Glycodelin A, Not Glycodelin S, Is Apoptotically Active

RELEVANCE OF SIALIC ACID MODIFICATION*

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Debadipta Mukhopadhyay†, Swathi SundarRaj, Anshula Alok‡, and Anjali A. Karande¶

From the Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

Glycodelin, previously known as PP14 (placental protein-14), is a keratin lipocalin secreted by the glandular epithelium of the endometrium upon progesterone stimulation and by the seminal vesicles. The isoform of the protein present in female reproductive tissue, glycodelin A (GdA), and the male counterpart, glycodelin S (GdS), have identical amino acid sequences, but strikingly different N-linked glycans. It is well documented in literature that GdA is an immunosuppressive protein, and we have shown that this activity is due to its ability to induce apoptosis in activated T cells. The precise role of GdS in seminal plasma is not known. In this study, we report that GdS is not apoptotically active. We observe that the apoptotic activity requires the presence of sialic acid residues on the complex glycans, as in the case of GdA; however, complex glycans of GdS are non-sialylated. We have expressed the wild-type protein in Pichia pastoris, which does not add sialic acid to the secreted proteins, and confirmed our observations that the protein is apoptotically inactive in the non-sialylated form. Our results indicate that differential glycosylation modulates the function of the different glycodelin isoforms.

Glycodelin, also known as PP14 (placental protein-14) (1), is a 162-amino acid glycoprotein classified under the lipocalin superfamily based on its 70% similarity to equine β-lactoglobulin (2). It is expressed in the reproductive system of primates by both the uterine endometrium and decidua (glycodelin A (GdA)³) (3) and by the glandular epithelium of the seminal vesicles (glycodelin S (GdS)) (4). The amino acid backbones of these two major isoforms being identical, the difference lies at the level of their glycosylation. Detailed characterization of the different types of complex glycans present on these two isoforms has been reported (5, 6), the major difference being sialylation and fucosylation. GdA is rich in sialylated complex glycans, whereas GdS is not sialylated, but has fucose- and mannoselike glycan structures.

GdA has been well documented to have multiple functions, viz., contraceptive (7), morphogenic (8), angiogenic (9), and, the most widely studied, immunosuppressive (10), by its ability to suppress the proliferation of activated T cells. Due to its role in immunoregulation, GdA has been subclassified as an immunocalin (11). Substantial evidence in literature indicates that GdA is an indispensable molecule in the maternal system for the establishment, maintenance, and progression of pregnancy (12). Also, its synthesis in the uterine endometrium and decidua is temporally regulated by progesterone (3). The steroid dependence, together with its immunosuppressive activity, suggests that GdA plays a role in down-modulating the T cell-mediated immune response against the fetal allograft in the maternal system. Recent data from our laboratory have unequivocally shown that immunosuppression by GdA is via induction of apoptosis in activated T cells (13), as demonstrated by several independent assays. Apoptosis of activated T cells at the materno-fetal interface may be a protective mechanism during pregnancy, as disturbances in programmed cell death of activated T cells in human decidua have been implicated in pregnancy loss (14).

In the case of immunocalins such as α₁-acid glycoprotein and α₁-microglobulin, which are members of acute-phase proteins, glycosylation plays a crucial role in their biological activity (11). The contraceptive activity of glycodelin is also dependent on the type of glycosylation present (15). The effect of differential glycosylation on the immunomodulatory activity of the protein has not been reported so far and is the subject of the study described in this work. As the primary amino acid sequences of the two forms (GdA and GdS) are identical, experiments were aimed at determining whether the latter also harbors apoptotic activity. GdS was found to be apoptotically inactive. Complete digestion of GdA with trypsin indicated that the isolated glycopeptides are not active and that the protein backbone is necessary for inducing apoptosis. Both glycodelins were deglycosylated using various glycosidases, and loss of GdA’s apoptotic activity was observed when the glycoprotein was desialylated. Thus, both the sialic acid residues on the N-linked glycans and the protein backbone are required for native GdA to show apoptotic activity.

EXPERIMENTAL PROCEDURES

Cells and Cell Lines

Peripheral blood mononuclear cells (PBMCs) were isolated using HISTOFAQUE (Sigma) from freshly drawn blood of normal healthy donors (male and female, 25–50 years old) according to the method described by Boyum (16). The human Jurkat (JR4) T cell line was obtained from the Cancer Research Institute (Mumbai, India), cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), and passaged three times/week.

