Glycodelin, also known as placental protein-14, is a multifunctional glycosylated protein secreted by the uterine endometrium during the early phases of pregnancy. It is a known suppressor of T cell proliferation, inducer of T cell apoptosis, and inhibitor of sperm zona binding. Unlike in contraceptive activity, where the glycans on the molecule have been shown to play a crucial role, mutagenesis of the asparagines at sites of N-linked glycosylation (Asn28 and Asn63) to glutamine shows that the apoptogenic activity of glycodelin A is executed by the protein backbone. Glycosylation at Asn28 appears to play a role in the extracellular secretion of the molecule, as mutation of Asn28 resulted in a significant decrease in the amount of secreted protein, and loss of both glycosylation sites reduced the secretion drastically. Our results also suggest that the loss of glycosylation does not affect the dimerization status of the molecule.

Analysis of the Role of Oligosaccharides in the Apoptotic Activity of Glycodelin A*

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Glycodelin A (GdA), also known as PP14 (placental protein-14), is a glycosylated dimeric 162-amino-acid protein secreted by human endometrium in the late secretory phase of the menstrual cycle and in the early phase of pregnancy under the regulation of the hormone progesterone (1). Glycodelin is glycosylated at two of the three putative glycosylation sites (Asn28 and Asn63) (2). Observations of several research groups suggest that GdA is immunosuppressive in function (3), and previous studies from our laboratory indicate that this effect is due to its capacity to induce apoptosis in T cells (4). Interestingly, glycodelin S (GdS), another isoform of glycodelin synthesized by the seminal vesicles and accumulating in the seminal plasma, is not apoptogenic (26) even though both isoforms share the same amino acid sequence. As the glycans on both isoforms are different and as glycosylation has been demonstrated to play an important role in the contraceptive activity of GdA (13), it appeared logical to investigate the relevance of glycosylation to the apoptogenic function. However, attempts to deglycosylate GdA and GdS under native conditions using enzymes were not successful. Therefore, we resorted to molecular biology approaches to mutate the glycosylation sites and to express the protein in a eukaryotic system. The functional analysis of protein thus expressed proved that the apoptogenic activity of the molecule resides in its protein backbone, with the oligosaccharides being either permissive or non-permissive in the manifestation of this observed property. Furthermore, we have demonstrated that glycosylation at Asn28 plays a role in the secretory rate of the protein, as the mutagenesis of the same drastically reduces protein secretion. We also have evidence to suggest that lack of glycosylation does not compromise the dimerization state of the molecule.

EXPERIMENTAL PROCEDURES

Cells and Cell Lines—Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood collected from healthy volunteers (male and female, 25–49 years old) using HISTOPAQUE-1077 (Sigma) according to the protocol described by Boyum (5). SF21 cells (Spodoptera frugiperda; Invitrogen) were maintained in TC-100 medium supplemented with 10% FBS at 37 °C in a 5% CO2 incubator and passaged twice/week.

Cloning the Glycodelin cDNA into the Baculoviral System—The Bac-to-Bac baculovirus expression system (Invitrogen) was used to clone the glycodelin cDNA. In brief, the full-length glycodelin cDNA with the secretory signal, obtained from the uterine endometrium (26), was cloned between BamHI and PstI into the shuttle vector pFASTBAC1 within the mini-Tn7 element. This vector was transformed into Escherichia coli DH10BAC cells, which harbor the bacmid (baculoviral genome)-carrying kanamycin resistance marker and an attachment site for the bacterial transposon Tn7 (mini-attTn7). The recombinant glycodelin bacmids thus obtained were screened by blue/white selection. Positive white clones were picked up and confirmed by PCR using a gene-specific forward primer and a bacmid-specific reverse primer (see Table I). The recombinant bacmid was isolated and transfected into the insect cell line SF21 using LipofectAMINE (Invitrogen) following the protocol provided with the Bac-to-Bac baculovirus expression system. The recombinant virus-containing medium was harvested at the end of 72 h. The virus was amplified further; its multiplicity of infection was determined, and it was stored at 4 or −70 °C.

