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# MECHANISM OF THE IMMUNOMODULATORY ACTIVITY OF GLYCODELIN

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**Abstract :** Glycodelin, a progesterone regulated protein synthesized by the endometrium (GdA) has been well documented to inhibit the proliferation of activated T-cells and is an indispensable molecule in the maternal system for the establishment, maintenance and progression of pregnancy. Data from our laboratory have unequivocally shown that the immunosuppression by GdA is via induction of apoptosis in activated T cells. Another isoform of glycodein, GdS, from the male reproductive system, in spite of sharing an identical amino acid sequence as that of GdA has been shown not to harbour the immunosuppressive activity of GdA. As the only difference between the two proteins is glycosylation, we proposed to study the role of the sugars in imparting apoptotic activity to Gd. Using the recombinant baculovirus system, Gd lacking glycosylation was expressed and from the experimental observations we could conclude that the activity of Gd lies in the protein backbone.

Recombinant Gd expressed in P pastoris, and Chinese hamster ovary cells, like the GdS did not exhibit apoptotic activity. A close analyses of the glycans associated with the Gd molecules from various sources suggested that though the apoptogenic activity of Gd lies in the protein backbone, the glycans modulate the activity by masking (as in case of GdS and most recombinant Gd expressed in our laboratory) or unmasking (as in case of GdA and baculovirus expressed Gd), the functional region of the molecule.

**Key words :** glycodelin T cells apoptosis glycans sialic acid

## INTRODUCTION

Glycodelin A (GdA) also known as placental protein 14 (PP14) is a glycosylated, dimeric, 162 amino-acid protein secreted by the human endometrium in the luteal phase of menstrual cycle and during pregnancy (1). Based on its 70% similarity with equine b-lactoglobulin Gd is classified under the lipocalin superfamily (2). It is expressed in the reproductive system of primates, both by the uterine endometrium and decidua

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(glycodelin A or GdA) (3), and by the glandular epithelium of the seminal vesicles (glycodelin S or GdS) (4). Both the isoforms are products of the same gene and therefore share the same amino acid sequence but differ significantly in the composition of their two N linked glycans at positions 28 and 63. GdA is rich in sialylated complex glycans whereas GdS is not sialylated, but has fucose and mannose rich glycans (5, 6).

GdA is a multifunctional protein, the functions attributed to the molecule are: contraceptive (7), morphogenic (8), angiogenic (9), and the most widely studied being immunosuppressive (10). Substantial evidence in literature indicates that GdA is reportedly 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 conclusively demonstrated that immunosuppression by GdA is via induction of apoptosis in activated T cells (13). It is believed that 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 case of immunocalins like α1-acid glycoprotein (AGP) or α1-microglobulin (A1M), which are members of the acute phase proteins (APP), 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). 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 (16) suggesting that the difference in the complex glycan on GdA may be responsible for the difference in the immunosuppressive activity. Total deglycosylation of Gd by chemical/enzymatic methods has not been possible without loss in the native conformation: however a significant decrease in the apoptotic activity of GdA was observed when the glycoprotein was desialylated (16). Molecular biology approaches were then adopted to mutate the glycosylation sites and express the protein in a eukaryotic system. The functional analyses of the protein thus expressed showed that the apoptogenic activity of the molecule resides in its protein backbone and that the role of the glycans is to modulate this activity (17).

## MATERIALS AND METHODS

### Cells and cell lines

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque (Sigma, USA), according to the method described by Boyum (18), from freshly drawn blood of normal healthy donors (male and female), (Age: 25–50 yr.). Jurkat (JR4) a human T cell line was cultured in RPMI supplemented with 10% FBS at 37C in a 5% CO2 incubator and passaged every three days.

The Sf21 (Spodoptera frugiperda) cells (Invitrogen Life Technologies, USA) were maintained in TC 100 medium (Sigma, USA) supplemented with 10% FBS (Sigma, USA) at 270C. Cell cultures were maintained as mono layers and passaged once every five days.

**Purification of glycodelin**

D9D4, a glycodelin specific monoclonal antibody (mAb) raised in our laboratory (unpublished data) was purified from the hybridoma culture supernatant by protein-A Sepharose (Sigma) chromatography (19). The antibody was periodate oxidized and crosslinked to Affi-Gel Hydrazide (Bio-Rad, USA) beads following manufacturer's instructions, to obtain an immunoaffinity column for purification of glycodelin. Amniotic fluid or seminal plasma were dialyzed against phosphate buffered saline (PBS), and passed through the immunoaffinity column. Bound protein was eluted with 0.1 M glycine-HCl, pH 2.5. and subjected to Western blot analysis using the monoclonal antibody B1C2 (20).