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D9D4, a glycodelin-specific monoclonal antibody (mAb) raised in our laboratory, was purified from the hybridoma culture supernatant by protein A-Sepharose (Sigma) chromatography (17). The antibody was periodate-oxidized and cross-linked to Affi-Gel hydrazide beads (Bio-Rad) following the manufacturer's instructions to obtain an immunoaffinity column for purification of glycodelin. Amniotic fluid or seminal plasma was subjected to Western blot analysis using mAb D9D4 (18). The proteins were also subjected to immunoblot analysis with mAb B1C2 (lanes 3 and 4).

Cloning and Expression of Full-length Glycodelin in Pichia pastoris

Glycodelin cDNA without the signal sequence and stop codon was amplified by PCR. Full-length glycodelin cDNA cloned into pBluescript SK+ (Stratagene) was used as the template with the following primer pair: 5'-GGGGATCCGAATTCGACATGCCCAGACC-3' (forward) and 5'-GAATTCGGATCCGAGACGGACCATTTCC-3' (reverse). The forward primer has BamHI and EcoRI restriction sites; and the reverse primer has a SalI site, but no stop codon. For cloning the PCR product into an expression vector (Invitrogen), both the vector and PCR product were digested with SalI and EcoRI, purified, and ligated. 50 μg of pPiCfl-ZoA-Gd was digested with BglIII. The linear DNA was then purified.

For making recombinant P. pastoris (Strain GS115) expressing glycodelin, the manufacturer's instructions were followed. Briefly, 10 μg of linearized pPiCfl-ZoA-Gd in 10 μl of water was used to electroporate 100 μl of the competent GS115 cells. The electroporated cells were immediately taken up in 1 ml of ice-cold sorbitol and transferred into a sterile 15-ml tube. The cells were kept at 30 °C for 30 min and plated onto 1% yeast extract, 2% peptone, and 2% dextrose agar plates containing 1 M sorbitol and 200 μg/ml Zeocin (Invitrogen). The transformants were grown for 3–4 days at 30 °C.

The colonies were screened with increasing concentrations of Zeocin up to 1 mg/ml. The recombinant glycodelin-expressing GS115 cells were grown in 5 ml of medium containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 0.00004% biotin, and 0.5% methanol per g of cells was added to the cells. The cells were incubated at 30 °C for 16 h. This pre-inoculum was divided into two 2-liter flasks containing 250 ml of the same medium, and the cells were grown for 48 h. The cell mass was determined, and 2 ml of medium containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 0.00004% biotin, and 0.5% methanol per g of cells was added to the cells. The cells were incubated at 30 °C for 120 h with vigorous shaking (250–300 rpm), with methanol being replenished every 24 h. At the end of methanol induction, the cells were separated from the supernatant by careful aspiration after centrifugation at 10,000 × g for 20 min at 4 °C. The supernatant was then dialyzed extensively against PBS and used for purification of full-length glycodelin expressed in P. pastoris (Pic-fl-Gd) by Ni²⁺-NTA (QIAGEN Inc.) chromatography.

Cell Proliferation Assay

PBMCs (0.2 × 10⁶ cells/200 μl) or cell lines (0.1 × 10⁶ cells/200 μl) in RPMI 1640 medium supplemented with 10% fetal bovine serum