Site-directed Mutagenesis of Asn28 and Asn63 (N28Q, N63Q, and N28Q,N63Q) in Glycodelin cDNA—The QuikChange method (Stratagene) followed by DpnI digestion was employed to make the glycosylation mutants. Briefly, sense-antisense primers (see Table I) carrying the site-specific mutation with an incorporated silent mutation for a unique restriction site were used. The primers for N28Q carried a BglII site, whereas the primers for N63Q abolished the pre-existing PvuII site in the DNA sequence. The pFASTBAC1-WT Gd vector was used as the template for the creation of single mutants. The pFASTBAC1-N28Q,N63Q vector was created by releasing the glycodelin cDNA from both single mutants (N28Q and N63Q) using BamHI and PstI. The released fragments were purified and subjected to digestion by AvaI, which digests the glycodelin cDNA between the mutated asparagines (N28Q and N63Q). To obtain the double mutant, the smaller 213-bp fragment carrying the N28Q mutation from the N28Q single mutant and the large 351-bp fragment carrying the N63Q mutation from the N63Q single mutant were swapped and religated to the BamHI/PstI double-digested pFASTBAC1 vector using T4 DNA ligase (New England Biolabs Inc.). Clones were screened by the unique restriction sites.
incorporated into them as well as by sequencing. The recombinant pFASTBAC1 clones were transformed in E. coli DH10BAC cells. The recombinant bacmids were purified, confirmed by PCR, and transfected into Sf21 cells using LipofectAMINE as described above. All restriction enzymes used were purchased from New England Biolabs Inc.

Expression and Purification of the Baculovirus-expressed WT and Mutant Proteins of Glycodelin (Gd-Bac)—Because of the presence of the mammalian secretory signal, Gd-Bac was secreted into the culture medium. It was purified by passing the culture supernatant through a glycodelin-specific monoclonal antibody (mAb) D9D4 immunoaffinity column. The column was washed with phosphate-buffered saline (PBS; 50 mM phosphate buffer [pH 7.2]) containing 150 mM NaCl, and the bound protein was eluted with 100 mM glycine HCl (pH 2.5). The fractions collected were immediately neutralized with 1 M Tris (pH 8.0), and protein-containing fractions were pooled and concentrated using a Centricon concentrator (Vivascience). The concentrate was dialyzed down, and the culture supernatants were assayed for the concentration of 10% FBS containing RPMI 1640 medium supplemented with 10% FBS and incubated for an additional 2 h. The cells were harvested and lysed on Whatman glass-fiber GF/C filters using a Nunc cell harvester. The filters were dried, and the [3H]thymidine incorporated was measured in a Wallac scintillation counter. All treatments were carried out in triplicates. In a 96-well culture plate, PBMCs seeded at a density of 0.2 ¥ 10^6 cells/200 µl of culture medium were activated with either phytohemagglutinin at 5 µg/ml or anti-CD3 mAb (OKT3) hybridoma supernatant at 1:1600 dilution. The cells were cultured with varying concentrations of Gd-Bac for 36 h, after which [3H]thymidine (5 ¥ 10^4 cpm) was added and incubated for an additional 12 h. The cell-incorporated radioactivity was measured as described above. All treatments were carried out in triplicates with the appropriate controls.

Acridine Orange/Ethidium Bromide Staining—Jurkat cells (1 ¥ 10^6) were cultured with 1 µg of either GdA or WT Gd-Bac for 16 h in 500 µl of RPMI 1640 medium and 5% CO2 at 37°C. The cells were then harvested, centrifuged at 300 ¥ g for 5 min, and resuspended in 25 µl of medium along with 1 µl of a dye mixture of acridine orange and ethidium bromide (100 µg/ml each in PBS). The cells were observed immediately under a Carl Zeiss fluorescence microscope using a blue filter. GdA served as the positive control, and untreated cells served as the negative control.

Fluorescence-activated Cell Scan Analysis of Gd-Bac-treated Jurkat Cells—Jurkat JR4 cells were plated at a density of 1 ¥ 10^6 in 500 µl of RPMI 1640 medium supplemented with 10% FBS and incubated for 12 h with 25 nM Gd-Bac and the appropriate controls. The cells were harvested, washed with PBS, and fixed in 70% alcohol for 1 h. After a wash with radioimmunossay buffer, the cells were resuspended in 250 µl of ethidium bromide staining solution (50 µg/ml EBr, 100 µg/ml EDTA, and 50 µg/ml RNase in PBS) for 1 h at 45°C and analyzed using a BD Biosciences FACScan with blue light.