**Cell proliferation assay**

PBMCs at $0.2 \times 10^6$ cells/200 µl, or Jurkat cells at $0.1 \times 10^6$/200 µl in RPMI 1640 supplemented with 10% FBS, were cultured with the proteins or with appropriate controls. The anti CD3 mAb OKT3 was used for inducing T-lymphocyte proliferation in PBMC cultures. $5 \times 10^4$ cpm of [$^3$H]thymidine [methyl T] (Board of Radiation and Isotope Technology, Mumbai, India) was added after 36 h incubation of the PBMC cultures and 24 h incubation of Jurkat cells, and incubated for a further 8 h. The cells were harvested and the [$^3$H]thymidine incorporated was measured in a scintillation counter (Wallac, Finland). All treatments were carried out in triplicates.

**Ethidium Bromide staining and Fluorescence Activated Cell Scan (FACS) analysis**

Cells ($1 \times 10^6$) cultured for 18 h with glycodelin were harvested and fixed in ice cold 70% ethanol for 30 min and subsequently stained with ethidium bromide solution [50 µg/ml ethidium bromide, 0.1 µg/ml of RNase A, 1% Triton X-100 and 40 mM sodium citrate in PBS) for 1 h. Analysis was carried out by fluorescence activated flow cytometer (Beckton Dickinson FACScan, USA). Blue light was used for excitation and emission was measured in the red region.

In order to determine whether glycodelin-induced apoptosis is triggered by activation of caspases, a pan-caspase inhibitor, N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone (z-VAD-FMK) was added to the cell culture along with glycodelin.

**Cloning the glycodelin cDNA into Baculoviral system**

The Bac-to-Bac Baculovirus expression system (Invitrogen Life Technologies, USA) was used to clone the glycodelin cDNA. The full length glycodelin cDNA with the secretory signal, obtained from the uterine endometrium (Mukhopadhyay et. al. unpublished) was cloned between BamHI and PstI into the shuttle vector pFASTBAC1 within the mini-Tn7 element. This vector was transformed into DH10BAC E. coli 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 were isolated and transfected into the insect cell line Sf21 using lipofectamine (Invitrogen) following the protocol provided with Bac-to-Bac Baculovirus expression system. The recombinant virus containing medium was harvested at the end of 72 h and the virus amplified further, multiplicity of infection (moi) determined. Fresh cultures of Sf21 cells were infected with the virus and culture supernatants harvested after 5 days and the proteins purified on the immunoaffinity column.

**Site directed mutagenesis of the Asn 28 and Asn 63 (N28Q, N63Q and N28,63Q) in glycodelin cDNA**

The Quick-change method (Stratagene, USA) using sense anti-sense primers carrying the site-specific mutation with an incorporated silent mutation for a unique restriction site were used. The wild type glycodelin pFASTBAC1 vector was used as the template for the creation of single mutants. The double mutant glycodelin pFASTBAC1 vector was created from both the single mutants (N28Q and N63Q). Clones were screened by the unique restriction sites incorporated in them as well as by sequencing. The recombinant pFASTBAC1 clones were transformed in DH10BAC E.coli cells. The recombinant bacmids were purified, confirmed by PCR and transfected into Sf21 cells using lipofectamine as described earlier. All restriction enzymes used were purchased from New England Biolabs, UK.

**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 Gd specific mAb D9D4 immuno-affinity column (unpublished data). The column was washed with PBS (50 mM phosphate buffer pH 7.2 containing 150 mM NaCl) and the bound protein eluted with 100 mM Glycine-HCl, pH 2.5. 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, Germany). The concentrate was dialyzed overnight against PBS, filter sterilized through 0.45 µ membrane filter (Sartorius, Germany) and protein concentration estimated (21).

**Neuraminidase treatment under nondenaturing conditions**

Glycodelins at a concentration of 1 mg/ml in 100 mM acetate buffer pH 5.5, were incubated with 20 mU/ml of neuraminidase at 37°C for 24 h. Then the proteins were resolved by 2D gel electrophoresis and subsequently immunoblotted with mAb B1C2. Alternatively the reaction mixture was neutralized with 1M Tris-HCl pH 8, sterilized and used in Jurkat cell proliferation assays.

