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Structural details and composition of *Trichomonas vaginalis* lipophosphoglycan in relevance to the epithelial immune function

Bibhuti N. Singh · Gary R. Hayes · John J. Lucas · Ulf Sommer · Nelly Viseux · Ekaterina Mirgorodskaya · Radiana T. Trifonova · Rosaria Rita S. Sassi · Catherine E. Costello · Raina N. Fichorova

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Abstract *Trichomonas vaginalis* causes the most common non-viral sexually transmitted infection linked to increased risk of premature birth, cervical cancer and HIV. This study defines molecular domains of the parasite surface glycoconjugate lipophosphoglycan (LPG) with distinct functions in the host immunoinflammatory response. The ceramide phospho-inositol glycan core (CPI-GC) released by mild acid had Mr of ~8,700 Da determined by MALDI-TOF MS. Rha, GlcN, Gal and Xyl and small amounts of GalN and Glc were found in CPI-GC. *N*-acetyllactosamine repeats were identified by *endo*-β-galactosidase treatment followed by MALDI-MS and MS/MS and capLC/ESI-MS/MS analyses. Mild acid hydrolysis led to products rich in internal deoxyhexose residues. The CPI-GC induced chemokine production, NF-κB and extracellular signal-regulated kinase (ERK)1/2 activation in human cervicovaginal epithelial cells, but neither the released saccharide components nor the lipid-devoid LPG showed these activities. These results suggest a dominant role for CPI-GC in the pathogenic epithelial response to trichomoniasis.

Keywords Trichomonad LPG · Mass spectrometry · Cytokines · NF-κB · ERK · Vaginal mucosal immunity

Abbreviations

CPI-GC	ceramide phospho-inositol glycan core
DHB	2,5-dihydroxy-benzoic acid
ESI	electrospray ionization
GPI	glycosylated phosphatidylinositol
FACE	fluorophore-assisted carbohydrate electrophoresis
FT MS	Fourier transform ion cyclotron resonance mass spectrometry
HPAE-PAD	high performance anion exchange chromatography-pulsed amperometric detector
LC	liquid chromatography
LPG	lipophosphoglycan
LPS	lipopolysaccharide
MALDI-TOF MS	matrix-assisted laser desorption ionization/time-of-flight mass spectrometry
NMR	nuclear magnetic resonance
PAS	periodic acid-Schiff staining
PBS	phosphate-buffered saline
PI-PLC	phosphatidylinositol-specific phospholipase-C
PSD	post-source decay
QoTOF	quadrupole orthogonal time-of-flight
SORI-CID	sustained off resonance irradiation-collision-induced decomposition
TFA	trifluoroacetic acid
TV	*Trichomonas vaginalis*
TF	*Tritrichomonas foetus*

B. N. Singh · G. R. Hayes · J. J. Lucas
Department of Biochemistry and Molecular Biology,
SUNY Upstate Medical University,
750 E Adams St, Syracuse, NY 13210, USA

U. Sommer · N. Viseux · E. Mirgorodskaya · C. E. Costello
Center for Biomedical Mass Spectrometry,
Boston University School of Medicine,
670 Albany St., Rm 511, Boston, MA 02118, USA

R. T. Trifonova · R. R. S. Sassi · R. N. Fichorova (✉)
Laboratory of Genital Tract Biology,
Department of Obstetrics, Gynecology and Reproductive Biology,
Brigham and Women's Hospital, Harvard Medical School,
221 Longwood Ave RF468, Boston, MA 02115, USA
e-mail: rfichorova@rics.bwh.harvard.edu

Introduction

Trichomonas vaginalis is an extracellular parasitic protozoan that causes one of the most common non-viral sexually transmitted infections, human trichomoniasis. Each year, over 180 million people are infected worldwide, including 8–10 million Americans [1–4]. Around the globe, infection rates have been reported to be as high as 67% in Mongolia [5], more than 60% in Africa, and 40% in indigenous Australian women [6]. *T. vaginalis* adheres to and damages vaginal epithelial cells [7, 8] and lives in the vagina of women and the urethra of men. In men, the infection is usually asymptomatic and self-limiting, although there may be an irritating urethritis or prostatitis [9, 10]. In women, the infection causes a wide range of inflammatory symptoms and has major health, economic and social consequences including preterm delivery, low birth weight, transmission of the parasite to newborn girls during delivery, infertility, cervical cancer and enhanced transmission rate of HIV [1, 4, 11–13].