Fig. 2. Functional characterization of glycodelin. A, GdA inhibits T cell proliferation. Panel a, PBMCs were stimulated with mAb OKT3 for 36 h in the presence of varying concentrations of GdA and then pulsed with [3H]thymidine for 12 h. Panel b, Jurkat cells were cultured for 24 h in the presence of varying concentrations of GdA and then pulsed with [3H]thymidine for 12 h. Cells were harvested on glass-fiber filters and lysed, and the radioactivity was measured. B, GdS does not inhibit T cell proliferation. Panel a, PBMCs were stimulated with mAb OKT3 for 36 h in the presence of varying concentrations of GdS and then pulsed with [3H]thymidine for 12 h. Panel b, Jurkat cells were cultured for 24 h in the presence of varying concentrations of GdS and then pulsed with [3H]thymidine for 12 h. Cells were harvested on glass-fiber filters and lysed, and the radioactivity was measured. The data presented are representative of at least three different experiments, and each bar represents [3H]thymidine counts of the sample in triplicates. C, apoptotic effect of glycodelin as determined by EtBr staining and FACS analysis. Jurkat cells were cultured with 200 nM glycodelin for 18 h, following which the cells were ethanol-fixed, stained with ethidium bromide, and analyzed using a FACSScan. Panel a, untreated; panel b, GdS; panel c, GdA.
were cultured with the protein of interest in Nunc 96-well plates along with the appropriate controls. The anti-CD3 mAb (OKT3) hybridoma culture supernatant at a dilution of 1:2500 was used for inducing T-lymphocyte proliferation in PBMC cultures. \[\text{methyl-}^3\text{H}\]Thymidine (5 \times 10^5 \text{ counts/min; Board of Radiation and Isotope Technology, Mumbai}) was added after a 36-h incubation of the PBMC cultures and a 24-h incubation of the cell lines and incubated for an additional 12 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 \[^3\text{H}\]thymidine incorporated was measured in a Wallac scintillation counter. All treatments were carried out in triplicates.

**FIG. 3.** Trypsin-digested GdA does not induce apoptosis. \(A\), complete trypsin digestion of GdA. 2 \(\mu\)g of GdA was trypsin-treated for 4 h at 37 °C to completely digest the protein backbone, electrophoresed on 12.5% denaturing polyacrylamide gel under reducing conditions, and silver-stained. \(B\), trypsin-treated GdA is unable to induce apoptosis. Jurkat cells were cultured for 18 h in the presence of trypsin alone, trypsin-treated GdA, or GdA alone. The cells were ethanol-fixed, stained with ethidium bromide, and analyzed using a FACScan.

**FIG. 4.** PNGase F treatment of glycodelin. \(A\), denaturing conditions. 1 \(\mu\)g of denatured glycodelin was incubated with PNGase F at 37 °C for 48 h, electrophoresed on SDS-polyacrylamide gel, and immunoblotted with mAb B1C2. \(B\), nondenaturing conditions. GdS (300 ng) or GdA (1 \(\mu\)g) was incubated with PNGase F at 37 °C for 48 h, electrophoresed on SDS-polyacrylamide gel, and immunoblotted with mAb B1C2. 2 \(\mu\)g of denatured glycodelin was incubated with PNGase F under nondenaturing conditions at 37 °C for 24 h and electrophoresed on 12.5% gel, and immunoblot analysis was carried out using mAb B1C2. Lane 1, GdS without PNGase F; lane 2, GdS with PNGase F; lane 3, GdA without PNGase F; lane 4, GdA with PNGase F. 300 ng of GdA was trypsin-treated for 4 h at 37 °C to completely digest the protein backbone, electrophoresed on 12.5% denaturing polyacrylamide gel under reducing conditions, and silver-stained.

**FIG. 5.** GdS does not inhibit Jurkat cell proliferation after Endo-H treatment. \(A\), Endo-H treatment of glycodelin. 2 \(\mu\)g of glycodelin was treated with Endo-H under nondenaturing conditions at 37 °C for 24 h and electrophoresed on 12.5% gel, and immunoblot analysis was carried out using mAb B1C2. Lane 1, GdS without Endo-H; lane 2, GdS with Endo-H; lane 3, GdA without Endo-H; lane 4, GdA with Endo-H. \(B\), effect of Endo-H-treated glycodelin on Jurkat cell proliferation. Jurkat cells were cultured in the presence of untreated or Endo-H-treated glycodelin (1 \(\mu\)M) for 24 h, pulsed with \[^3\text{H}\]thymidine for 12 h, and harvested on glass-fiber filters; and the radioactivity incorporated was measured using a liquid scintillation counter.