**Two dimensional gel electrophoresis**

Two dimensional gel electrophoresis was carried out following the method of Celis et. al. (22). Briefly, ~2 µg protein was mixed

with lysis buffer (9.8 M urea, 2% Nonidet P 40, 2% Ampholine pH 3.5–9.5 (Amersham Pharmacia Biotech, Sweden), 100 mM dithiothreitol) and isoelectric focussing was performed for 4 h at 400 V, in 1.5 mm tube gels, pre-focussed for 30 min at 250 V, using mini-PROTEAN 2D-Cell (Bio-Rad, USA). For the 2nd 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% bromophenol blue and electrophoresed. At the end of 2D separation the gels were either silver stained or used for immunoblotting.

## RESULTS

### Purification of Glycodelin from various sources

GdA was purified from human amniotic fluid (Figure 1A) and GdS from human seminal plasma (Figure 1B) by single step affinity chromatography on mab D9D4-affigel hydrazide column. The purified proteins were electrophoresed on 15% polyacrylamide gels (Figure 1) and identified by Western blotting using another Gd specific mab (Figure 1).

### Proliferation assays

Purified GdA inhibited both, OKT3 mAb induced proliferation of PBMC (Figure 2A-a] as well as Jurkat cell proliferation (Figure 2A-b] in a dose dependent manner as reported earlier (10, 13). In case of GdA, the 50% inhibitory concentration is 600 nM for PBMCs and 150 nM for Jurkat cells. Interestingly, GdS purified by the identical procedure did not show inhibition of

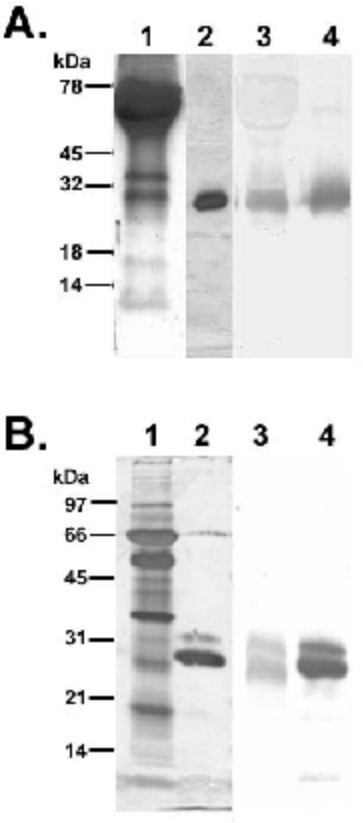


Fig. 1: Purification of glycodelin. [A] Molecular characterization of GdA. 5 µl amniotic fluid (lanes 1 and 3) and 2 µg GdA purified on mAb D9D4 Affgel hydrazide column (lanes 2 and 4) were electrophoresed on a 12.5% denaturing polyacrylamide gel under reducing conditions, were stained with Coomassie brilliant blue (lane 1) or silver (lane 2). The proteins were transferred to nitrocellulose membrane and subjected to immunoblot analysis with mAb B1C2 (lanes 3 and 4). B. Molecular characterization of GdS. 2 µl seminal plasma (lanes 1 and 3) and 2 µg GdS purified on mAb D9D4 Affigel hydrazide column (lanes 2 and 4) were electrophoresed on a 12.5% denaturing polyacrylamide gel under reducing conditions, were stained with silver (lanes 1 and 2). The proteins were subjected to immunoblot analysis with mAb B1C2 (lanes 3 and 4).

OKT3 mAb induced PBMC proliferation (Figure 2B-a) or Jurkat cell proliferation (Figure 2B-b).