RESULTS

Isolation of Recombinant Baculoviruses Carrying the WT and Site-directed Mutants of the Glycodelin Gene—The presence of WT glycodelin cDNA in the bacmids was confirmed by PCR using a glycodelin gene-specific forward primer and a bacmid-specific reverse primer (Table I), and the clone was confirmed by the specific PCR product of ~1000 bp (Figs. 1 and 2F). Restriction digestion of the pFASTBAC1-N28Q clone with BglII revealed the presence of a 1163-bp insert in addition to the pre-existing 470-bp insert (Fig. 2A), confirming the presence of the primer-incorporated N28Q mutation. Digestion of the pFASTBAC1-N63Q clone with PvuII and HindIII failed to release an insert of 300 bp due to the loss of a pre-existing PvuII site (Fig. 2B), confirming the incorporation of the mutation. Fig. 2C shows the products of Aval digestion of N28Q and N63Q glycodelin cDNAs, which were later used in the construction of the double mutant. The double mutant clone was confirmed using the restriction enzymes as mentioned for confirming both single mutants (Fig. 2, D and E). In addition, the pFASTBAC1 clones were further confirmed by sequencing the

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2 M. Mukhopadhyay, P. Gangatikar, and A. A. Karande, unpublished data.
glycodelin cDNA. The recombinant bacmids of the mutants were confirmed as described for the WT bacmid by PCR using the same primers (Fig. 2F). The WT and mutant recombinant viruses were rescued from the corresponding bacmid DNA by transfection into Sf21 cells. The generated viruses were amplified and titrated.

Molecular Characterization of Gd-Bac—The molecular masses of all the recombinant proteins expressed in insect cells were assessed by SDS-PAGE and identified by Western blotting using anti-glycodelin mAb B1C2. The apparent molecular mass of native GdA is 28 kDa. It is well known that, in insect cell lines, differential glycosylation occurs, which results in expressed recombinant protein with multiple molecular species (9). WT Gd-Bac separated as four distinct molecular species, with maximum expression of the protein species corresponding to 26 kDa and the fastest moving species corresponding to 18 kDa (Fig. 3, A and B). The molecular mass of the predominant species in both recombinant N28Q Gd-Bac and N63Q Gd-Bac was 24 kDa. The double mutant protein separated as a doublet, with the upper band corresponding to 21 kDa and the lower band corresponding to 18 kDa (Fig. 3, A and B). The gel filtration chromatography analysis of WT Gd-Bac showed that it exists only as a dimer of ~45 kDa (Fig. 1D). Peptide N-glycosidase F digestion of WT Gd-Bac showed the convergence of the multiple bands into a doublet (Fig. 1C). The upper band could be that of the peptide N-glycosidase F-resistant form due to the presence of proximal α1,3-fucose linkages (10). The lower band in the double mutant could be due to degradation of the molecule.

Gd-Bac Protein Secretion Is Dependent on Glycosylation—
Mutation of the Asn28 glycosylation site resulted in almost 50% reduction of protein secretion by the cells compared with the WT protein (Table II). Loss of both Asn28 and Asn63 resulted in almost 80% reduction of protein secretion, as shown in the Table II. These results suggest that glycosylation at Asn28 is important for the proper folding and transport of the molecule. As there was a further decrease in protein secretion in the case of the double mutant, it can be concluded that both glycosylations are necessary for efficient secretion of the molecule by the insect cells. Interestingly, mutation of Asn63 to glutamine did not affect the secretion of the protein (Table II), which underlines the importance of Asn28 over Asn63 in proper folding and secretion of the protein.

WT Gd-Bac and the Glycosylation Mutants Are Apoptotically Active—To investigate the immunosuppressive activity of the recombinant protein, PBMC proliferation assay was carried out. As shown in Fig. 4A (panels a and b), WT Gd-Bac inhibited phytohemagglutinin- as well as anti-CD3 mAb-induced proliferation of human peripheral T cells in a dose-dependent manner, comparable with that seen with GdA. The 50% inhibition (IC50) of proliferation was brought about by 120 nM WT Gd-Bac. Gd-Bac inhibited the proliferation of Jurkat JR4 cells as well (Fig. 4A, panel c). Inhibition of proliferation was found to be comparable with that exhibited by GdA, with the IC50 for WT Gd-Bac being 40–50 nM and that for GdA being 30–40 nM. This shows that the recombinant glycodelin expressed in the baculoviral system is biologically active.

Having confirmed that the recombinant glycodelin expressed in baculoviral system is active, the glycosylation mutants N28Q Gd-Bac, N63Q Gd-Bac, and N28Q,N63Q Gd-Bac were assayed for immunosuppressive activity. Mutants N28Q, N63Q, and N28Q,N63Q were also found to be inhibitory for Jurkat JR4 cell proliferation, as shown in Fig. 5. The molar protein concentrations that exhibited 50% inhibition (IC50) were ~30–45 nM for N28Q, N63Q, and N28Q,N63Q.

Jurkat cells cultured with either WT Gd-Bac or the glycosylation mutant proteins were stained with propidium iodide and subjected to FACScan analysis. The apoptotic population was identified by the pre-G0/G1 peak. Treatment with the recombinant proteins increased the apoptotic population to ~28% compared with 6% as seen with the control (Fig. 6).

