Differences in Glycosylation Patterns of Heat Shock Protein, gp96: Implications for Prostate Cancer Prevention


Department of Microbiology and Immunology, New York Medical College, Valhalla, New York

Abstract

Heat shock protein gp96 induces a tumor-specific protective immunity in a variety of experimental tumor models. Because the primary sequences of the glycoprotein, gp96 are identical between tumor and normal tissues, the peptides associated with gp96 and/or the posttranslational modifications of gp96, determine its immunogenicity. Gp96-associated peptides constitute the antigenic repertoire of the source tissue; thus, purified gp96-peptide complexes have clinical significance as autologous cancer vaccines. However, the role of altered glycosylation and its contribution in the biological as well as immunologic activity of gp96 still remains uncharacterized. We examined the cancer-specific glycosylation patterns of gp96. To this end, monosaccharide compositions of gp96 were compared between normal rat prostate and two cancerous rat prostate tissues, nonmetastatic androgen-dependent Dunning G and metastatic androgen-independent MAT-LyLu, as well as two human nonmetastatic prostate cancer cell lines, androgen-dependent LnCaP and androgen-independent DU145. Marked differences were observed between the gp96 monosaccharide compositions of the normal and cancerous tissues. Furthermore, gp96 molecules from more aggressive cellular transformations were found to carry decreasing quantities of several monosaccharides as well as sum total content of neutral and amino sugars. We believe that the unique glycosylation patterns contribute to cellular phenotype and that the posttranslational modifications of gp96 may affect its functional attributes. (Cancer Res 2005; 65(14): 6466-75)

Introduction

Gp96 is a glycoprotein belonging to a class of conserved proteins known as heat shock proteins. Gp96 is located within the endoplasmic reticulum where it associates with cellular peptides (1–3). During cellular stress such as heat shock, viral and bacterial infections, and exposure to UV radiation, heat shock protein synthesis increases (4). Heat shock proteins are chaperones and their functionality is related to the proper folding of nascent polypeptides and protecting polypeptides from denaturing during cell stress (4).

The ability of gp96 to associate with cell-specific peptides led to the concept of using autologous gp96 as vaccines to treat disease (5, 6). One particular area for the potential use of gp96 as a vaccine is in cancer therapy (5, 6). Purified gp96-peptide complex(es) isolated from specific cancer tissues is able to elicit a cancer-specific immune response and tumor regression which has been observed in several animal models as well as human clinical trials (6–9). The immunogenic property of gp96 is believed to be intrinsic to the gp96-peptide complex and not to gp96 or peptide alone (10). The immune response mounted against cancer cells is primarily a CTL-mediated immune response (11, 12). This cell-mediated immune response is elicited by binding of the gp96-peptide complex to specific receptors on antigen-presenting cells such as CD91 (13, 14). The tumor-specific peptide is then represented through MHC class I complex, leading to the cell-mediated immune response. Nevertheless, it is not yet known whether the posttranslational modifications of gp96 play any role in this tissue-specific process leading to cancer-protective immune response.

One of the main posttranslational modifications of proteins is glycosylation that can either occur on serine or threonine residues (O-linked) or asparagine residues (N-linked; ref. 15). Protein glycosylation is more abundant and structurally diverse than all other types of posttranslational modifications combined (16). Proteins that follow the secretory pathway from the Golgi apparatus to the endoplasmic reticulum can be covalently modified with various carbohydrates such as O-glycans and N-glycans (17). The addition of these glycosyl moieties involves enzymes, known as glycosyltransferases, specific for the sugar nucleotide donors to be attached covalently to the polypeptide backbone (18). Gp96 is composed of 803 amino acids with five potential N-linked glycosylation sites (19). Although gp96 is known to be an N-linked glycosylated protein, the glycan of this molecule has not been widely studied. One of the previous studies (20) on the molecular heterogeneity of gp96 proposed that glycosylation of gp96 has no role in creating the heterogeneity of gp96 and the mobility difference of three to four closely spaced bands that migrate in the range between 96 and 110 kDa. However, a subsequent study (21) indicated differential glycosylation of cyclic nature to be the cause for gp96 heterogeneity. The role of glycosylation in generation of gp96 molecular heterogeneity still remains poorly understood.

It is known that malignant transformation of cells leads to profound alterations in cellular glycosylation patterns of specific proteins (22). Examples of altered glycosylation patterns that occur during malignant cell transformation include greater Lewis antigen expression, enhanced glycan branching, and increased sialylation (22). In addition, one glycosylating activity known to be involved in tumor progression is N-acetylgalactosaminyl transferase V (GlcNAc-TV), which promotes cell motility through biosynthesis of β-1,6-GlcNAc branching (18). Yet, it remains to be confirmed whether glycosylation of gp96 can be changed due to cellular transformation because little is known regarding the nature of the glycosylation of gp96.