Deglycosylation of Glycodelin

**Peptide N-Glycosidase F (PNGase F) Treatment under Denaturing Conditions**—Glycodelins (1 mg/ml) in PBS containing 1% SDS and 10 mM \(\beta\)-mercaptoethanol were heated at 100 °C for 2 min. Nonidet P-40 was then added so that the ratio of Nonidet P-40 to SDS was 7:1 in the final reaction. The enzyme (0.5 milliunits/ml) was added, and the mixture was incubated at 37 °C for 48 h. To prepare the samples for SDS-PAGE, they were precipitated with equal volumes of 50% trichloroacetic acid containing 10 mM sodium deoxycholate, boiled in SDS sample buffer, and electrophoresed on 12.5% polyacrylamide gel under denaturing conditions. The proteins were transferred onto a nitrocellulose membrane and probed with glycodelin-specific mAb B1C2 (18).

**PNGase F Treatment under Nondenaturing Conditions**—Glycodelins (1 mg/ml) in PBS were incubated with 0.5 milliunits/ml PNGase F at
37 °C for 48 h. The proteins were electrophoresed on 12.5% SDS-polyacrylamide gel and subsequently immunoblotted with mAb B1C2.

Endoglycosidase H (Endo-H) Treatment under Nondenaturing Conditions—Glycodelins (1 mg/ml) in 100 mM acetate (pH 5.5) were incubated with 20 milliunits/ml Endo-H at 37 °C for 24 h. The proteins were then electrophoresed on 12.5% SDS-polyacrylamide gel and subsequently immunoblotted with mAb B1C2. The reaction mixture was neutralized with 1 mM Tris-HCl (pH 8), sterilized, and used in Jurkat cell proliferation assays.

Neuraminidase Treatment under Nondenaturing Conditions—Glycodelins (1 mg/ml) in 100 mM acetate (pH 5.5) were incubated with 20 milliunits/ml neuraminidase at 37 °C for 24 h. The proteins were then resolved by two-dimensional gel electrophoresis and subsequently immunoblotted with mAb B1C2. The reaction mixture was neutralized with 1 mM Tris-HCl (pH 8), sterilized, and used in Jurkat cell proliferation assays.

Trypsin Digestion of Glycodelin

Complete trypsin digestion of GdA was carried out by incubating GdA with trypsin at a molar ratio of 20:1 in 20 mM Tris-HCl (pH 8.5) containing 2 mM MgCl₂. After 4 h of digestion at 37 °C, completion of the reaction was checked by 12.5% SDS-PAGE. At the end of the trypsin digestion, the reaction mixture was used in a Jurkat cell assay, and apoptosis was measured by ethidium bromide staining and fluorescence-activated cell scan (FACS) analysis.

Partial trypsin digestion of glycodelin was done by incubating glycodelin with trypsin at a molar ratio of 1000:1 in 20 mM Tris-HCl (pH 8.5) containing 10 mM MgCl₂ at 37 °C. The end of varying time intervals, an aliquot of the reaction mixture was boiled in SDS sample buffer and stored at −20 °C. At the end of the experiment, all the samples were electrophoresed on 12.5% SDS-polyacrylamide gel, and the proteins were visualized by silver staining. The apoptotic activity of GdA after partial trypsin digestion was determined in Jurkat cells.

Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was carried out following the method of Celis et al. (19). Briefly, −2 μg of protein was mixed with lysis buffer (9.8 M urea, 2% Nonidet P-40, 2% Ampholine pH 3.5–9.5 (Amer sham Biosciences), and 100 mM dithiothreitol), and isoelectric focusing was performed for 4 h at 400 V in 1.5-mm tube gels pre-focused for 30 min at 250 V using a mini-PROTEAN 2D-Cell (Bio-Rad). For the second dimensional separation, the tube gels were placed on 12.5% SDS-polyacrylamide gels, sealed with 1% agarose in SDS-PAGE running buffer with 0.01% bromphenol blue, and electrophoresed. At the end of the two-dimensional separation, the gels were either silver-stained or used for immunoblotting.

Far-UV Circular Dichroism

CD spectra were measured and recorded with a Jasco J-715 spectropolarimeter equipped with a data processor. Protein solutions (0.5 mg/ml) were prepared in PBS. Spectra were taken at 25 °C in the far-UV region (200–280 nm) using a 0.5-cm quartz cuvette.

