity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*p<0.05
Actin FDL
Actin EGFP
U6 FDL
U6 EGFP
ie FDL
ie EGFP
(C)
Relative specific activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*p<0.05
*
Actin FDL
Actin EGFP
U6 FDL
U6 EGFP
ie FDL
ie EGFP


(A)
*p<0.05
*
*
Relative specific activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Actin FDL
Actin EGFP
U6 FDL
U6 EGFP
ie FDL
ie EGFP
(B)
Relative intensity
1.0
0.8
0.6
0.4
0.2
0.0
Actin FDL
U6 FDL
ie FDL
U6 EGFP
Actin FDL
ie FDL
U6 FDL
U6 EGFP
BmFDL
BmGAPDH

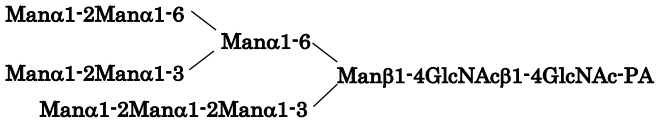
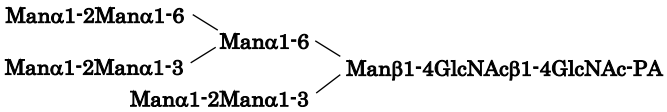
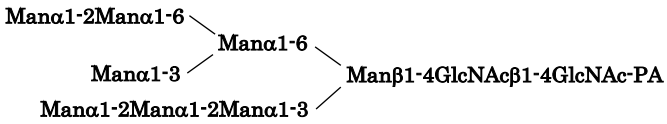
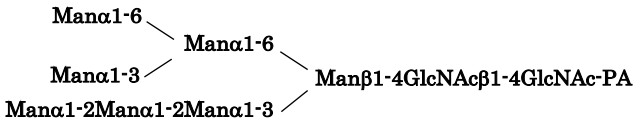
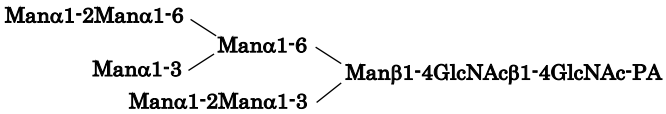
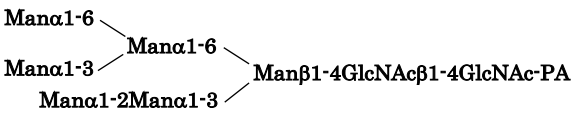
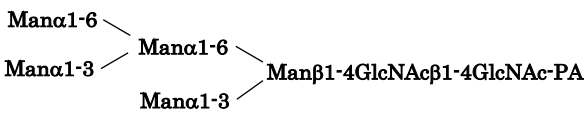
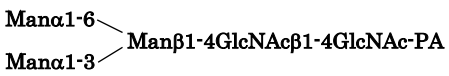
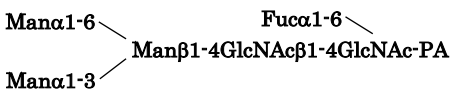
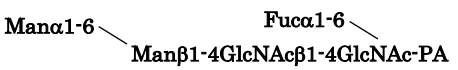
Structure	Ratio (%)		
	Actin	IE2	U6
	2.6	5.5	
	1.3	1.7	
	2.1	5.6	
	0.5	1.0	
	1.5	2.7	
	0.9	1.6	
	4.6	5.1	4.7
	2.2	1.8	4.0
	3.6	3.3	3.3
	15.5	9.9	46.8
	65.0	61.5	40.0
Others	0.3	0.3	1.2

Fig. 5. Kato et al.