The *T. vaginalis* virulence factors responsible for the remarkable evasion of the host immunity and the severity of the pathologic inflammatory reaction in trichomoniasis remain elusive. Although women may develop specific antibodies to *T. vaginalis* antigens, the infections do not provide lasting immunity, and reinfection is common [1, 4]. Undefined phenotypic variations, release of proteases and other unknown factors have been attributed to the chronic nature and re-incidence of infections [1, 14], as well as the host inflammatory responses during infection [15, 16]. In other parasitic protozoa, cell surface glycoconjugates, *e.g.*, glycolipids, glycoproteins, and glycosylated phosphatidyl inositol (GPI) glycolipids have been implicated in cell adhesion, host cell invasion and evasion of host immune responses [17–20]. The importance of carbohydrate moieties in the pathogenesis of trichomoniasis is suggested by earlier studies showing that pretreatment of trichomonads with periodate abolishes the adhesion of parasites to their respective host cells [7, 21]. We isolated and partially characterized *T. vaginalis* lipophosphoglycan (LPG) and obtained evidence for its essential role in the parasite adherence and the proinflammatory activation of epithelial cells from all three distinct anatomic compartments of the lower human female genital tract—endocervix, ectocervix and vagina [22].

LPG is the most abundant glycosylated molecule in the pathogenic trichomonads *T. vaginalis* and *Tritrichomonas foetus* ($2–3\times10^6$ copies/parasite). *T. foetus* causes bovine trichomoniasis; this represents the only animal model for human trichomoniasis. *T. vaginalis* and *T. foetus* LPGs are anchored on the cell surface *via* a phospho-inositol ceramide (CPI) [23, 24]. LPG contains mostly carbohydrate and the lipid component, and is devoid of any peptide [22, 24, 25]. To a certain extent, trichomonad LPGs are analogous to the LPGs from other parasites, but the *T. vaginalis* and *T. foetus* LPGs are clearly distinct from them in their monosaccharide compositions and other structural features [22, 26, 27]. Unlike the LPG from *Leishmania* (which has distinct promastigote and amastigote stages), the LPGs from trichomonads (which have only one stage) do not undergo structural modifications during parasite development. Furthermore, unlike the previously described GPI-anchored molecules, TV-LPG contains no mannose and our earlier data has suggested the presence of the phospho-inositol linked ceramide [23, 24] and poly-*N*-acetyllactosamine repeats (Galβ1–4GlcNAc) in the LPG molecule [22, 26]. We previously demonstrated that trichomonad LPGs are involved in the adhesion of parasites to their respective host cells in a species-specific manner, a behavior which may be related to the unique monosaccharide compositions of *T. vaginalis* and *T. foetus* LPGs [21, 22]. Bastida-Corcuera *et al*. [28] produced *T. vaginalis* mutants defective in LPG and showed that these mutants have reduced adherence and cytotoxicity to cervical epithelial cells, confirming our own biochemical observations [21, 22].

Along with the partial compositional characterization of *T. vaginalis* LPG, we recently reported the first evidence for TV-LPG's role in triggering a selective and species-specific production of cytokines by human female genital tract epithelial cells [22]. Here we further define the biochemical nature of *T. vaginalis* LPG and determine the specific molecular domain required for triggering immunoinflammatory pathways in human vaginal and cervical epithelia.

Materials and methods

Parasites and LPG

T. vaginalis isolates (UR1) obtained from a symptomatic patient were cultured in Diamond's modified media (pH 6.0) with 10% heat-inactivated horse serum (HyClone Laboratory) at 37°C, as reported earlier [7]. Parasites were harvested in late log phase (24 h) by centrifugation and washed twice with phosphate-buffered saline (PBS, pH 7.4) and suspended in methanol/chloroform (1:2) followed by extraction of the LPG with solvent E [23]. LPGs from *T. vaginalis* and *T. foetus* were isolated and purified on an octyl-Sepharose column as previously described [23].

Generation of saccharides and ceramide phospho-inositol glycan core (CPI-GC) fractions of LPG

LPG was digested by various chemical and enzymatic treatments and the resultant sub-domains presented sche-

matically in Fig. 1 were purified and characterized as described below.