Fluorescence Measurements and Stern-Volmer Plots

Fluorescence studies were performed using a PerkinElmer Life Sciences LS55 luminescence spectrometer. Fluorescence emission from
tryptophan was measured using excitation at 295 nm to minimize the loss of intensity due to energy transfer to tyrosine. Slits with a bandpass of 5/10 nm were used in the excitation/emission channels. The proteins were dissolved in PBS or in PBS containing different concentrations of urea. The sample temperature was kept at 27 °C. The quenching constant was determined by fitting the data to the Stern-Volmer relation: $F/F_0 = 1 + K_{SV}[Q]$, where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of the quencher, respectively; $[Q]$ is the molar concentration of the quencher, and $K_{SV}$ is the Stern-Volmer (or dynamic) quenching constant (20).

**Partition Coefficient ($K_{AV}$) Determination of Glycodelin**

50 μg of glycodelin was chromatographed on a Superdex 200 HR 10/30 gel filtration column using AKTA design systems (Amersham Biosciences). The flow rate was maintained at 0.5 ml/min, and the elution of the proteins was monitored by measuring the UV absorption at 280 nm. The partition coefficient of the proteins in the gel phase was calculated using the following formula: $K_{AV} = (V_e - V_0)/(V_t - V_0)$, where $K_{AV}$ is the partition coefficient of the protein, $V_e$ is the elution volume of the protein, $V_t$ is the bed volume of the column, and $V_0$ is the void volume of the column.

**Ethidium Bromide Staining and FACS Analysis**

Cells (1 × 10⁶) treated with glycodelin or with the appropriate controls were cultured for 12 h and harvested by centrifugation at 300 g for 5 min at room temperature. The cells were resuspended in 100 μl of PBS, treated with 1 ml of ice-cold 70% ethanol, and incubated on ice for 30 min. The cells were then centrifuged at 800 × g for 5 min at 4 °C. After a wash with PBS, 500 μl of staining solution (50 μg/ml ethidium bromide, 0.1 mg/ml RNase A, 1% Triton X-100, and 40 μg/ml sodium citrate in PBS) was added to the cells and incubated for 1 h. Analysis was carried out using a fluorescence-activated flow cytometer (BD Biosciences FACScan). Blue light was used for excitation, and emission was measured in the red region.

**RESULTS**

**GdA Is Not Apoptogenic on T Cells**—GdA and GdS were purified from amniotic fluid and seminal plasma, respectively, by immunoaffinity chromatography using the mAb D9D4 column. The purity of the two proteins was determined by silver staining, and their identity was confirmed by Western blotting using mAb B1C2 (Fig. 1) (18). Whereas GdA resolved as a single band at 28 kDa, GdS resolved as a doublet at 27 and 30 kDa. Both bands of the doublet reacted with glycodelin-specific mAbs.

The pure proteins were tested for their ability to inhibit Jurkat cell and anti-CD3 mAb (OKT3)-induced PBMC proliferation. Purified GdA was able to inhibit both Jurkat cell proliferation and mAb OKT3-induced proliferation of PBMCs in a dose-dependent manner (Fig. 2A), as reported previously (10, 13). In the case of GdA, the 50% inhibitory concentration was 600 nM for PBMCs (Fig. 2A, panel a) and 150 nM for Jurkat cells (panel b). Interestingly, GdS purified by the identical procedure did not show inhibition of mAb OKT3-induced proliferation (Fig. 2B, panel a) or Jurkat cell proliferation (panel b). Purified GdA inhibits Jurkat cell proliferation by inducing apoptosis in these cells (13), as confirmed by EtBr staining and FACS analysis (Fig. 2C, panel c). As expected, GdS was unable to induce apoptosis in Jurkat cells (Fig. 2C, panel b).

**Complex Glycan Structures of GdA Do Not Induce Apoptosis Independent of the Protein Backbone**—Previously, GdS and GdA were shown to be functionally distinct with respect to their contraceptive activity (15), and this difference has been attributed solely to the different glycan structures present on the two proteins (6). To observe the effect of the complex sugars on T cells in the absence of the protein backbone, GdA was completely digested with trypsin (Fig. 3A), and Jurkat cells were cultured with the digested product. As shown in Fig. 3B, although intact GdA induced apoptosis in Jurkat cells, the complex glycans present on GdA did not in the absence of the protein backbone.