This study seeks to determine the monosaccharide compositions of various gp96 samples purified from normal and cancerous...
prostate tissues to examine whether differences in gp96 glycosylation exist between noncancerous and cancerous prostate in general, as well as specifically between the different types of prostate cancer. To achieve these objectives, purified gp96 samples were obtained from five different tissue sources. The sole noncancerous source was the normal rat prostate tissue. Additionally, two rat prostate tumor tissues, Dunning G (nonmetastatic/androgen dependent) and MAT-LyLu (metastatic/androgen independent), were used. We also analyzed gp96 from two nonmetastatic human prostate cancer cell lines, androgen-dependent LnCaP and androgen-independent DU145. We ascertained the monosaccharide compositions of gp96 samples using a technique that combines high pH anion exchange chromatography with pulse amperometric detection (HPAE-PAD) on both trifluoroacetic acid (TFA) and HCl hydrolysates of all gp96 samples for reliable determination of their monosaccharide compositions. Finally, some general features of gp96 glycosylation that are likely related to the progression of the transformation process are discussed in this report.

Materials and Methods

Growth of Cell Lines In vivo and In vitro
LnCaP, DU145, Dunning G, and MAT-LyLu were grown in culture using RPMI 1640 supplemented with 10% fetal bovine serum (Gemini, Calabasas, CA), 50 IU/mL of penicillin (Mediatech, Herndon, VA), 50 μg/mL streptomycin (Mediatech), and 2 mmol/L of L-glutamine (Mediatech). Tumor tissues were then centrifuged at 9,000 rpm for 10 minutes after which the supernatant was centrifuged at 27,000 rpm for 90 minutes. The supernatant was then subjected to successive 50% and 80% ammonium sulfate precipitations, after which the supernatant was applied to concanavalin A (ConA)-Sepharose column (Pharmacia, Uppsala, Sweden). Glycoproteins were eluted with 10% methyl-α-D-mannose pyranoside (Sigma Chemical Co., St. Louis, MO). This high concentration of mannose was used to elute bound gp96. The collected fractions were then applied to PD-10 columns (Pharmacia) equilibrated with 10 mmol/L sodium phosphate buffer (pH 7.0) containing 300 mmol/L NaCl. After the PD-10 column, samples were loaded on to a DEAE-Sephacel (Pharmacia) column equilibrated with 10 mmol/L sodium phosphate buffer (pH 7.0) containing 300 mmol/L NaCl and bound gp96 was eluted in seven separate fractions with 10 mmol/L sodium phosphate buffer (pH 7.0) containing 700 mmol/L NaCl.

Silver Staining and Western Blot Analysis of Gp96

Silver stain. gp96 (5 μg) was loaded and resolved on a 12% SDS-PAGE gel (24). The gel was then stained with Gel Code Silver SNAP Stain Kit (Pierce, Rockford, IL).

Western blot. gp96 (5 μg) was loaded and resolved on a 12% SDS-PAGE gel (5, 24). After 2 hours, the proteins were transferred at 200 mA for 2 hours on a polyvinylidene difluoride membrane (PVDF, Millipore, Bedford, MA). The membrane was blocked with 4% milk and subsequently incubated overnight with rat anti-gp94 (gp96) antibody (NeoMarkers, Fremont, CA) at 4°C. The blot was then developed with goat anti-rat IgG conjugated to horseradish peroxidase (HRP, Pierce) using Super Signal West Pico Chemiluminescent Substrate (Pierce).

In vivo Glycosylation of Gp96

LnCaP, DU145, MAT-LyLu, and Dunning G packed cell pellets (1 × 10⁶) were suspended in radioimmunoprecipitation assay buffer [RIPA: 50 mmol/L, Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.2% sodium deoxycholate, 0.1% SDS, 0.5% NP40, and 1 mmol/L phenylmethylsulfonyl fluoride], kept on ice for 30 minutes, and vortexed intermittently. Cell lysates were centrifuged at 4°C for 20 minutes at 14,000 rpm and the supernatant was collected as total cytosolic extracts. Protein concentration was determined by Bio-Rad protein assay. Protein samples were subjected to anion exchange chromatography with pulse amperometric detection (HPAE-PAD) on both trifluoroacetic acid (TFA) and HCl hydrolysates of all gp96 samples for reliable determination of their monosaccharide compositions. Finally, some general features of gp96 glycosylation that are likely related to the progression of the transformation process are discussed in this report.