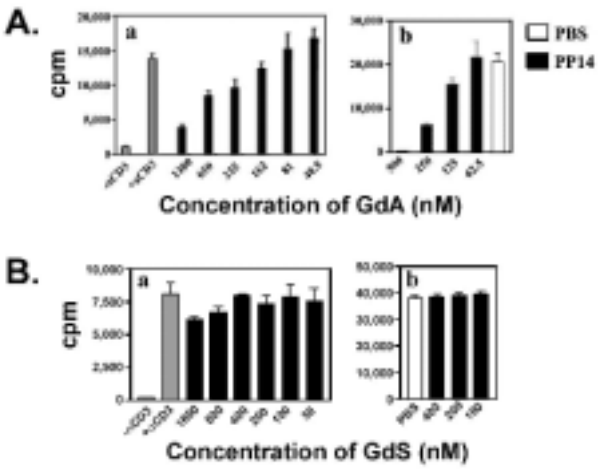


Fig. 2 : [A] GdA inhibits T cell proliferation. (a) PBMCs were stimulated with OKT3 mAb for 48 h in the presence of varying concentrations of GdA, later pulsed with [3H]thymidine for 12 h. (b) Jurkat cells were cultured for 24 h in the presence of varying concentrations of GdA and then pulsed with [3H]thymidine. Cells were harvested on glass fibre filters, lysed and the radioactivity measured. [B] GdS does not inhibit T cell proliferation. (a) PBMCs were stimulated with OKT3 mAb for 48 h in the presence of varying concentrations of GdS, later pulsed with [3H]thymidine for 12 h. (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 fibre filters, lysed and the radioactivity measured. Data presented are representative of at least three different experiments and each bar represents [3H]thymidine counts of the sample in triplicates.

**Generation of apoptosis in PBMCs and Jurkat cells :**

PI staining and FACS. Cells cultured with glycodelin were stained with PI and subjected to FACS analysis. All cells showed DNA loss as indicated by the pre-G0/G1 peak. In the case of lymphocytes, 14% of the cell population was apoptotic after treatment with glycodelin as compared to untreated cells, which showed only 4% apoptosis (data not shown). The apoptotic population in Jurkat cells increased to 54% on culturing with glycodelin for 18 h as compared to the 7% in untreated cells (Fig. 3a-B). When the pan caspase inhibitor z-VAD-FMK was added along with glycodelin, apoptosis was completely inhibited (Figure 3b-C).

AcOr/EtBr staining. Morphological observations of apoptosis was carried out by dual staining the cells with EtBr and AcOr after exposure to glycodelin for 18 h. As can be seen in Figure 3c, Jurkat cells

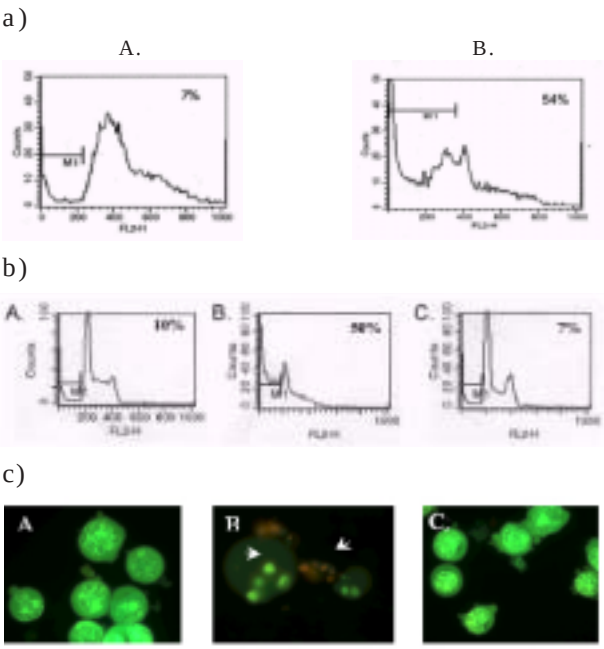


Fig. 3 : [a] FACScan analysis Jurkat of cells after culturing in absence (A) or in presence (B) of glycodelin for 18 h followed by staining with ethidium bromide and analysed by FACScan. FL2H fluorescence intensity (red channel).[b] GdA induced apoptosis is caspase dependent: Jurkat cells were cultured with 500 nM GdA in presence or absence of pan caspase inhibitor Z-VAD-FMK (25 µM) for 18 h. The cells were harvested, fixed with ethanol, stained with EtBr and analyzed by FACS. A. vehicle control (0.5% DMSO); B. cells treated with GdA; C. cells treated with GdA in presence of 25 µM Z-VAD-FMK. [c] Apoptotic effect of glycodelin as determined by EtBr staining: Jurkat cells were cultured with glycodelin for 18 h following which cells were stained with ethidium bromide acridine ornage (a) Untreated, (b) GdS and (c) GdA.

underwent chromatin condensation with appearance of apoptolic bodies that is characteristic of apoptosis after treatment with GdA (Figure 3c-B). GdS was unable to exhibit this activity (Figure 3c-C).