Mild acid-released fractions LPG was hydrolyzed with 100 mM TFA containing DTT (1 mg/mL) at 100°C for 3 h, under a N_2 atmosphere. The resultant sample was dried with 2-propanol and resuspended in a small volume of 0.1% TFA. The sample was placed on a C18 Sep-Pak® column (Waters Corp., Milford, MA, USA). Saccharides (outer branch) were eluted with 0.1% TFA and the CPI-GC was eluted sequentially with 30%, 50%, and 70% *n*-propanol. Aliquots from the outer branch saccharide fraction and the CPI-GC fraction were dried and later used to stimulate human cervicovaginal epithelial cells, as described below. Additional aliquots of the same fractions were dried and used for monosaccharide and MS analyses. SDS-PAGE followed by PAS staining was used to verify the release of the CPI-GC from LPG. For monosaccharide composition analysis, aliquots of the outer branch saccharide and CPI-GC fractions were hydrolyzed with 4 M TFA at 125°C for 1 h and the monosaccharides were separated and quantified on a PA1 column (Dionex Corp., Sunnyvale, CA, USA) by HPAE-PAD as reported earlier [22]. The CPI-GC fraction released by TFA treatment is referred to later as "native" CPI-GC in contrast to "post-enzymatic" CPI-GC derived by *endo*-β-galactosidase digestion of "native" CPI-GC.

Glycosidase treatment "Native" CPI-GC was treated with β-galactosidase (Jack bean), β-*N*-acetylhexosaminidase (Jack bean), and *endo*-β-galactosidase (*Bacteroides fragilis*) which were purchased from Oxford Glyco Systems (Oxford, UK). In a few experiments, *endo*-β-galactosidase (*Escherichia freundii*) was also obtained from Seikagaku Corp. (Tokyo, Japan; Associates of Cape Cod, Inc., East Falmouth, MA, USA). The incubation conditions recommended by the manufacturers were employed in a total volume of 0.2 mL. The released saccharide(s) and the "post-enzymatic" CPI-GC (glycan lipid core) were separated on a C18 Sep-Pak as described above. The saccharides were labeled with the fluorophore, 8-aminonapthalene-1,3, 6-trisulfonic acid (ANTS), and subjected to Glyko-FACE analysis for sequencing, as described by the manufacturer. Selected individual bands were cut and purified by elution from the gel slices in H_2O and re-analyzed. The monosaccharide compositions of the released fractions were quantified by HPAE-PAD. Individual saccharide fractions were further analyzed by MALDI-TOF MS, MALDI-FTMS and LC/MS/MS.

The *endo*-β-galactosidase released saccharide fraction and the post-enzymatic CPI-GC fraction were used to stimulate human cervicovaginal epithelial cells as described below.

The following two enzymes were used to examine the release of Rha or Xyl from native LPG, CPI-GC and the mild acid released saccharide fractions: (1) rhamnosidase B from *Bacillus* Sp (specific for releasing terminal α-L(+) Rha from rhamnosyl disaccharides) obtained from Dr. K. Murata (Kyoto University, Japan) [29]; and (2) β-D-xylosidase from *Selenomonas ruminatium* (which catalyzes

