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The role of glycosylation in the function of a 48-kDa glycoprotein from carrot

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Abstract

Carrot extracellular dermal glycoprotein (EDGP) may play an important role in plant defense systems and in signal transduction. Our experiments show that differences in p*I* values of EDGP isoforms are caused by differences in amino acid sequence and not by heterogeneity in phosphorylation. The binding affinity of native EDGP for a 4-kDa hormone-like peptide from soybean was approximately one-third that of deglycosylated EDGP, and deglycosylation of EDGP caused complete loss of its ability to inhibit xyloglucan-specific *endo*-β-1,4-glucanase. Experiments using tunicamycin-treated carrot cell cultures showed that glycosylation is essential for correct EDGP folding and secretion, and that tunicamycin does not affect EDGP gene transcription.

Keywords: EDGP; Protein inhibitor family; Gene duplication; Phosphorylation; N-linked glycosylation; Glycan function

Asparagine-linked carbohydrates affect a wide variety of protein-mediated functions and characteristics including cell–cell recognition, host–pathogen interactions, and tissue distribution [1]. N-linked oligosaccharide chains affect the thermodynamics of folding of nascent polypeptides, and glycans are important in interactions between incompletely folded glycoproteins and specific ER chaperones [2].

In carrots, extracellular dermal glycoprotein (EDGP) is expressed in response to biotic or abiotic stress [3,4]. It protects plant cell walls by inhibiting the activity of xyloglucan-specific *endo*-β-1,4-glucanase (XEG) from the fungus *Aspergillus aculeatus* [5], suggesting that EDGP plays an important role in the plant's defensive strategy. EDGP binds insulin and a 4-kDa hormone-like peptide from soybean (4-kDa peptide) in vitro [6,7], suggesting that it may also be important in signal transduction.

Amino acid sequence comparison shows that EDGP homologues are widely present in plants and may belong to an inhibitor protein superfamily [8]. EDGP isoforms may have different isoelectric point (p*I*) values [9] owing to their slight differences in amino acid sequence or to different posttranslational modifications. The *Arabidopsis* genome contains multiple copies of a gene for an EDGP homologue. In the tomato genome, the EDGP homologue XEGIP (XEG inhibitor protein) is encoded by a single-copy gene, but five other genes of significant sequence similarity are also present [5]. The number of EDGP gene copies in the carrot genome is unknown.

All EDGP homologues have putative N-linked glycosylation sites, and most Cys residues are conserved. Posttranslational modifications of EDGP include N-terminal modification, disulfide bond formation (six pairs), and glycosylation (at four sites). We previously examined disulfide bond formation in EDGP and demonstrated that it significantly affects its ability to bind hormone-like peptides [7]. We also determined the molecular weight and composition of EDGP glycans,

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but the roles of EDGP glycans and glycosylation in EDGP function are still unclear.

In the present study, we examined the source of heterogeneity in EDGP isoforms and found that the carrot genome contains at least two EDGP genes encoding slightly different amino acid sequences. To study the role of glycosylation, we removed the glycans with trifluoromethanesulfonic acid (TFMS) or used tunicamycin to inhibit N-linked glycosylation in vivo. Our results demonstrate that glycosylation is essential for the folding, secretion, and physiological functions of EDGP.

Materials and methods

Two-dimensional electrophoresis and phosphoprotein detection. Extracellular dermal glycoprotein (EDGP) was purified from 3-week suspension cultures of carrot cells as previously reported [7]. EDGP (15 μg) was mixed with 15 μg casein (Sigma–Aldrich, USA) and subjected to two-dimensional electrophoresis (2-DE) with a 13-cm IPG strip (Amersham Biosciences, USA). After electrophoresis, the gel was stained using a Pro-Q Diamond Phosphoprotein Gel Stain kit (Molecular Probes, USA) to detect phosphoryl groups, and proteins on the gel were visualized with RUBY Gel Stain (Bio-Rad, USA). Electrophoresis and staining procedures were performed according to the manufacturers' instructions. Furthermore, EDGP isoforms on the 2-D gel were separately digested with chymotrypsin and subjected to analysis by LC-ESI-Q-Tof mass spectrometry according to the method of Shang et al. [7].

cDNA cloning. Genomic DNA was extracted from suspension cultures of carrot cells using the cetyltrimethylammonium bromide method [10]. Total RNA was prepared with TRIzol Reagent (Invitrogen, USA) and reverse-transcribed to cDNA with an RT-PCR kit (Takara, Japan). The cDNA was used as a template for PCR with EDGP primers F (5′-AACCATGGCTCAGCCATCTTTCCGACCA TC-3′) and R (5′-AAAAGCTTTCATGAGGTAAAATTAAAGT TCG-3′). The resultant EDGP cDNA was labeled with digoxigenin (Dig Labeling and Detection kit; Roche, USA) and analyzed by Southern blotting as described previously [11].