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of 1 mL/min and passed through a PA-10 chromatographic column for separation of the monosaccharides. The resolved sugars were then quantitated through a pulse amperometric ED50 detector. With the help of the Chromeleon (Dionex) software package, a chromatogram was produced based on the ED$_{50}$ read-out of each hydrolysate analysis; this program was used for analysis and comparison of all PAD chromatograms reported in this study. The chromatograms of the hydrolysates were compared with a reference chromatogram produced from a standard monosaccharide mixture (Dionex) containing 100 pmol each of fucose (Fuc), galactosamine (GalN), glucosamine (GlcN), galactose (Gal), glucose (Glc), and mannose (Man) to determine the concentrations of each of the monosaccharides present in all hydrolysates. Actually, the GalN and GlcN peaks in the chromatograms of the hydrolysates respectively represent $N$-acetyl galactosamine (GalNAc) and $N$-acetyl glucosamine (GlcNAc) released from the glycoproteins because both of these sugars undergo quantitative decylation immediately following their release (26).

It is also worth mentioning that, although HPAE-PAD analysis was done using both the TFA and HCl hydrolysates of all gp96 samples, as per the currently accepted norm of glycobiology (26), only the TFA hydrolysate data were used for quantitation of the neutral sugar content of the gp96 samples, whereas their amino sugar contents were calculated solely based on the data obtained from the HCl hydrolysates. TFA hydrolysis yields accurate quantitation of the neutral sugar (i.e., Fuc, Gal, Glc, and Man) as well as amino sugar (i.e., GalN and GlcN) contents for neutral sugars like Fuc, Gal, Glc, and Man but not for amino sugars like GalNAc and GlcNAc, whereas the reverse is true for HCl hydrolysis.

**Results**

**Purification of gp96 from tumor tissues.** Whole tumor tissues from rat and human origin, as well as a noncancerous rat prostate tissue, were used to purify gp96 for glycosylation studies. The whole tumor tissues used in the study are as follows: two transplantable rat prostate cancer tumors, Dunning G (nonmetastatic and androgen dependent) and MAT-LyLu (metastatic and androgen independent); and two transplantable human prostate cancer tumors, LnCaP (nonmetastatic and androgen dependent) and DU145 (nonmetastatic and androgen independent). Dunning G–induced tumors are less aggressive, whereas MAT-LyLu, although spontaneously derived from Dunning G, readily metastasizes to the lung and lymph nodes and forms primary tumors within 2 weeks with as low as 10,000 cells implanted intradermally.

Because gp96 is an N-linked glycoprotein, a ConA-Sepharose column was used to selectively bind glycosylated proteins (specifically Man and Glc residues) to separate them from nonglycosylated proteins. The glycosylated proteins eluted from the ConA column were then applied to a size exclusion PD-10 column for buffer exchange. Gp96 was finally purified on a DEAE-Sepharose column and eluted in seven separate fractions, among which fractions 3 and 4 showed the highest concentrations of purified protein as shown by the elution profile (Fig. 1A). The purity of isolated gp96 was determined via silver staining of SDS-PAGE gel (Fig. 1B) and Western blot analysis (Fig. 1C) using a monoclonal rat anti-gp94 (gp96) antibody (NeoMarkers) in several different cell lines and tissues. All cell lines/tissues tested showed presence of gp96. The protein corresponding to gp96 seems in the 96-kDa region visible between the 116- and 66-kDa bands of the MW standards present on the silver-stained SDS-PAGE gel (Fig. 1F); no other band was visible in this gel. These gp96 bands were confirmed by Western blotting (Fig. 1C). These Western blot and silver stained SDS-PAGE analyses assured us that only gp96 was being used for glycan analysis.

The expression of glycosylated gp96 was assessed in human and rat prostate cancer cell lines by immunoprecipitation. Gp96 was immunoprecipitated using rat monoclonal antibody and subjected to SDS-PAGE and Western blot analysis. Glycosylated gp96 was detected by using ConA-HRP. A single migrating species representing glycosylated gp96 is present in all cell lines (Fig. 1D). Inhibition of in vivo glycosylation of gp96 was shown in one human cell line (LnCaP) and one rat prostate cancer cell (MAT-LyLu) using...
Glycosylation of gp96

Protective effect of tumor-derived gp96 as a vaccinating agent. It has been observed in several experimental models that purified preparations of gp96 can protect against subsequent challenges of the tumor cells from which it is derived (23). To test if gp96 isolated from MAT-LyLu cells protects against MAT-LyLu-induced tumors in Copenhagen rats, we vaccinated a group of rats (three rats per group) with 40 μg of purified MAT-LyLu gp96 per rat and compared this experimental group with a control group that were vaccinated with PBS. A representative figure is shown here of at least three independent experiments. The vaccination schedule was days 0 and 7 and live cell challenge with 10,000 MAT-LyLu cells per rat at day 7. Tumors were visible in PBS vaccinated rats by week 2 and palpable in gp96-vaccinated rats by week 4 (Fig. 2). This shows that tumor-derived gp96 vaccinated rats had a latency of 2 weeks in tumor development. By week 5, all rats in both the PBS and gp96 vaccinated group had tumors but a significant decrease in tumor size was visible between both groups. There was an apparent 2-fold decrease in tumor size between both groups by week 6 illustrating the protective role of tumor derived gp96 against the tumor. The tumor rejection property of gp96 is greater under constant vaccination pressure as shown earlier (27). Although repeat vaccinations yielded better antitumor effects, removal of vaccination pressure results in an enhanced rate of growth which was true in the experiments presented here as well.1 Results shown here are representative of three separate experiments.