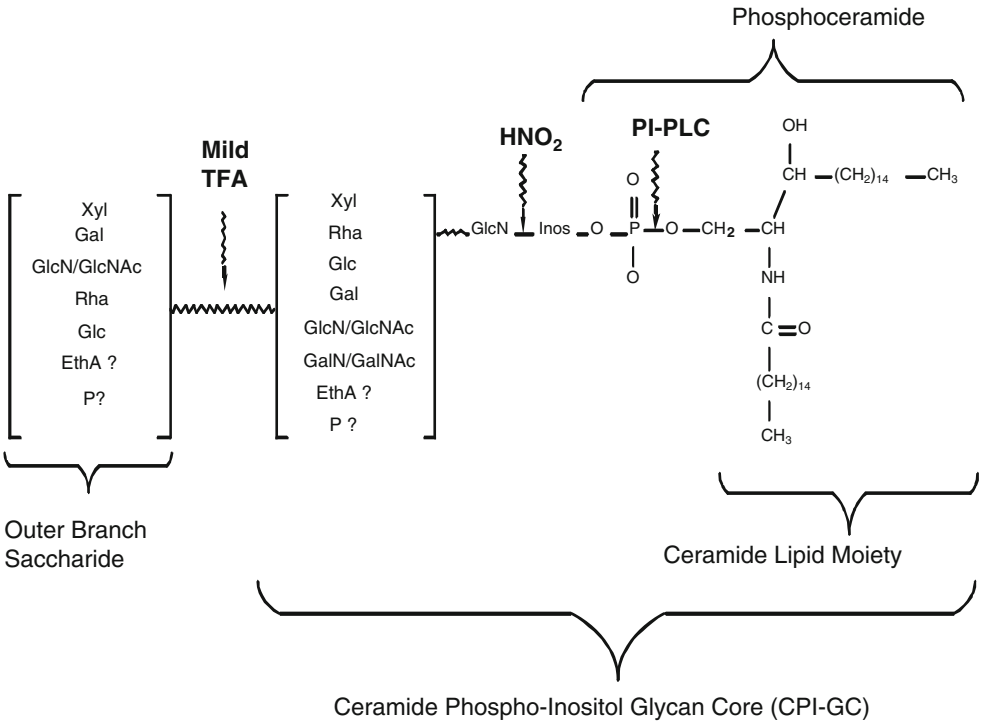

Fig. 1 Schematic of *T. vaginalis* LPG showing the major breakdown products *e.g.* outer saccharide branch and ceramide phospho-inositol glycan core (CPI-GC) cleaved by mild TFA hydrolysis

the hydrolysis of the β1–4 linkage of long polymers of xylose) kindly provided by Dr. D. B. Jordan (USDA, ARS, IL, USA) [30].

PI-PLC treatment of LPG To separate the lipid moiety from the oligosaccharide-inositol-phosphate core (saccharide-I-P) as previously shown [23, 24], intact LPG was treated with PI-PLC from *Bacillus thuringiensis* (obtained from PROzyme Corp., San Leandro, CA, USA) per the manufacturer's instructions for 20 h at 37°C. After the reaction was stopped with hot ethanol, the solution (which contained the complete non-lipoid saccharide) was dried and placed on C18 Sep-Pak and eluted with 0.1% TFA. The eluant which contained the oligosaccharide-I-P, *i.e.*, the LPG devoid of the ceramide moiety, was collected and dried and was later subjected to functional analysis in stimulation of cervicovaginal epithelial cells, as described below.

Periodate treatment Intact LPG was treated with periodate (10 mM) in sodium acetate buffer pH 4.5 for 4 h, as described [7, 21] and the reaction was stopped by the addition of excess glycerol to destroy unreacted periodate. The reaction mixture was dried and dissolved in 0.1% TFA. It was further purified on a C18 Sep-Pak column and the lipid-containing fraction that was eluted with *n*-propanol (30–70%) was collected and examined to determine its effect in upregulation of cytokines in our experimental human cell model.

Mass spectrometry

CPI-GC The mass of the native CPI-GC was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Reflex IV mass spectrometer (Bruker Corp., Billerica, MA, USA), using a UV laser (337 nm, 3-ns pulse width) and 2,5-dihydroxybenzoic acid as the matrix for analysis of samples deposited on a stainless steel target. The data were acquired using Flexcontrol, and were displayed using MoverZ freeware (Proteometrics, LLC; now within Genomic Solutions, Ann Arbor, MI, USA).

Glycans The sugars released by mild acid or enzymes, were analyzed (without derivatization) by MALDI-TOF MS and PSD of the positive ions on a Vision 2000 reflectron TOF MS [Finnigan Corp. (now Thermo-Fisher Scientific), Bremen, Germany] [31] and also by MALDI-FT MS with SORI-CID on an Ion-Spec system (IonSpec Corp., Irvine, CA, USA; now Varian FTMS, Lake Forest, CA, USA), with an external MALDI source and a 7-T actively shielded magnet. For both types of MALDI-MS analysis, the matrix was 2,5-DHB. FTMS data are displayed using the BUDA open source software developed by P. B. O'Connor at Boston University Center for Biomedical Mass Spectrometry and available on its websites (www.busm.bu.edu/msr and www.busm.bu.edu/cardiovascularproteomics).

To increase sensitivity and provide more informative fragmentation, the released glycans were permethylated [31, 32] and further characterized by MALDI-TOF MS, and MALDI-FTMS. For both types of MALDI analysis of the permethylated glycans, 2,5-DHB was used as the matrix. In later experiments, nanospray MS/MS, or capLC-MS/MS was employed for samples that were reduced with sodium borohydride (to avoid chromatographic separation into two anomeric forms) then purified with a HyperSEP Hypercarb column (Thermo-Fisher Scientific, Waltham, MA, USA) and permethylated. These derivatized glycans were analyzed online with a QStar Pulsar *i* QoTOF (quadrupole orthogonal time-of-flight) MS (Applied Biosystems/Sciex, Toronto, ON, Canada), using protocols we have described recently [32]. Samples were introduced *via* self-pulled glass-tips, or Thermo Hypersil–Keystone porous graphite chromatography (PGC) columns (2.1×50 mm) on a turbo spray ion source, and QAnalyst software [32]. Tandem MS experiments were performed on $[M+H]^+$ and $[M + Na]^+$ ions; several runs were repeated, excluding values up to *m/z* 900 from independent data acquisition to avoid solvent clusters. For LC/MS experiments, the flow rate through the analytical column was maintained at 30 μL/min through stepwise gradients of different lengths, ranging from 30% B to 100% B (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile/isopropanol 1:1) and an after-column syringe feed of 2 mL/min of 5% formic acid was employed. MS/MS spectra were interpreted using QAnalyst software and a self-made calculation table for reduced and permethylated glycans.