**Enzymatic desialylation of GdA reduces its apoptotic activity**

At both the N-linked glycosylation sites (N28 and N63) 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 are more acidic than the calculated pI (5.36) of the protein, as sialic acid adds additional negative charge to the molecule (Figure 4A-a). However, GdS resolved essentially as a single spot at the calculated pI (5.36) (Figure 4A-c). Upon neuraminidase treatment of GdA, the most acidic spot disappeared and the protein resolved as two closely moving spots near the calculated pI (Figure 4A-b), indicating that the protein has been desialylated, though not entirely. When the desialylated GdA was tested for its ability to inhibit Jurkat cell proliferation, the protein was significantly less active compared to the no enzyme control (Figure 4B) indicating that the apoptotic activity of GdA is dependent on the presence of the sialic acid residues on this protein, which are absent in GdS.

The recombinant glycodelin gene was expressed in CHO and P pastoris. The WT and glycosylation mutant glycodelin gene was expressed in the Sf21 and Tni insect cells using the baculovirus system. The secreted proteins were purified from the culture supernantants by affinity chromatography on D9D4-Sepharose column.

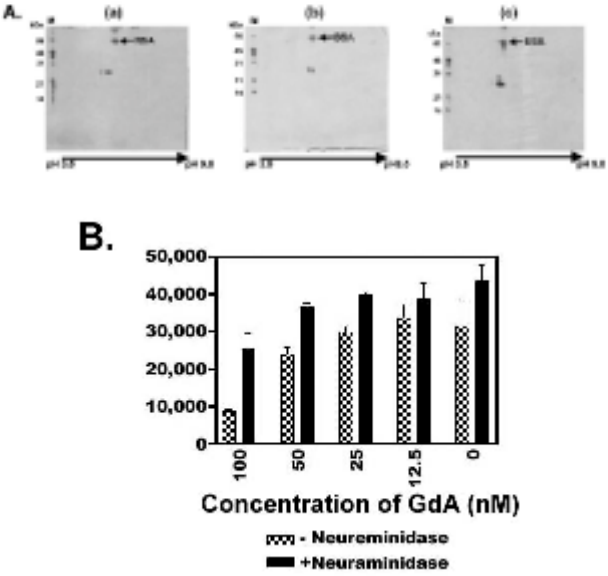


Fig. 4 : A. Two dimentional gel electrophoresis after neuraminidase treatment of GdA. (a) 0.25 µg GdA without enzyme, (b) 0.25 µg GdA after neuraminidase treatment and (c) 0.25 µg of GdS separated by 2D gel electrophoresis. Bovine serum albumin serves as an internal control. After the second dimensional separation the proteins were visualized by silver staining.

B. Neuraminidase treated GdA exhibits lower activity. Jurkat cells were cultured for 24 h in the presence of varying concentrations of neuraminidase treated or untreated GdA and then pulsed with [3H]thymidine. Cells were harvested on glass fibre filters, lysed and the radioactivity measured.

All the purified recombinant proteins were electrophoresed and identified using Western blotting as above [data not shown].

**Full length glycodelin expressed in Pichia pastoris and Chinese Hamster Ovary cells is not apoptogenic**

Glycodelin was expressed as a C-terminal hexa histidine tagged fusion protein in Pichia pastoris strain GS115. The protein was purified from the culture supernatant using Ni-NTA chromatography. The identity of the protein was confirmed

by Western blotting using glycodelin specific mAb B1C2 (20). The yeast expressed Gd was unable to inhibit T cell proliferation, as tested on Jurkat cells (data not shown) not surprising as it is well established that Pichia pastoris is unable to add sialic acid residues (23), confirming the observation that sialic acid modification is required for the T cell inhibitory activity of the protein. The recombinant Gd expressed in CHO cells did not harbour apoptogenic activity. The sialyl transferase activity in CHO cells is low and also the linkage is different from that seen in the human system (24).

**Wildtype (WT) Gd-Bac as well as the glycosylation mutants are apoptotically active**

To investigate the immunosuppressive activity of the recombinant protein, PBMC proliferation inhibition assay was carried out. WT Gd-Bac inhibited PHA (Figure 5A-a) as well as anti-CD3 mAb (Figure 5A-b) induced proliferation of human peripheral T cells in a dose dependent manner, comparable to that seen with GdA. Gd-Bac inhibited the proliferation of Jurkat JR4 cells as well (Figure 5A-c). This showed that the recombinant glycodelin expressed in baculoviral system is biologically active. Further, Jurkat cells cultured with WT Gd-Bac for 18 h were stained with EtBr and AcOr and morphological analyses were carried out to determine the apoptotic population. As can be seen in Figure 5B, cells treated with GD-bac underwent apoptosis as seen with cells treated with GdA.