Monosaccharide compositions of gp96 isolated from different rat prostate tissues. For monosaccharide composition determination, data obtained from TFA hydrolysates were used for quantitation of the neutral sugars in gp96 samples, whereas their amino sugar contents were calculated solely based on the data obtained from the HCl hydrolysates. All TFA and HCl hydrolysates were analyzed in triplicates. For all gp96 samples, the three PAD chromatograms obtained from a particular hydrolysate seemed very similar to each other indicating the reproducible nature of this analysis. Therefore, only the set of chromatograms produced by the first batch of analysis of all TFA and HCl hydrolysates is shown here. For monosaccharides show that the Fuc contents (Table 1) of the TFA hydrolysate of the rat gp96 samples, which were obtained from one normal and two cancerous (nonmetastatic Dunning G and metastatic MAT-LyLu) rat prostate tissues. A comparison among the three profiles shows that all of the rat gp96 samples carry the four neutral monosaccharides (Fuc, Gal, Glc, and Man) but it also indicates the clear differences between the samples in terms of the contents of the monosaccharides. These differences can be confirmed by contrasting the samples’ neutral monosaccharide contents (Table 1) of the TFA hydrolysate.

Separate scrutiny of the four individual species of neutral monosaccharides show that the Fuc contents (Table 1; considering the mean values in mmol/μg of gp96) of the rat samples progressively decrease from normal prostate (5.0) through Dunning G (2.5) through MAT-LyLu (0.30).

Like Fuc, the Gal and Glc contents of rat gp96 show a similar downward trend from normal prostate (5.38/5.00) through Dunning G (2.50/3.75) through MAT-LyLu (1.63/1.63). However, the Man content does not display such an across-the-board decreasing pattern. Although this content decreases from normal prostate (17.50) through Dunning G (8.75), it subsequently increases in MAT-LyLu (15.0). In addition, the Man contents of rat gp96 are much higher (17.50-8.75) than those of the other neutral sugars (5.38-0.3).

The gp96 samples’ total neutral and amino sugar contents as well as the grand total contents of all neutral and amino sugar components are shown in Table 3. These total contents were determined by adding up the mean content values of individual monosaccharide species. As a cautionary note, our reported grand totals of the neutral and amino sugar contents of the gp96 samples should not be considered as the total monosaccharide contents of those glycoproteins because a gp96 molecule may also contain monosaccharides (like sialic acid), which cannot be categorized as either neutral or amino sugars and is not presented in the analysis here.

Figure 3A illustrates the first set of the HPAE-PAD chromatograms representing the monosaccharide contents of the TFA hydrolysates of the rat gp96 samples, which were obtained from one normal and two cancerous (nonmetastatic Dunning G and metastatic MAT-LyLu) rat prostate tissues. A comparison among the three profiles shows that all of the rat gp96 samples carry the four neutral monosaccharides (Fuc, Gal, Glc, and Man) but it also indicates the clear differences between the samples in terms of the contents of the monosaccharides. These differences can be confirmed by contrasting the samples’ neutral monosaccharide contents (Table 1) of the TFA hydrolysate.

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1 Unpublished data.

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Figure 2. Protective effect of purified gp96 as a vaccine. Mean tumor volumes (in cm³) were plotted against the time elapsed (in weeks) after the challenge of animals with live MAT-LyLu cells. The control group and the gp96-vaccinated group of rats had latency periods for tumor development of 2 and 4 weeks, respectively. By the sixth week, a 2-fold increase in tumor volume was observed between the control and the experimental groups illustrating the protective role of gp96 in decreasing the tumor size.
A quick comparison of the HPAE-PAD chromatograms/profiles (Fig. 3B) representing the amino monosaccharide contents of the HCl hydrolysates of the same three rat gp96 samples shows distinct differences between the samples’ amino sugar compositions (Table 2). Rat gp96 samples’ contents of GalNAc are similar to those of Fuc, Gal, and Glc in that GalNAc progressively decreases from normal prostate (3.50) through Dunning G (2.00) through MAT-LyLu (1.38). In contrast, the GlcNAc content of gp96 does not decrease from normal prostate (13.75) through Dunning G (15.00) but it shows a steep drop in MAT-LyLu (1.63). Overall, rat gp96’s GlcNAc contents seem somewhat similar to the Man content because both do not decrease all the way across from normal prostate through MAT-LyLu and also because both generally range high (near 15.00).