Epithelial cell lines and treatment protocols

Well established immortalized human vaginal, ectocervical and endocervical epithelial cell lines [22, 33–40], were grown in keratinocyte serum-free medium supplemented with 50 μg/mL bovine pituitary extract, 0.1 ng/mL epidermal growth factor, penicillin/streptomycin, and $CaCl_2$ as described [33]. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). The cell lines have been derived from normal endocervical, ectocervical, and vaginal epithelium and closely represent the phenotypes of their tissues of origin [33]. These cell lines produce distinctive repertoires of immunological mediators under basal, as well as stimulated conditions, in the absence of TLR4 expression [37] and maintain closely the phenotypic characteristics of their tissues of origin [40]. They have

been widely employed as *in vitro* models for studying cervical/vaginal physiology and infection [28, 34, 37–39, 41–44].

Confluent cell monolayers in 96-well plates were exposed to escalating doses of LPG, equivalent doses of LPG sub-domain fractions and live *T. vaginalis* for indicated period of time. Recombinant human IL-1β (R&D Systems, Minneapolis, MN, USA) served as a positive control for epithelial proinflammatory activation. Nontoxic experimental doses of LPG were selected based on MTT viability assays and ranged from 500 to 15 μg/mL. These doses were equivalent to 2.5×10^7–0.8×10^6 parasites/mL, or about 10^{-1} parasites/epithelial cell in our experimental model, which approximates parasite load during trichomonad infection [22]. The concentration of each LPG fraction was normalized based on the known amount of LPG used for the isolation of this fraction. At the end of each stimulation period, cell culture supernatants were collected for cytokines, and the cells were used for MTT viability assay (Promega, Madison, WI, USA) as described in detail elsewhere [22] or were lysed for total protein assessment by BCA assay (Pierce, Rockford, IL, USA) and phosphoprotein analysis.

Endotoxin measurement

Endotoxin contamination of LPG and LPG fractions was ruled out using the EndoSafe Test System (Charles River Laboratories, Charleston, SC, USA) based on the *Limulus Amoebocyte* Lysate test [45] with sensitivity <0.05 EU/mL. A negative control and the recovery of a known amount of endotoxin spike are inherent in each sample testing in this system. Each sample was tested in quadruplicate with a coefficient of variation <10%.

Measurement of inflammatory mediators and phosphoproteins

Electrochemiluminescence (ECL) immunoassays and Imager 2400 (both from Meso Scale Discovery, Gaithersburg, MD, USA) determinations were used to measure IL-8 in cell culture supernatants and phosphoproteins in cell lysates, as previously described [22, 46]. For IL-8 analysis, epithelial cells were stimulated for 24 h. For protein phosphorylation analysis, epithelial cells were stimulated for 30–90 min, then rinsed with ice-cold PBS containing 1 mM Na_3VO_4 (Sigma, St. Louis, MO, USA) and lysed in 20 mM Tris lysis buffer (100 μL lysate per 10^5 cells), containing the phosphatase inhibitors 2 mM PMSF and 0.01M NaF (Sigma) and Roche protease inhibitor cocktail (Fisher). The lysates were normalized to total protein concentration measured by BCA assay prior to analysis for phospho (p) extracellular signal-regulated ERK1/2 (Thr/Tyr: 202/204; 185/187), pNF-κB/p65 (Ser538) and pMEK (MAPK/ERK) 1 and 2 (Ser217/221).

Activation of nuclear factor (NF)-κB

The endocervical epithelial cell line End1/E6E7 was stably transfected with pHTS-NFκB firefly luciferase reporter vector (Biomyx Technology, San Diego, CA, USA) using the genejuice transfection method [22] and hygromycin selection (Biomyx). The reporter cell line was seeded at 1×10^5 cells/mL in 96-well plates and treated as described above. At the end of each stimulation period, cells were washed with PBS, lysed in Glo lysis buffer 0.1 mL/well and luciferase activity was measured using the Bright-Glo Luciferase Assay System per the manufacturer's instructions (Promega). The luminescence signal was read using Victor2 1420 multilabel microplate counter with Wallac software 2.01 (Perkin Elmer Life Sciences, Waltham, MA, USA).