**Enzymatic Deglycosylation of Glycodelin**—The primary structures of GdA and GdS are identical (21); hence, the difference in their apoptotic activity could be because of their differential post-translational modification, most likely glycosylation. To study the role of the glycans in the apoptotic activity of glycodelin, the protein was treated with PNGase F, which catalyzes the hydrolysis of the asparagine–N-acetylgalactosamine bond, thus completely deglycosylating the protein. Although the enzyme deglycosylated both glycodelins under denaturing conditions (Fig. 4A), it was not able to deglycosylate the proteins under native conditions (Fig. 4B), presumably because of inaccessibility of the glycosidic linkage site.

Endo-H removes high mannose-type N-glycans from proteins. GdS contains high mannose glycans at Asn28 (6). Endo-H treatment of GdS led to a slight mobility shift from 27 to 24 kDa for the lower band and from 30 to 27 kDa for the upper band (Fig. 5A), indicating that Endo-H deglycosylates GdS at Asn28. It...
can be concluded that the difference in the mobilities of the two bands of GdS is because of the different extent of glycosylation present on Asn63. As expected, GdA was not sensitive to Endo-H (Fig. 5A), as it does not contain high mannose glycans (5). Partial deglycosylation of GdS did not impart apoptotic activity to the protein, as tested by the Jurkat cell proliferation assay (Fig. 5B).

Enzymatic Desialylation of GdA Reduces Its Apoptotic Activity—At both N-linked glycosylation sites (Asn28 and Asn63), GdA has been reported to have sialic acid (5), which is absent in GdS (6). In two-dimensional gel electrophoresis, GdA moved as multiple spots, which were more acidic than the calculated pI (5.36) of the protein, as sialic acid adds additional negative charge to the molecule (Fig. 6A, panel a). However, GdS resolved essentially as a single spot at the calculated pI (5.36) (Fig. 6A, panel c). Upon neuraminidase treatment of GdA, the most acidic spot disappeared, and the protein resolved as two closely moving spots near the calculated pI (Fig. 6A, panel b), indicating that the protein had been desialylated, although not entirely. When the desialylated GdA was tested for its ability to inhibit Jurkat cell proliferation, the protein was significantly less

![Table 1](image)

**Table 1**
Calculated Stern-Volmer constants (described under "Experimental Procedures") for the glycodelins as determined from the slopes of the curves at different urea concentrations (n = 6)

| [Urea] | K<sub>SV(GdA)</sub> | K<sub>SV(GdS)</sub> |
|--------|----------------|----------------|
| 0 M    | 3.931 ± 0.1328 | 5.847 ± 0.1282 |
| 2 M    | 4.234 ± 0.1832 | 6.534 ± 0.1281 |
| 4 M    | 7.681 ± 0.1241 | 8.115 ± 0.0877 |
| 6 M    | 8.792 ± 0.1844 | 9.090 ± 0.0639 |
| 8 M    | 9.799 ± 0.1929 | 10.040 ± 0.2032 |

Fig. 8. GdA and GdS are conformationally distinct. A, far-UV CD spectra of glycodelin. GdA or GdS (0.5 mg/ml) in PBS was subjected to CD measurement, and the spectra were recorded. B, limited trypsin digestion of glycodelin. 1 µg of GdA or GdS was digested with trypsin at a molar ratio of 1000:1 for different time points at 37 °C. The proteins were electrophoresed on 12.5% SDS-polyacrylamide gel and silver-stained. Lane M, molecular mass markers; lanes 1, 3, 5, and 7, GdA; lanes 2, 4, 6, and 8, GdS. C, determination of Stern-Volmer constants for glycodelin at different urea concentrations. Shown are the Stern-Volmer plots for GdA and GdS at 0 (panel a), 2 (panel b), 4 (panel c), 6 (panel d), and 8 (panel e) M urea. Mol. Ellip., molar ellipticity.
Full-length Glycodelin Expressed in P. pastoris Is Not Apoptotically Active—As it is well established that P. pastoris is unable to add sialic acid residues (22), glycodelin was expressed as a C-terminal hexahistidine-tagged fusion protein in P. pastoris strain GS115. The protein was purified from the Pichia culture supernatant using Ni²⁺-NTA chromatography (Fig. 7A, panel a). The identity of the protein was confirmed by Western blotting using glycodelin-specific mAB B1C2 (Fig. 7A, panel b) (18). The purified protein was dimeric, as determined by fast performance liquid chromatography (data not shown). Pic-fl-Gd was unable to inhibit T cell proliferation, as tested in Jurkat cells (Fig. 7B), confirming the observation that sialic acid modification is required for the T cell inhibitory activity of the protein. In this regard, it is important to note that the N-terminal 23 amino acid-deleted dimeric glycodelin expressed in P. pastoris in our laboratory (23) was apoptotically active (13).