Furthermore, the total neutral sugar content of gp96 (Table 3) decreases sharply from normal prostate (32.88) through Dunning G (17.50) but not subsequently in MAT-LyLu (18.56). On the contrary, the total amino sugar content of gp96 stays comparable between the first two tissues (17.75 versus 17.00) but drops precipitously in MAT-LyLu (3.01). Nevertheless, most interestingly, the grand total contents of the amino and neutral monosaccharides display the progressively diminishing order among the three tissues (50.63 versus 34.50 versus 21.57).

Figure 3. Comparison of the monosaccharide compositions of the rat gp96 samples isolated from normal and cancerous prostate tissues. HPAE-PAD analyses of the TFA hydrolysates (A) and the HCl hydrolysates (B) of the various rat gp96 samples were used for specific determination of their neutral sugar composition and amino sugar composition, respectively (see Materials and Methods). In each panel of HPAE-PAD chromatograms, the reference profile representing the standard monosaccharide mixture is shown at the bottom with the peaks for all six monosaccharides being marked above; each reference peak contains 100 pmol of respective monosaccharide. For all of the other three chromatograms of both panels, which represent the TFA/HCl hydrolysates of the three rat gp96 samples, the labels on the right indicate the respective source tissues. The neutral sugar compositions of these gp96 samples, which were quantitated based on the HPAE-PAD analysis of the HCl hydrolysates, are reported in Table 1. The amino sugar compositions of these samples, which were derived from the analysis of the HCl hydrolysates, are reported in Table 2. In both sets of chromatograms, all peaks used for quantitative purpose are marked by *. Mean ± SD of three separate determinations (Tables 1 and 2).
Thus, both rat cancers, Dunning G and MAT-LyLu, seem to carry much less of sugars than the normal rat prostate. More remarkable is the difference in gp96’s sugar content between the two cancerous rat gp96 samples, particularly from the perspective of aggressiveness of the transformations. As stated earlier, Dunning G is clearly much less aggressive than MAT-LyLu. Therefore, the less aggressive transformation seems to carry more sugar in gp96. In addition, if we include the normal rat prostate in this scheme and arrange all three rat tissues used in this study in increasing order of aggressiveness of proliferation, the ranking should be as normal prostate followed by Dunning G followed by MAT-LyLu.

**Monosaccharide compositions of gp96 isolated from two human prostate cancer tissues.** Having observed differences in sugar content of gp96 isolated from rat cancer cells with diverse phenotype, we wanted to extend our analysis to two human prostate cancer cells LnCaP (nonmetastatic/androgen dependent) and DU145 (androgen independent). HPAE-PAD analyses of the TFA (A) and the HCl hydrolysates (B) of the gp96 samples were used for specific determination of their neutral and amino sugar composition, respectively. The standard chromatogram of a mixture of monosaccharides is shown at the bottom and each reference peak contains 100 pmol of respective monosaccharide. The other two chromatograms of each set represent either the TFA or the HCl hydrolysates of the two human gp96 samples. The neutral sugar compositions (TFA hydrolysates) are reported in Table 1, while the amino sugar compositions (HCl hydrolysates) are reported in Table 2. In both sets of chromatograms, all peaks used for quantitative purpose are marked by *. Mean ± SD of three separate determinations (Tables 1 and 2).
and DU145 (nonmetastatic/androgen independent). In general, androgen-independent prostate cancers are more aggressive and more refractory to treatment strategies than hormone-dependent prostate cancers (29). In this context, it is pertinent to point that the unlike the previously analyzed pair of rat cancers, both the human cancers were chosen to be nonmetastatic so that they are distinguishable solely based on one variable of measuring aggressiveness (i.e., androgen dependence versus androgen independence). This was done to narrow down the extraneous factors’ interference on gp96 glycosylation.

A comparison of the neutral sugar profiles of the panel (Fig. 4A) showing analysis of the TFA hydrolysates, as well as the amino sugar profiles of the panel (Fig. 4B) representing the HCl hydrolysates, suggested that the gp96 glycosylation patterns of LnCaP and DU145 are profoundly different. This notion was further confirmed by examination of these two human gp96 samples’ neutral (Table 1) and amino (Table 2) sugar contents.

Gp96’s Fuc, Gal, and Glc contents (Table 1) decrease from LnCaP (1.63/3.50/5.00) through DU145 (~0.0/2.75/1.63). However, in contrast, the Man content of gp96 actually displays a slight increase from LnCaP (15.00) through DU145 (17.5). In addition, as in rat gp96, the Man content in the human gp96 in much higher than that of the other neutral monosaccharides. Thus, as per the neutral sugar contents of gp96, the human cancer profiles seem quite similar to those of the rat cancers.