Enzyme inhbition assays. The inhibition of XEG by EDGP was assayed with the PAHBAH method [12]. Briefly, a solution containing XEG and EDGP at a molar ratio of 1:2 was incubated for 5 min at room temperature, and then 50 μl of 2 mg/ml xyloglucan was added. After a 1-h incubation at room temperature, 1% of 4-hydroxybenzoic hydrazide (Sigma–Aldrich, USA) solution was added, and the solution was heated in boiling water for 5 min. The reducing ends of hydrolyzed xyloglucan were quantitated by measuring the absorbance at 410 nm.

Deglycosylation of EDGP. The glycans of EDGP cannot be removed with glycosidase, such as PNGase F and endoglycosidase F1 [7]. Therefore, chemical deglycosylation was performed according to the method of Edge et al. [13] and Edge [14]. Briefly, 200 μg of EDGP was dialyzed against water and then lyophilized. A 9:1 (v/v) mixture of TFMS and anisole was added to the sample, which was incubated at −80 °C for 5 min and then at −20 °C for 4 h. Excess TFMS was neutralized with 60% pyridine solution followed by the addition of 100 mM NH_4HCO_3, pH 8.0. The resultant precipitate was collected at 15,000*g* for 10 min at 4 °C, dissolved in 50 mM sodium acetate buffer, pH 5.0, containing 0.3% sodium *N*-lauroyl sarcosinate, and subjected to SDS–PAGE. Western blotting with an anti-EDGP antibody and surface plasmon resonance (SPR) analysis were performed as previously described [7].

Tunicamycin treatment of carrot cells. Three-week cultures of carrot cells were centrifuged at 100*g* for 15 min at 4 °C, and the cell pellet was resuspended in fresh MS medium [15]. The pH was adjusted to 7.0, tunicamycin (ICN Biomedicals, USA) was added to a final concentration of 1 μg/ml, and the cells were allowed to continue growing. After 3 h, a 1-ml aliquot of the suspension culture was removed and centrifuged to collect the cells. The cells were lysed by addition of SDS–PAGE loading buffer [60 mM Tris–HCl, pH 6.8, 5% (v/v) of 2-mercaptoethanol, 10% (v/v) glycerol, and 2.5% (w/v) SDS] and heated in boiling water for 10 min. The sample was then subjected to SDS–PAGE and Western blotting with anti-EDGP antibody.

After the remaining cells had been cultured for 4 days with tunicamycin, a 150-ml aliquot of the suspension culture was removed and centrifuged at 1000*g* for 20 min, and both the media containing secreted proteins and cell pellets were saved for further processing. The media were filtered through a 0.45-μm filter (Millipore, USA), brought to 90% saturation with solid $(NH_4)_2SO_4$, and incubated for 4 h at 4 °C. The solution was centrifuged at 48,000*g* for 20 min, and the resultant pellet was dissolved directly in SDS–PAGE loading buffer for SDS–PAGE and Western blotting analyses.

The cell pellet from the 4-day tunicamycin-treated culture was homogenized and blended with 100 mM Tris–HCl buffer, pH 8.0, containing 200 mM NaCl. The suspension was centrifuged at 15,000*g* for 15 min, and the resultant supernatant (containing soluble proteins) and pellet (containing insoluble proteins) were mixed separately with SDS–PAGE loading buffer for SDS–PAGE and Western blotting.

After the remaining cells had been cultured for 10 days with tunicamycin, a 5-ml aliquot of the suspension culture was removed and the carrot cells were collected by centrifuge at 100*g* for 15 min. The cell pellet was homogenized and processed as described above. Total RNA was also prepared from the 10-day tunicamycin-treated cells and was subjected to electrophoresis on 1.2% agarose, 1.8% formaldehyde gels. Northern blotting analysis was performed with digoxigenin-labeled EDGP cDNA. A similar procedure was used for Southern blotting analysis, except that 50% formaldehyde in hybridization buffer and a hybridization temperature of 50 °C were used.