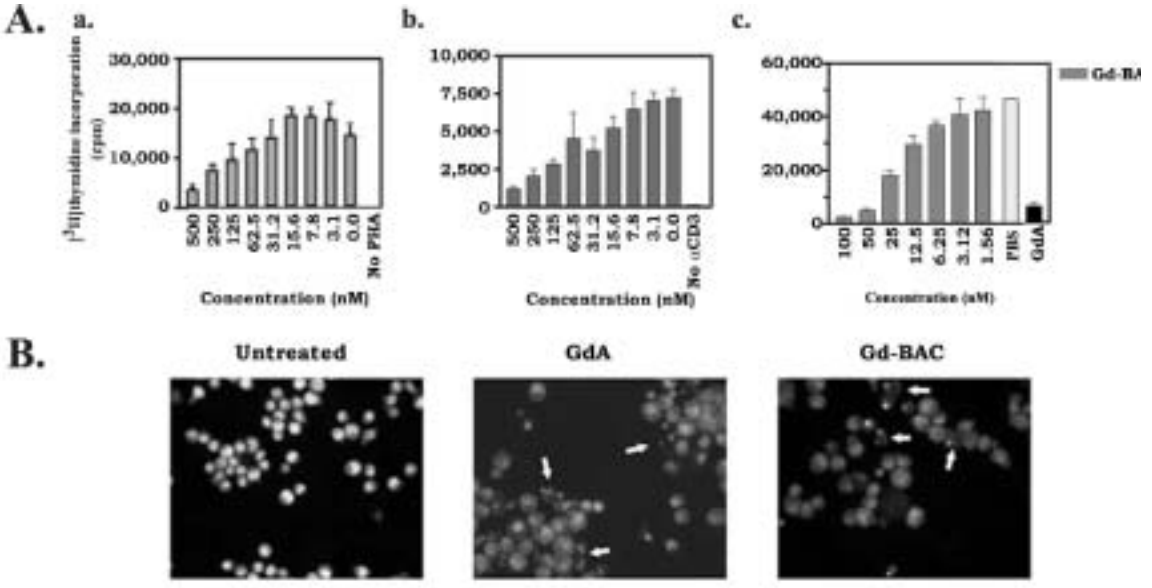


Fig. 5 : (A) WT Gd-Bac inhibits proliferation of activated T cells. PBMCs seeded at a density of 0.2 × 106/200 µl culture medium were activated with either phytohaemagglutinin (PHA) (a) or anti-CD3 mAb (b). The cells were cultured with varying concentrations of Gd-Bac for 36 h after which 3H-thymidine was added, further incubated for 12 h and the cell incorporated radioactivity was measured. All treatments were carried out in triplicates. (c) WT Gd-Bac inhibits proliferation of Jurkat cells as well. Jurkat cells seeded at a density of 0.1 × 106/200 µl medium were incubated with varying concentrations of WT Gd-Bac for 24 h followed by incubation with 3H-thymidine for 8 h and were harvested on glass fibre filter. (B) Ethidium bromide-Acridine orange staining of Gd-Bac treated Jurkat cells showing apoptotic population in cells treated with Gd-Bac. PBS serves as the negative control and panel GdA, the positive control.