GdA and GdS Have Subtle Conformational Differences—To investigate if there is any conformational difference between the two glycodelins at the secondary structure level, the far-UV CD spectra of the two proteins were measured (Fig. 8A). Both proteins showed a peak near 219 nm and a shoulder around 210 nm, indicating that the secondary structures of the two proteins are very similar and composed of predominantly β-sheets, as reported previously (24).

In the absence of any gross structural dissimilarity, subtle conformational differences between the two proteins were probed by limited trypsin digestion. GdS was more susceptible to trypsin digestion compared with GdA (Fig. 8B), suggesting that the two proteins may have differences in their tertiary structures. The difference in conformation between the two proteins was confirmed by measuring their Stern-Volmer constants (K_SV) (Fig. 8C and Table I).

Glycodelin has three tryptophan residues, which are responsible for the intrinsic tryptophan fluorescence when excited at 295 nm. From Fig. 8C and Table I, it is clear that, upon treatment with up to 2 M urea, the K_SV values for GdA and GdS were different; but higher concentrations of urea denatured the protein such that the difference was no longer apparent. These results indicate that, at the tertiary structure level, GdA and GdS have slight differences in their folding.

To probe whether the differences in the trypsin sensitivity and K_SV of GdA and GdS are due to the presence or absence of sialic acid, GdA was desialylated with neuraminidase. The trypsin sensitivity and K_SV of the desialylated protein were measured and compared with those of the native protein. Neither the trypsin sensitivity (data not shown) nor the K_SV (Fig. 9) of the desialylated GdA differed from that of the native protein. Thus, if there is a conformation alteration due to the presence of sialic acid, it does not affect the trypsin sensitivity or the tryptophan environment of the protein.

It was interesting to observe that the presence of sialic acid residues in GdA did affect the hydrodynamic volume of GdA, which was found to decrease upon neuraminidase treatment, as determined by Superdex 200 fast performance liquid chromatography (Table II). GdA has a larger hydrodynamic volume than GdS. Upon removal of sialic acid residues, GdA eluted at the GdS value. This result indicates that the difference in the hydrodynamic volumes of the two proteins is solely due to the presence and absence of sialic acid residues.

**DISCUSSION**

Seminal plasma is well known to have immunosuppressive properties (25), and it is assumed that Gds, being structurally similar to GdA (26), contributes to this activity (15). However, our data clearly demonstrate that purified GdS is not immunosuppressive, as tested in the assays utilized in our study (Fig. 2), thereby raising the question as to why GdS (unlike GdA) lacks anti-proliferative/apoptotic activity. GdA and GdS are identical in their amino acid sequence, the only difference between them being at the glycosylation level (6). Glycodelins have complex-type glycan structures at two of the three putative glycosylation sites (5, 6), viz. Asn⁵⁸ and Asn⁶³. On both glycan structures, GdA has sialic acid residues, which are absent in GdS. On the other hand, GdS has high mannose glycans at Asn⁵⁸ and fucose-rich glycan structures at Asn⁶³ (6). The glycans present on GdA have been implicated in the contraceptive activity of the protein (15). It appeared possible that these glycans on GdA could bind to a cell-surface lectin receptor and induce apoptosis in T cells. To observe the effect of the GdA-specific glycans on T cells, Jurkat cells were treated with trypsin-digested GdA (Fig. 3). Apoptosis was not induced by the glycans alone in the absence of the protein.