Results

EDGP isoforms

Although 2-DE of EDGP resulted in at least four distinct spots, no phosphorylation was detected (Fig. 1A), indicating that the different p*I* values of EDGP isoforms might be owing to different amino acid sequences. This possibility is consistent with the results of Southern blotting analysis (Fig. 1B), which confirmed that the EDGP gene is present in more than one copy in the carrot genome. The amino acid sequences of the EDGP isoforms encoded by these genes were compared by MS/MS analysis of EDGP chymotryptic fragments, which identified two ions. As shown in Fig. 1C, the first ion (*m*/*z* 2026.04) corresponds to R^{389}-TSIVIGG HQLEDNLVQF^{406}, which is identical to the sequence reported previously [7]. The second ion (*m*/*z* 2069.98), however, corresponds to the sequence RGSSIVIGGH QLEDNLVQF, which differs from the first sequence at the underlined residues (Fig. 1D). This difference in sequence confirms that EDGP isoforms are encoded by different EDGP genes. Despite the differences in their amino acid sequences, all of these isoforms inhibit the activity of XEG [5].

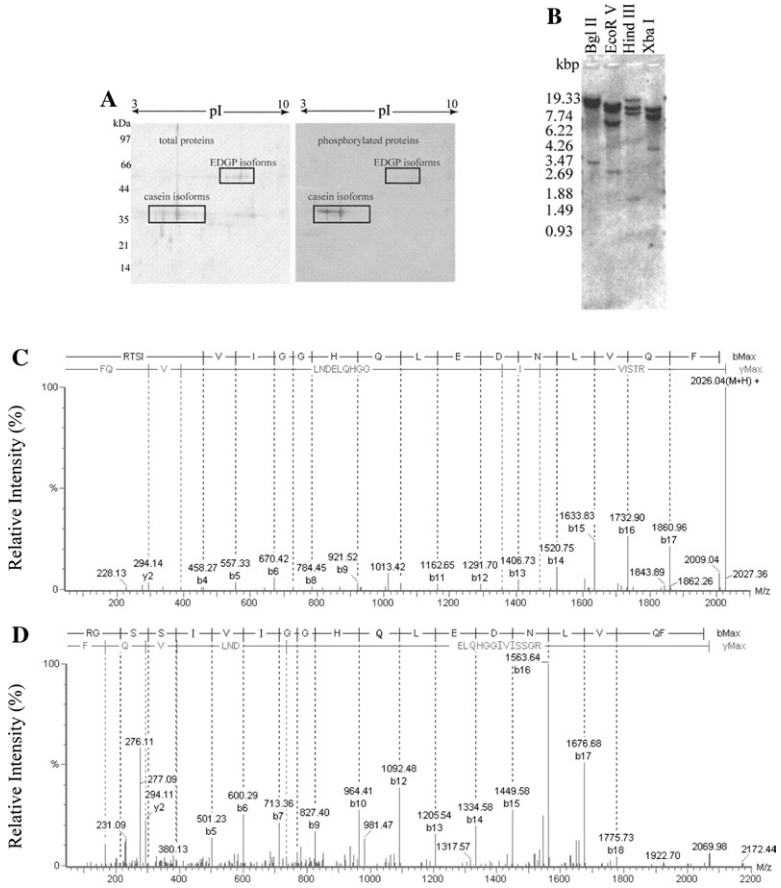

Fig. 1. Analysis of EDGP isoforms with different p*I* values. (A) Two-dimensional maps of EDGP and casein. Total proteins (left gel) and phosphorylated proteins (right gel) were visualized as described in Materials and methods. The p*I* range of 3–10 is indicated on the horizontal axis. (B) Genomic DNA (10 μg/lane) was digested with the indicated restriction enzymes, and the DNA gel blot was hybridized with the full-length EDGP cDNA probes. (C) MS/MS analysis showed that an ion with *m/z* 2026.04 corresponded to RTSIVIGGHQLEDNLVQF and (D) an ion with *m/z* 2069.96 corresponded to RGSSIVIGGHQLEDNLVQF, where underlined letters mean different amino acid residues between two sequences.