Likewise, based on gp96’s contents of individual amino sugars (Table 2), the human cancers show overall resemblance to the rat cancers. First, the human gp96 samples have relatively low GalNAc content in general and it decreases from LnCaP (1.5) through DU145 (0.18). Second, extremely high and very low GlcNAc contents of gp96 correlate with the less aggressive LnCaP (25.00) and more aggressive DU145 (0.63), respectively.

Additionally, the total neutral sugar content of gp96 (Table 3) show some decrease from LnCaP (25.13) through more aggressive DU145 (21.88), which is unlike the trend seen with the corresponding rat samples, Dunning G and MAT-LyLu. Nevertheless, as per the total amino sugar contents and also according to the sum total contents of all six monosaccharides, a sharp decrease is observed between LnCaP (26.50/51.63) and DU145 (0.81/22.69), which resembles the patterns of these contents in the rat gp96 samples.

In conclusion, based on most individual monosaccharide content data, as well as the cumulative total contents of all six neutral and amino sugars of gp96, the human cancers displayed remarkable similarity with the rat samples. Overall, the greater the aggressive nature of the cancer, the lesser the glycosylation of its gp96. These studies need to be extrapolated to the generation of the glycosyl tree and analysis of the array of glycosylation (30).

**Discussion**

Gp96 is important, a chaperone with a fundamental role in various biological processes, including antigen presentation and immune response to cancer cells as well as other key housekeeping functions (23, 31). However, in spite of existing indications that glycosylation may critically influence various functionalities of gp96 (21), its glycan structure thus far remained uncharacterized due to a lack of analyses of gp96 glycosylation by contemporary techniques such as mass spectrometry and HPAE-PAD. In addition, a possible variation of gp96 glycosylation was indicated to be the cause of immunogenicity of a cancer-associated form of gp96 but its glycan has not been analyzed further (21). Therefore, the current investigation is a comprehensive attempt towards analysis of the gp96 glycan contents. Primarily, this study was designed to ascertain whether gp96 glycosylation differs between the normal tissues and the cancers, as well between the various types of cancers of both rat and human.

The monosaccharide composition analyses of the rat gp96 samples indicated substantial differences in the monosaccharide compositions of gp96 between the normal prostate and the two noncancerous rat prostate tissues. Such difference between the cancerous and noncancerous rat tissues is not surprising because malignancy is known to cause alterations in protein glycosylation (22), as changes of the contents of a particular species of sugar such as GlcNAc were shown to be associated with activities like GlcNAc-TV (18) and GlcNAc-TIII (32). The latter study indicated

<p>| Table 1. Neutral sugar compositions of gp96 |</p>
<table>
<thead>
<tr>
<th>Source of gp96</th>
<th>Sugar residue content/µg protein</th>
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<tbody>
<tr>
<td></td>
<td>Fuc (nmol)</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
</tr>
<tr>
<td>Normal rat prostate</td>
<td>5.00 ± 0.500</td>
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<tr>
<td>Dunning G</td>
<td>2.50 ± 0.250</td>
</tr>
<tr>
<td>MAT-LyLu</td>
<td>0.30 ± 0.087</td>
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<tr>
<td>Human</td>
<td></td>
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<tr>
<td>LnCaP</td>
<td>1.63 ± 0.087</td>
</tr>
<tr>
<td>DU145</td>
<td>ND (−0)</td>
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</tbody>
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NOTE: All values are in nmol ± SD. Quantitation of all neutral monosaccharide contents was done by HPAE-PAD analyses of the TFA hydrolysates derived from rat (Fig. 3A) and human (Fig. 4A) gp96 samples. For each compositional data, the mean of the three values obtained by triplicate analysis has been reported along with the corresponding SD. All associated probability values were checked by ANOVA to ensure that all monosaccharide composition data are within statistically significant range of variation (P < 0.05). As stated in Materials and Methods, due to batch-to-batch variation of the reference, the compositional data reported here may not be absolute. However, the contents are reliable relative to each other because the same batch of the reference was used throughout the study.

Abbreviation: ND, not detectable.
that the invasiveness of transformations may decrease with increasing cellular levels of GnTIII, the β-1,4-GlcNAc transferase type III. GnTIII adds a "bisecting" GlcNAc, which is a branched or antennal residue. It has been shown that the transfection of the GnTIII gene into B16 melanoma cells results in a suppression of invasive ability and lung colonization. The phenomenon has been postulated to be due to the higher cellular level of "bisecting" GlcNAc that leads to up-regulation of E-cadherin on cell surface. This elevated E-cadherin surface expression results in greater cell-cell adhesion, as well as cell matrix adhesion. Aberrations in sialyl Lewis A, sialyl Lewis X, and E-cadherin are related to cancer metastasis and angiogenesis (33, 34). Furthermore, the glycosylation patterns broaden the substrate profile of metalloproteinases that allows escape of a cancer subset through the matrix resulting in metastasis (35).