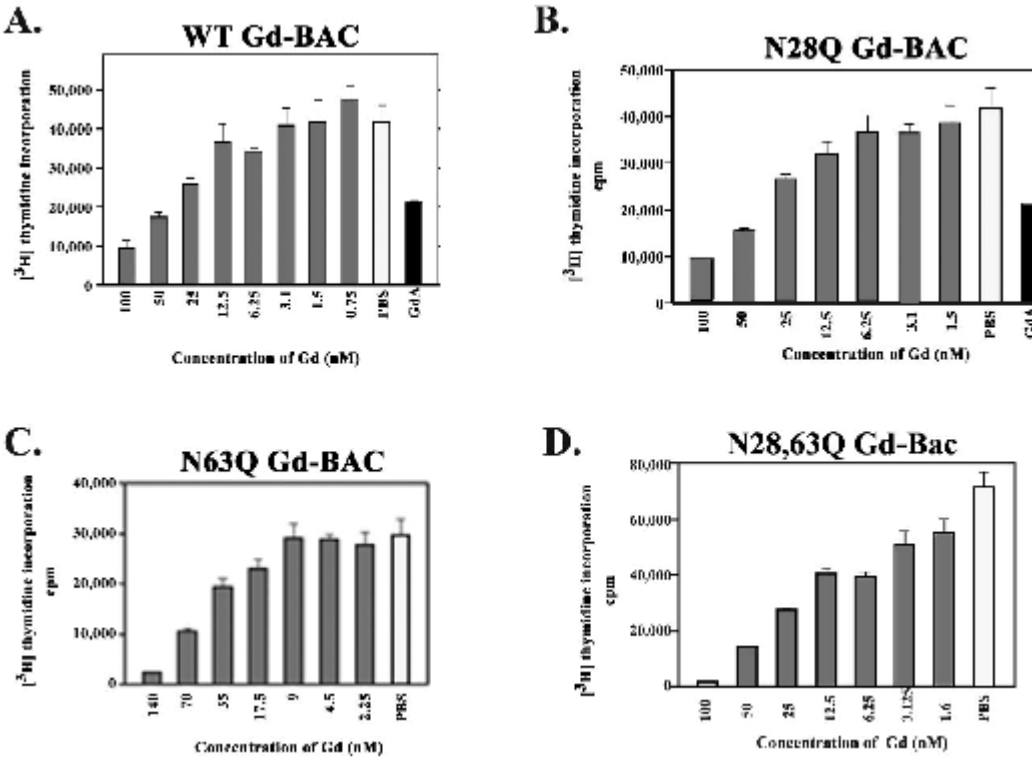


Fig. 6 : The recombinant glycosylation mutants inhibit proliferation of Jurkat cells. Jurkat cells seeded at a density of 0.1 million per 200 µl medium per well were cultured with varying concentrations of the WT and mutant Gd-Bac proteins for 24 h after which 3H-thymidine was added and incubated for further 8 h. Cells were harvested and the incorporated radioactivity was measured.

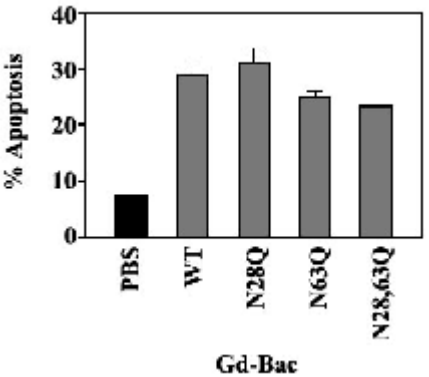


Fig. 7 : The recombinant glycosylation mutants induce apoptosis in Jurkat cells. Jurkat cells were cultured with the Wt or mutant Gd-Bac proteins for 12 h after which the cells were ethanol fixed, stained with propidium iodide and analyzed for percentage of cells undergoing apoptosis by FACscan.

Having confirmed that Gd-bac is comparable to GdA, the glycosylation mutants N28Q Gd-Bac, N63Q Gd-Bac and N28,63Q Gd-Bac proteins were assayed for immunosuppressive activity. The mutants N28Q (Figure 6B), N63Q (Figure 6C) and N28,63Q (Figure 6D) were also found to be inhibitory for Jurkat JR4 cell proliferation. 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 determined by the pre-G0/G1 peak. All the glycosylation mutants of Gd induced apoptosis in Jurkat cells comparable to that seen with

the WT Gd-bac confirming that the apoptogenic activity of Gd lies in the protein backbone.

## DISCUSSION

The fetal allograft survives maternal rejection during normal pregnancy but an allograft at sites other than the uterus is rejected efficiently by the maternal immune system. Therefore, studies on immunological events at the feto-maternal interface have been the major focus for researchers in this area. Experimental observations suggest that endometrial glycodelin is involved in the immunoregulation that down-regulates the maternal immune response to foreign fetal antigens. It has been postulated that glycodelin action is through immunosuppression, which is essential for protection of the embryo from the mother's immune response (10). As has been reported earlier glycodelin purified from the amniotic fluid in our laboratory was found to inhibit the proliferation of peripheral blood lymphocyte either by PHA or by cross-linking of CD3 receptors by antibody (13). Studies were then initiated to look into the molecular events that are triggered by GdA. At first, the PI staining studies were initiated to determine whether GdA blocks the cell cycle at any one stage of cell cycle, leading to anergy. The presence of the pre G0/G1 cell population indicated clearly that GdA induces apoptosis in PBMCs and Jurkat cells (Figure 2A). That GdA had an apoptotic effect on T cells was further confirmed by DNA fragmentation studies (data not shown] and EtBr/AcOr staining (Figure 2C). The final confirmation came from our studies with the pan caspase inhibitor that inhibited GdA induced apoptosis in Jurkat cells (Figure 2B).