The roles of glycans of EDGP

TFMS-treated EDGP exhibited higher mobility than native EDGP in SDS–PAGE, confirming that the glycans were removed (Fig. 2A). The 4-kDa peptide binding affinities of native, reduced, and deglycosylated EDGP preparations were compared by SPR analysis. As shown in Fig. 2B, the affinity of native EDGP for the 4-kDa peptide was about 20-fold higher than that of reduced EDGP and about one-third that of deglycosylated EDGP. The latter result suggests that the binding site(s) was made accessible by deglycosylation.

Native, reduced, and deglycosylated EDGP were also assayed for their inhibitory activity against XEG (Fig. 2C). Native EDGP inhibited XEG, as expected. But neither reduced EDGP nor deglycosylated EDGP exhibited any inhibitory activity, suggesting that both the tertiary structure and the glycans of EDGP are essential for its inhibitory function.

Role of glycosylation in EDGP folding

Tunicamycin, a potent antibiotic that blocks the formation of the dolichol-bound glycan precursor [16], was used to study the role of EDGP glycosylation. Carrot cells that had been tunicamycin-treated for 3 h contained both glycosylated and unglycosylated EDGP, whereas untreated carrot cells contained only glycosylated EDGP (Fig. 3A). The glycosylated EDGP found in tunicamycin-treated carrot cells may have been synthesized before tunicamycin could completely inhibit N-linked glycosylation.

The secreted proteins found in the media, and the soluble and insoluble cellular proteins of a culture that had been tunicamycin-treated for 4 days were analyzed by Western blotting with an anti-EDGP antibody (Fig. 3B). The media from both the treated and untreated cultures contained secreted, glycosylated EDGP, but not unglycosylated EDGP, suggesting that glycosylation is

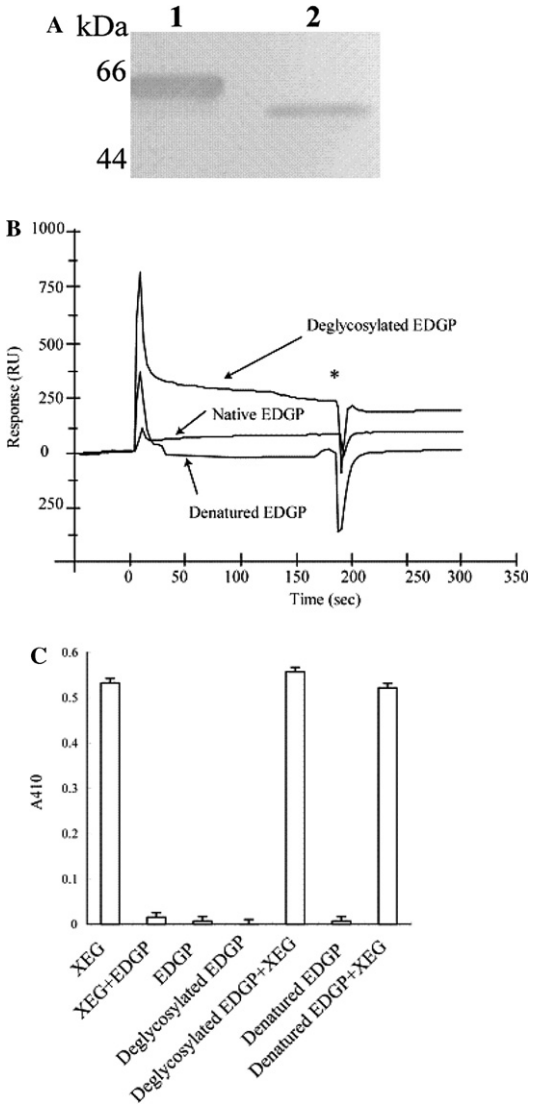

Fig. 2. The roles of glycans of EDGP. (A) SDS–PAGE followed by Western blotting analysis confirmed that the glycans of EDGP have been removed by TFMS treatment. Lane 1, native EDGP; lane 2, TFMS-treated EDGP. (B) SPR analysis showed that deglycosylation increased the binding activity of EDGP to the 4-kDa peptide. Phases before the asterisk (*) represent the association sensorgrams while those after asterisk are the dissociation sensorgrams. Denatured EDGP means that native EDGP was reduced with DTT and modified with iodoacetic acid. (C) Inhibition assays showed that deglycosylated EDGP lost its ability to inhibit XEG. Samples were analyzed as indicated on the *x*-axis. Each test was repeated three times and the average values were used.