More interestingly, the monosaccharide contents of rat and human gp96 display an overall decrease with increasing aggressiveness of the transformation process. This pattern was observed by comparing monosaccharide composition data of gp96 between the rat tumors Dunning G (less aggressive) and MAT-LyLu (metastatic more aggressive), as well as between two nonmetastatic human cancers LnCaP (androgen dependent) and DU145 (androgen independent). The aggressiveness is also observed in their faster rate of proliferation; for instance, the doubling time of Dunning G (~48 hours) is much higher than MAT-LyLu (~6-8 hours). This trend of decreasing gp96 glycosylation with increasing aggressiveness of transformation agrees with the trend of gp96 glycosylation observed between the normal rat prostate and Dunning-G/MAT-LyLu, because the noncancerous tissue is much less aggressive than any of the cancers based on the cellular proliferation phenotypes of these tissues. Therefore, in sum, the trend of sugar analysis leads to the correlation that the higher the cellular proliferation, lesser is the content of gp96 glycosylation.

Correlation of gp96 glycosylation profile with progression of transformation phenotype might enable identification of certain motifs of gp96 glycosylation that promote malignancies. Precedence of altered glycosylation patterns, such as GlcNAc-branched N-glycans and terminal Lewis antigen sequences, being linked to cell transformation and cancer progression (18) already exists. Whether these glycosylation patterns can alter functional activity of clinically relevant molecules, such as gp96, has ramifications for both basic and clinical research. This is not without precedence as it has been reported earlier that MHC class II-restricted T-cell responses against tumor-associated carbohydrate structures have been observed (36). Cancer-specific carbohydrate antigens, such as α-galactosyl epitopes, have been shown to function in opsonization of cancer cells (37, 38) as well as humoral responses directed against cancer cells (39–42). Enhancement of humoral response by gp96 or optimal adjuvant activity of gp96 that is mitigated by altered tissue specific glycosylation patterns needs an in depth examination.

In this study, we identified several specific monosaccharide residues of gp96, particularly Fuc, Gal, Glc, and GalNAc to a larger extent, as well as Man and GlcNAc to a smaller extent, which are either up-regulated or down-regulated among rat metastatic (MAT-LyLu), nonmetastatic (Dunning G), and noncancerous rat prostate as well as between two human prostate cancer lines (LnCaP and DU145). It seems that Fuc, Gal, Glc, and GalNAc (as opposed to Man and GlcNAc) display a steady pattern of decrease from rat prostate through Dunning G through MAT-LyLu, as well as from human cancers LnCaP through DU145. It is possible that these changes in gp96 monosaccharide compositions might be triggering some common mechanisms promoting proliferation. Because glycosylation is a posttranslational modification, it may confer distinct functional activity that is related to peptide chaperoning, peptide binding, and/or antigen presentation.

Changes in gp96 glycan contents may be reflective of altered tissue specific glycosylation cellular machinery and because some of our analysis suggests a correlation with progression of transformation, it is tempting to speculate that the glycosylation pattern of conserved proteins, such as gp96, may be signatures of tissue/cell phenotype. Altered glycosyl patterns that are a reflection of cellular glycobiology have been related to vaccine production, optimal adjuvant activity, and humoral response (36–39). A striking decrease in the GlcNAc content of gp96 was seen in MAT-LyLu compared with Dunning G, as well as in DU145 vis-à-vis LnCaP. In both pairs of malignancies, the latter member is androgen independent whereas the former is androgen dependent. The androgen-dependent cells may display a more complete sugar profile due to activation of hormone-associated growth regulating pathways, which remain silent or down-regulated in their androgen-independent counterparts. Taken together, we hypothesize that glycosylation patterns of gp96 differ between the various tumors and that the presence or absence of specific glycosyl motifs can affect cancer phenotype. This study is the first significant to attempt the identification of the gp96 glycan motifs/epitopes that can be presumably related to cell phenotype. Detailed structural and functional characterization of such gp96 glycan moieties would help better elucidation of gp96 glycosylation's proposed involvement in its function and eventual cell characteristics.

Additionally, the glycosylation patterns of various clinically relevant samples of gp96 need a detailed evaluation in view of these molecules potential use in development of autologous cancer vaccines. The use of gross tumor protective assays, although elucidate the tumor rejection property of gp96, cannot evaluate the biological function of gp96 in its entirety. Tumor protective effect was observed for both Dunning G (24) and in MAT-LyLu (this study). The degree of response was however greater for MAT-LyLu; however, at this stage, this cannot be attributed to the glycosylation

### Table 2. Amino sugar compositions of the gp96

<table>
<thead>
<tr>
<th>Source of gp96</th>
<th>Sugar residue content/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GalNAc (nmol)</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
</tr>
<tr>
<td>Normal rat prostate</td>
<td>3.50 ± 0.125</td>
</tr>
<tr>
<td>Dunning G</td>
<td>2.00 ± 0.088</td>
</tr>
<tr>
<td>MAT-LyLu</td>
<td>1.38 ± 0.063</td>
</tr>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>LnCaP</td>
<td>1.50 ± 0.087</td>
</tr>
<tr>
<td>DU145</td>
<td>0.18 ± 0.012</td>
</tr>
</tbody>
</table>