Lymphocytes appear in the decidua of the first trimester of pregnancy and disappear towards the third trimester (25). Those cells that recognize non-self paternal antigens of the trophoblast would need to be deleted from the local environment, should the embryo survive. Death by apoptosis would be the preferred choice as apoptotic cells are quickly phagocytosed by macrophages via a receptor mediated process (26) ensuring that there is no release of intracellular contents (containing inflammatory components) in the surrounding tissue which would prove to be harmful to the developing fetus. Glycodelin s one of the many factors required for destroying activated T cells in order to abrogate the deleterious maternal response to paternal antigens.

Glycodelin A (GdA) and glycodelin S (GdS) are products of the same gene and secreted by the glandular epithelia of the endometrium (3) and the seminal vesicles (4) respectively. Both are glycosylated on the same two asparagines, N28 and N63: however, their glycan structures are distinctly different (5, 6). GdS was purified under identical conditions as GdA and tested on Jurkat cells. GdS did not inhibit Jurkat cell proliferation and did not exhibit apotogenic activity even at ten fold higher concentrations as that of GdA. As the difference between the two isoforms is their glycan composition, it was logical to determine whether the glycans on GdA dictate also its immunosuppressive characteristics. Enzymatic deglycosylation of

GdA using PNGaseF under native conditions however failed as it resulted in denaturation of the protein (16), therefore we resorted to recombinant DNA technology to address this question. The Gd cDNA was cloned into baculoviral system and expressed in Sf21 insect cells and the secreted protein purified. The recombinant protein was found to harbor antiproliferative activity on PBMCs and Jurkat JR4 cells (Figure 5A). It also exhibited apoptotic activiy (Figure 5B) as has been reported earlier in case of GdA (13, 16).

With an active wild type (WT) protein in hand we went ahead to determine the relevance of either or both the N-linked glycosylation of Gd. The asparagines at position 28 and 63 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 antiproliferative activity. Interestingly all the mutant proteins exhibited activities comparable to that of WT Gd-Bac and native protein (Figures 6 & 7), showing clearly that the immuno-suppressive activity of Gd resides in the protein backbone.

If the anti-proliferative activity of GdA is dictated by its primary structure, then the obvious question is, why is GdS not active in spite of 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 (15) on GdS perhaps keeps this region inaccessible. This hypothesis is supported by our observations that full-length Gd expressed in P.pastoris is inactive where there is high level of glycosylation and lack of sialylation (16).

The fact that the E.coli expressed GST-tagged Gd is apoptotically inactive (unpublished observation) highlights the necessity of an endoplasmic reticular transit for proper folding and disulfide bond formation. Our data also show that full length P.pastoris (16) and Human Embryonic kidney (HEK) cell line expressed Gd are inactive and importantly the baculovirus expressed Gd is active (17). This suggests that certain type of glycosylation can be permissive to the Gd molecule to execute its apoptogenic activity while other type of glycosylation hinders the apoptogenic activity by perhaps covering the apoptogenic region. The fact that GdA is apoptotically active while GdS is apoptotically inactive needs to be discussed in this light. It could be that the presence of large number of negatively charged sialic acid residues at sites N28 and N63 of the GdA molecule might result in charge repulsion between each other, thereby exposing the apoptogenic region while the absence of the same in GdS can result in the oligosaccharide chains keeping the apoptogenic region inaccessible. The minor differences in the conformation between GdA and GdS due to the presence and absence of sialic acid residues has been demonstrated (16). But in the case of Sf21 cell expressed Gd the extent of glycosylation is simple with only mannose 3 structures [Jayachandran et al, unpublished data] that cannot mask the apoptogenic region. It is very well known that glycans play a very important role in maintaining the stability, conformation and biological activity of glycoproteins (27, 28). Oligosaccharides are

essential recognition sequences in cell mediated adhesions in inflammatory and immune responses (29). The role of oligosaccharides in modulating the half-life and bio-potency of biomolecules is indeed well known (27, 28, 30). The role played by the glycans in glycodelin needs to be unravelled further for getting insights into functional constraints imposed on this molecule. In conclusion, the apoptogenic activity of the GdA resides in the protein backbone of the molecule. However the glycans play a major role in modulating the same.

## ACKNOWLEDGEMENTS

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