Morphological observation of apoptosis was carried out by dual staining of the cells with EtBr and acridine orange after culturing the cells with recombinant WT Gd-Bac for 16 h. As
shown in Fig. 4B, Jurkat cells underwent chromatin condensation with WT Gd-Bac, which is very characteristic of apoptosis seen with GdA as shown in the positive control panel.

**DISCUSSION**

GdA and GdS are products of the same gene and are secreted by the glandular epithelia of the endometrium (11) and the seminal vesicles (12), respectively. Although both are glycosylated at the same two asparagines (Asn28 and Asn 63), their glycan structures are distinctly different (13, 14). Several functions have been ascribed to GdA, one of which, the contraceptive activity (not shared by GdS), has been reported to be associated with its glycosyl groups (13). Another function of GdA well documented in the literature is immunosuppression (3) and induction of apoptosis in T cells (4). Recent studies from our laboratory demonstrated clearly that GdS lacks apoptotic

| Gd-Bac     | Concentration (ng/liter) |
|------------|--------------------------|
| WT         | 5–6                      |
| N28Q       | 2–3                      |
| N63Q       | 5–6                      |
| N28Q,N63Q  | <1                       |

GdA well documented in the literature is immunosuppression (3) and induction of apoptosis in T cells (4). Recent studies from our laboratory demonstrated clearly that GdS lacks apoptotic

**FIG. 2.** Confirmation of pFASTBAC1-N28Q, pFASTBAC1-N63Q, and pFASTBAC1-N28Q,N63Q clones by restriction digestion. A, pFASTBAC1-N28Q clones digested with BglII. The presence of the N28Q mutation confers an additional BglII site, as shown by the 1163-bp band in lane 2. B, N63Q clones digested with PvuII and HindIII. The presence of the N63Q mutation abolishes the pre-existing PvuII site, as shown by the loss of the 300-bp band in lane 2. C, Avai digests of N28Q and N63Q glycodelin cDNAs, which were released by BamHI and PstI digestion of the pFASTBAC1-N28Q and pFASTBAC1-N63Q vectors. Lane M, molecular size markers; lane 1, Avai digests from N28Q; lane 2, Avai digests from N63Q. D and E, confirmation of the double mutant by restriction digestion with BglII and PvuII, respectively. Lanes 1 and 2 are the clones selected; lane 1 has the positive clone. The presence of N28Q mutant confers an additional BglII site, as shown by the 1163-bp band; and the presence of the N63Q mutant abolishes the pre-existing PvuII site, as shown by the loss of the 300-bp band. F, PCR confirmation of the bacmid clones. PCR was performed with the bacmid-specific sense and antisense primers. The presence of the ~2500-bp band corresponds to the recombinant bacmid, and the 300-bp band indicates the non-recombinant bacmid. PCR was performed with the gene-specific sense and bacmid-specific antisense primers. The presence of the ~1000-bp band indicates the positive clone. Lane 1, N28Q; lane 2, N63Q; lane 3, double mutant; lane M, molecular size markers.

**FIG. 3.** A, Coomassie Blue-stained 12.5% SDS-polyacrylamide gel showing the purified recombinant Gd-Bac proteins. Lane 1, WT Gd-Bac; lane 2, N28Q; lane 3, N63Q; lane 4, N28Q,N63Q; lane M, molecular mass markers. B, Western blot of the recombinant Gd-Bac proteins with mAb B1C2. Lane 1, WT Gd-Bac; lane 2, N28Q; lane 3, N63Q; lane 4, N28Q,N63Q.

**TABLE II**

| Gd-Bac     | Amount of Gd-Bac secreted per liter of culture supernatant as determined by competitive enzyme-linked immunosorbent assay |
|------------|--------------------------------------------------------------------------------------------------------------------------|
| WT         | 5–6                                                                                                                      |
| N28Q       | 2–3                                                                                                                      |
| N63Q       | 5–6                                                                                                                      |
| N28Q,N63Q  | <1                                                                                                                      |
FIG. 4. A, WT Gd-Bac inhibits proliferation of activated T cells and of Jurkat cells. In a 96-well culture plate, PBMCs seeded at a density of $0.2 \times 10^6$ in $200 \mu l$ of culture medium were activated with either phytohemagglutinin (PHA; panel a) or anti-CD3 mAb hybridoma supernatant (αCD3; panel b). The cells were cultured with varying concentrations of Gd-Bac for 36 h, after which $[^3H]$thymidine ($5 \times 10^4$ cpm) was added and incubated for an additional 12 h. The cell-incorporated radioactivity was measured. All treatments were carried out in triplicates. In panel c, Jurkat cells seeded at a density of $0.1 \times 10^6$ in $200 \mu l$ of medium were incubated with varying concentrations of WT Gd-Bac for 24 h, followed by incubation with $[^3H]$thymidine for 12 h and harvesting on glass-fiber filters. B, acridine orange/ethidium bromide staining of Gd-Bac-treated Jurkat cells showing the apoptotic population in cells treated with Gd-Bac. PBS served as the negative control, and GdA served as the positive control.