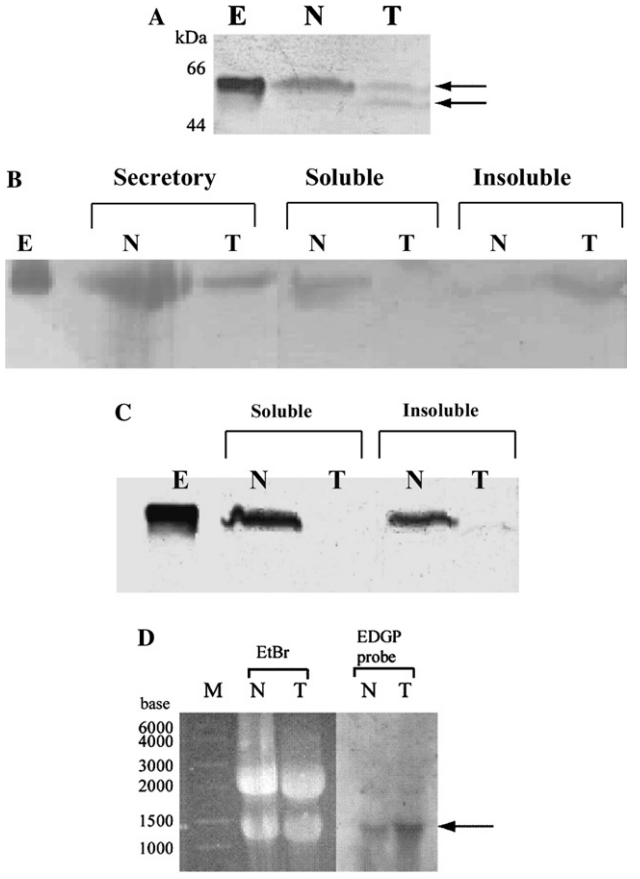

Fig. 3. Roles of glycosylation in EDGP folding and secretion. 'E' means purified EDGP, 'N' represents proteins from normal carrot cell, and 'T' indicates proteins from tunicamycin-treated carrot cell. (A) Glycosylated and unglycosylated EDGP were detected in 3-h tunicamycin-treated carrot cells while only glycosylated one was detected in normal carrot cells. Arrow indicates the glycosylated or unglycosylated EDGP. (B) After 4-day culture with tunicamycin, the distribution of EDGP in carrot cell was analyzed with Western blotting. 'Secretory' means proteins from 4-day-cultured carrot suspension medium, 'soluble' represents soluble cell lysates, and 'insoluble' indicates those proteins resolved only in SDS–PAGE loading buffer. (C) After 10-day culture with tunicamycin, EDGP from tunicamycin-treated cells was checked with Western blotting analysis. (D) Total RNA (10 μg/lane) from 10-day-cultured normal or tunicamycin-treated carrot cell was separated with formaldehyde-containing agarose gel. 'EtBr' represents that the gel was stained with ethidium bromide after electrophoresis; 'EDGP probe' means that total RNA was hybridized with digoxigenin-labeled EDGP cDNA after transferring to nylon membrane. Arrow indicates the EDGP mRNA.

essential for EDGP secretion. In addition, glycosylated EDGP extracted from untreated carrot cells was detected predominantly in the soluble fraction. The small amount of glycosylated EDGP found in the insoluble fraction may have been a result of incomplete homogenization of the cells or of incomplete removal of the soluble fraction from the insoluble pellet. In contrast, the soluble fraction of the cell extract from the tunicamycin-treated cultures contained no EDGP; instead, EDGP was detected only in the insoluble fraction. The aggregation of unglycosylated EDGP extracted from tunicamycin-treated carrot cells indicates that glycosylation in the ER is necessary for correct folding of the EDGP polypeptide.

Cultures that had been treated with tunicamycin for 10 days contained little EDGP aggregate (Fig. 3C). In general, incorrectly folded proteins in the cell are degraded with the unfolded protein response (UPR) and ER-associated degradation (ERAD) system [17,18].

When the production of misfolded proteins exceeds the capacity of the cell to degrade them, these polypeptides often form intracellular aggregates before they can be rapidly degraded [19]. This scheme is consistent with the proposition that lack of glycosylation causes misfolding of the EDGP polypeptide, which is followed by aggregation of the misfolded polypeptides and degradation with UPD and ERAD.