NOTE: All values are in nmol ± SD. Quantitization of all amino monosaccharide contents was done by HPAE-PAD analyses of the HCl hydrolysates, derived from rat (Fig. 3B) and human (Fig. 4B) gp96 samples. For each compositional data, the mean of the three values obtained by triplicate analysis has been reported along with the corresponding SD. All associated probability values were checked by ANOVA to ensure that all monosaccharide composition data are within statistically significant range of variation (P < 0.05).
pattern as tumor rejection in an intact animal involves in vivo immune response and presumably a multitude of antigens. For MAT-LyLu, the continuous transport of antigens in the lymph node by virtue of MAT-LyLu being metastatic may be one reason. The contribution of altered glycosylation pattern to specific immune response can be estimated if the peptide binding ability of two distinct gp96s with altered sugar contents are examined with a defined peptide. Experiments to examine this concept are under way using the VSV8 peptide.

Whereas disaccharides and other pentasaccharide trees have a role in determining cancer cell phenotype, the variations in monosaccharides, such as sialic acid and GlcNAc, cannot be overlooked. Sialic acid has a major role in cell growth and apoptosis that may be mediated by Fas (43, 44). These changes are significant than total Man content (50). These are direct extrapolations of our results that can be tested in experimental models and have significance for both the biological and clinical activity of this important cellular protein. Altered glycans can have significant B-cell responses in vivo, a hitherto ignored immunologic activity related to gp96-induced cancer responses. We plan to investigate this phenomenon in greater detail using defined peptides such as the VSV8 peptide and gp96 isolated from different sources and by using deglycosylated gp96 and testing for its immunologic activity in animal models.

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We thank George Osae of Dionex for his help in sugar analyses.

### Table 3. Combined monosaccharide compositions of gp96

<table>
<thead>
<tr>
<th>Source of gp96</th>
<th>Monosaccharide content/µg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total of neutral sugars (nmol)</td>
</tr>
<tr>
<td>Rat Normal rat prostate</td>
<td>32.88</td>
</tr>
<tr>
<td>Dunning G</td>
<td>17.50</td>
</tr>
<tr>
<td>MAT-LyLu</td>
<td>18.56</td>
</tr>
<tr>
<td>Human LuCaP</td>
<td>25.13</td>
</tr>
<tr>
<td>DU145</td>
<td>21.88</td>
</tr>
</tbody>
</table>

NOTE: The total neutral sugar content of each gp96 sample is the sum of the four mean values for the four neutral sugar components, as shown in Table 1. The total amino sugar content is the sum of the two mean values for the two amino sugar components, as shown in Table 2. By adding the total neutral sugar content of a gp96 sample with its total amino sugar content, the sample's grand total content of all neutral and amino sugars was obtained.

found on the surface of platelets and endothelial cells thus allowing cancerous cells to metastasize beyond the point of origin (49). These changes on specific glycoproteins, such as gp96, and the consequent effect on their functional activity have not been reported and this study is a step in that direction with future emphasis in examining the sialic acid content and biochemical differences that may be observed.

Different patterns of gp96 glycosylation in different types of cancers have a notable relevance for development of autologous cancer vaccines. The mutual association between gp96 and peptide provides a direct route for delivering the bound immunogenic peptide to the MHC class I complex of the antigen-presenting cell via a specific receptor, CD91 (13, 14). Gp96 with altered glycosyl motifs may differ in their affinity to cell surface receptors and this will affect the representation of the cell derived peptides. This may account for the variability of clinical response and/or ability to be purified on ConA-Sepharose columns. The differences in purification yields from different tissues may be a function of glycans; however, these results are not a mere function of Man content. The yields of purified gp96 from different tissues were variable and it is worth mentioning that the ConA recognition is not only through Man but a pentasaccharide core and until the glycosyl tree is determined the issue of the relationship between the sugar and protein purification cannot be clarified. The pentasaccharide core is β-GlcNac (1→2)-α man (1→3)-(β-GlcNac)-α-man (1→6)-Man and it is this structural tree that may be relevant in the affinity of binding of the different glycoproteins that may be more significant than total Man content (50). These are direct extrapolations of our results that can be tested in experimental models and have significance for both the biological and clinical activity of this important cellular protein. Altered glycans can have significant B-cell responses in vivo, a hitherto ignored immunologic activity related to gp96-induced cancer responses. We plan to investigate this phenomenon in greater detail using defined peptides such as the VSV8 peptide and gp96 isolated from different sources and by using deglycosylated gp96 and testing for its immunologic activity in animal models.

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