FIG. 5. Glycosylation mutants inhibit proliferation of Jurkat cells. Jurkat cells seeded at a density $1 \times 10^5$ in $200 \mu l$ of medium were cultured with varying concentrations of the WT (A) and mutant Gd-Bac (B–D) proteins for 24 h, after which $[^3H]$thymidine was added and incubated for an additional 12 h. Cells were harvested, and the radioactivity incorporated was measured.
activity as well (26). It was therefore logical to determine whether the glycans on GdA also dictate its immunosuppressive characteristics. Enzymatic deglycosylation of GdA using peptide N-glycosidase F under native conditions failed (26); therefore, we resorted to recombinant DNA technology to address this question.

The glycodelin cDNA was cloned into the baculoviral system and expressed in SF21 insect cells, and the secreted protein was purified (Fig. 1B). The recombinant protein was found to harbor anti-proliferative activity in PBMCs (Fig. 4A, panels a and b) and Jurkat JR4 cells (panel c). It also exhibited apoptotic activity (Fig. 4B), as has been reported previously in the case of GdA (4). With an active WT protein in hand, we went ahead to determine the relevance of either or both of the N-linked glycosylations of glycodelin. Asn289 and Asn653 were mutated to glutamine to abolish glycosylation, and glutamine was chosen to minimize the alteration of the native conformation. The mutant proteins thus expressed and purified were tested for anti-proliferative activity. Interestingly, all the mutant proteins exhibited activity comparable with that of WT Gd-Bac and the native protein, showing clearly that the immunosuppressive activity of glycodelin resides in the protein backbone. It has been shown previously in the case of α1-microglobulin, another member of the lipocalin superfamily, that the unglycosylated form of the recombinant protein retains biological activity (15).

If the anti-proliferative activity of GdA is dictated by its primary structure, then the obvious question is why GdS is not active despite an identical amino acid sequence. A logical conclusion could be that the glycans present on GdA allow exposure of the apoptogenic region for binding to the target cells. High fucosylation and no sialylation (13) on GdS perhaps keep the molecule to execute its apoptogenic activity, whereas other types of glycosylation hinder the apoptogenic activity perhaps by covering the apoptogenic region. The fact that GdA is apoptotically active and GdS is apoptotically inactive needs to be discussed in this light. It could be that the presence of a large number of negatively charged sialic acid residues at Asn289 and Asn653 of the GdA molecule might result in charge repulsion between each other, thereby exposing the apoptogenic region, whereas the absence of the same in GdS can result in the oligosaccharide chains keeping the apoptogenic region inaccessible. The difference in the hydrodynamic volume between GdA and GdS due to the presence and absence of sialic acid residues has been demonstrated (26). But, in the case of SF21 cell-expressed glycodelin, the extent of glycosylation may be not large enough to form a protuberant structure to bring about masking of the apoptogenic region.

It is well known that glycans play a very important role in maintaining the stability, conformation, and biological activity of glycoproteins (17, 18). Oligosaccharides are essential recognition sequences in cell-mediated adhesions in inflammatory and immune responses (19). The role of oligosaccharides in modulating the half-life and biopotency of biomolecules is indeed well known (17, 18, 20). It has been shown in the case of thyroid-stimulating hormone that the deglycosylated form is 10 times more potent in vitro, but is 10 times less active in vivo due to rapid clearance (21). The same is seen in the case of interferon-γ, where the oligosaccharylated form has full antiviral and anti-proliferative activity (22–24), but its half-life tends to be short due to rapid clearance or to high susceptibility to proteases (24). It could be that the sialylated GdA has a longer half-life in circulation, as in the case of erythropoietin, where it has been shown that the removal of sialic acid groups leads to rapid clearance of the molecule from circulation (25).

From this study, it can be concluded that the apoptogenic activity of GdA resides in its protein backbone. However, it should be noted that the unglycosylated form of glycodelin is not physiological. Hence, the role played by the glycans on glycodelin needs to be unraveled further to obtain insights into functional constraints imposed on this molecule.

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*Glycosylation and Apoptotic Activity of Glycodelin*