To examine the possibility that the trace amount of EDGP found in tunicamycin-treated carrot cells was a result of inhibited transcription of EDGP genes or of degradation of EDGP mRNA, Northern blotting was performed. The resultant data showed (Fig. 3D) that tunicamycin treatment did not affect EDGP gene transcription or lead to complete degradation of EDGP mRNA. Overall, the data, therefore, demonstrate that glycosylation is essential for correct folding of EDGP.

Discussion

We previously demonstrated that an EDGP isoform with a p*I* of 9.5 is not phosphorylated and that no EDGP isoforms carry charged monosaccharides [7]. In the present study, we have shown that the different pI values of multiple EDGP isoforms from carrot suspension culture medium are not caused by phosphorylation (Fig. 1A). However, it is still unclear whether EDGP located in plasma membrane has phosphoryl group(s), which might be important for understanding the role of EDGP in signal transduction system. An evidence from MS/MS analysis showed that EDGP isoforms are encoded by genes of different sequences (Fig. 1). EDGP homologues from soybean, tomato, and *Arabidopsis* are also encoded by multi-copy genes, an observation that is consistent with the putative importance of EDGP in defense against pathogens and signal transduction.

Native EDGP can be dissolved and denatured in 8 M urea at a high concentration. Removal of glycans from EDGP with TFMS results in aggregation, and these aggregates are difficult to dissolve in urea but do dissolve easily in ionic surfactants (data not shown). These observations suggest that, in native EDGP, the glycans protect hydrophobic surface patches from aggregation due to hydrophobic interactions. Hydrophobicity profiles of EDGP performed with the program Masslynx 3.5 (Micromass, UK) showed that EDGP glycans are attached to the protein only in hydrophobic regions of secondary structure, with the exception of the glycan linked at asparagine 274. After oxidation to promote the formation of disulfide bonds, recombinant EDGP expressed in *Escherichia coli* is difficult to dissolve in buffer without ionic surfactant (data not shown), indicating that glycans may be essential for its solubility.

Hanada and Hirano [20] identified three regions of a 43-kDa receptor-like protein from soybean that are involved in binding the 4-kDa peptide. In an amino acid sequence alignment of EDGP and the 43-kDa protein, two of these 4-kDa peptide-binding regions are near EDGP glycosylation sites (Asn 110 and Asn 274). In the 43-kDa protein, however, these regions are not glycosylated. These data may explain that the binding affinity of the 43-kDa protein for the 4-kDa peptide is higher than that of EDGP, and deglycosylation of EDGP increases its binding affinity about threefold. Zhong et al. [21] reported that glycosylation of the receptor-like protein does not affect its ligand binding affinity or its translocation to the membrane, however, it rather affects signal transduction. A 4-kDa peptide-like hormone has not been identified in carrot [22], and therefore the effect of glycans on EDGP binding in its physiological context is not completely understood.

Deglycosylation of EDGP leads to loss of XEG inhibitory activity, possibly owing to a resulting defect in protein stability or a resulting disruption in the interaction with XEG. Supporting the former possibility, some studies have shown that glycosylation can provide rigidity to residues proximal to the glycosylation site, which may enhance overall protein stability [23,24]. On the other hand, the composition of EDGP glycans is similar to that of xyloglucan [7,9], which may support the latter possibility.

A homologue of EDGP from lupine seeds, conglutin γ, is prevented from correct secretion by treatment with tunicamycin [25]. Similarly, we found that tunicamycin treatment also prevents correct secretion of EDGP (Fig. 3B). Hence, glycosylation appears to be vital for EDGP-like proteins. Glycosylation can facilitate the formation of correct secondary structure [23] and disulfide bonds [26,27]. Since glycosylation is not possible in *E. coli*, lack of glycosylation may partially explain the difficulty of expressing recombinant EDGP in *E. coli*.

In conclusion, we have shown that glycosylation is essential for correct folding and secretion of EDGP. This information will be important in determining the physiological functions of EDGP-like proteins. Some questions concerning EDGP glycosylation remain, however. Glycosylation sites may play different roles in protein folding and activity [23,28]. Which glycosylation site(s) plays the essential role in EDGP? Is glycan post-processing in the Golgi apparatus necessary? Further experiments are needed to address these questions.

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