This observation led us to speculate that the homodimer of GdA presents the glycosyl groups to the cell-surface receptors.
in an orientation that leads to receptor oligomerization and apoptosis. To test this possibility, we studied the effect of complete removal of the glycosyl groups from the folded proteins on their apoptotic activity. PNGase F was unable to remove the glycosyl groups from glycolipids under non-denaturing conditions (Fig. 4). Next, removal of the glycosylation by Endo-H was attempted. As expected, only the high mannose glycans were removed from GdS, whereas GdA was unaffected by this treatment (Fig. 5A). The removal of high mannose glycosylation from Asn28 of GdS did not impart apoptotic activity to GdS (Fig. 5B), suggesting that lack of apoptotic activity of GdS is not the effect of the specific glycans present on Asn28. Moreover, it has recently been demonstrated in our laboratory that the protein backbone alone, in the absence of the glycans, is apoptotically active (31).

Although the protein backbone is sufficient for the apoptotic activity, this activity is probably modulated by the different glycans in the native proteins. When GdA was desialylated using neuraminidase and tested in Jurkat cells, the apoptotic activity was significantly reduced (Fig. 6). Supporting this is the observation that Pic-f-gD was inactive (Fig. 7). It is well known that P. pastoris does not add sialic acid residues to the expressed proteins (22). Our results show that the sialylated oligosaccharides on GdA allow the manifestation of apoptotic activity innately present in the protein backbone. Therefore, we investigated the possibility that the GdA-specific glycans interact with the protein backbone to induce a conformation that exposes a masked apoptogenic region.

GdA and GdS are very similar at the secondary structure level, as detected by far-UV CD (Fig. 8A). To observe if there is any difference at the tertiary structure level, limited trypsin digestion and tryptophan fluorescence studies were performed. GdS was more susceptible to trypsin digestion compared with GdA (Fig. 8B), and the two isoforms had different tryptophan environments, as evident from their Stern-Volmer constants (Fig. 8C and Table I), indicating that there is a subtle conformational difference between the two isoforms. They also differ in their hydrodynamic volume, as measured by gel filtration chromatography (Table II).

When we studied the contribution of the sialic acid residues to maintaining this difference in conformation, desialylation of GdA neither increased its susceptibility to trypsin (data not shown) nor changed its Stern-Volmer constant (Fig. 9). These data indicate that GdA-specific glycans do not interact with the protein backbone to induce an apoptogenic conformation, or do so without affecting the tryptophan environment of the protein. However, desialylation of GdA decreased its hydrodynamic volume to a value close to that of GdS (Table II). Such a change in hydrodynamic volume upon removal of sialic acids has been reported for another lipocalin, α1-acid glycoprotein (27). Reversion of the hydrodynamic volume of desialylated GdA to a value close to that of GdS suggests that the addition of sialic acids exposes an apoptogenic region on the glycolipid backbone that is masked in their absence.

The presence of sialic acid residues in glycoproteins is generally associated with blocking interactions with other proteins (28). In fact, our data indicate the reverse, considering that the apoptotic activity resides in the protein backbone (31). It is difficult to explain exactly why the loss of sialic acid residues correlates with the loss of apoptotic activity. Two possibilities emerge from this observation. Either removal of sialic acids increases the accessibility of the glycolipid protein backbone (as discussed above), or the sialic acid residues in glycolipids themselves play a key role in mediating this activity via some signal transduction mechanism based on sialic acid recognition. Cell-surface lectins that specifically bind to sialic acid-containing glycoproteins (Siglecs) are known to be expressed by cells of hematopoietic origin and play a role in regulating immune responses (29). It is possible that the sialylated native GdA induces apoptosis by a similar mechanism.

Our data clearly demonstrate that there is a very subtle conformational difference between GdA and GdS. Although there was no detectable change in the trypsin susceptibility or Stern-Volmer constant upon removal of sialic acids from GdA, GdS is a significant anti-apoptogenerative activity. Sialic acid-dependent modulation of the cell surface has been reported previously (27, 30). We also observed a reduction of the hydrodynamic volume after removal of sialic acids from GdA. However, it is important to note that recombinant glycolipid devoid of any glycosylation expressed in S21 cells is apoptotically active (31), establishing that the information in the protein backbone alone is sufficient for apoptotic activity. Thus, the absence of apoptotic activity in GdS may be due to masking of the apoptogenic region on the protein backbone. This study points out that the function of the protein is elegantly altered by its differential glycosylation in different organs.