Statistical analysis

One-way ANOVA with Dunnett's Bonferroni's multiple comparison tests or *t*-test analysis was performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). *P* values <0.05 were considered significant.

Results

Structural features of the LPG fragment

Previous data indicated the presence of terminal β1–4-linked Gal residue(s) [47] in the *T. vaginalis* LPG structure, based on lectin RCA-1 binding to intact LPG [47]. Because of the complexity of LPG, its polydisperse nature and the unusual monosaccharide compositions [22], we designed a strategy to separate outer-carbohydrate and glycan-lipid subdomains to further study their composition and functional importance. Figure 1 shows a schematic summary of current knowledge of the complex LPG molecule, representing the generation of various fragments and their compositions.

The fraction referred to as the outer branch in Fig. 1 was released by mild acid treatment, as reported for *Leishmania* LPGs [17–19]. The nature of linkage between the outer branch saccharide fraction and the CPI-GC that is cleaved by mild TFA is not known at this point. So far, the hydrolysis observed upon treatment with mild acid, indicates that phosphate linkage(s) connect the CPI-GC core and the TFA released fractions; the possible presence of other types of linkages is still under investigation. Glyko-FACE analysis of this outer-branch fraction revealed several saccharide fragments equivalent to a size range of 2 to more than 20 standard glucose units (data not shown).

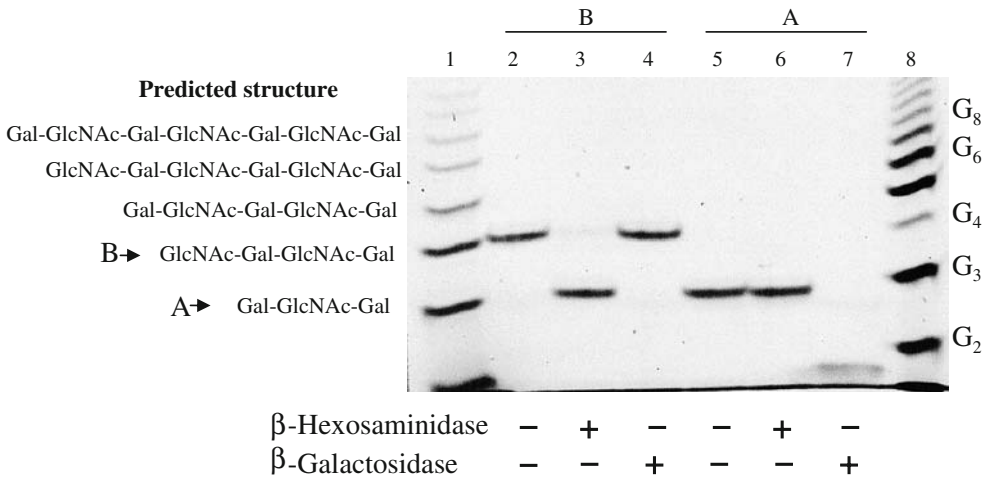

Fig. 2 FACE analysis and predicted structure of *endo*-β-galactosidase digest of *T. vaginalis* LPG. Lane *1*—total digest; lane *8*—standard glucose oligomers. Bands *B* and *A* (marked with *arrows*) from lane *1* were eluted and further digested with exoglycosidases shown in lanes *2–4* (band *B*) and *5–7* (band *A*)

This fraction was biologically inert, as shown below (Figs. 8, 9 and 10).

Individual treatments of intact TV-LPG with the exoglycosidases, β-galactosidase and β-*N*-acetylhexosaminidase resulted in release of Gal and GlcNAc, respectively, suggesting the presence of terminal β-Gal and β-GlcNAc. Release of these residues was verified by HPAE-PAD analysis. No monosaccharides were released from the intact LPG by treatment with any of the other glycosidases tested, including rhamnosidase and xylosidase.

Incubation of intact LPG with *endo*-β-galactosidase resulted in the release of four to five oligosaccharide fragments ranging in size from 2.8 glucose units to 6 glucose units as determined by Glyko-FACE analysis (Fig. 2). The band migrating at ~3.5 glucose units (Fig. 2, band B) contained saccharides with terminal GlcNAc while the band at ~2.8 glucose units contained terminal Gal. These data suggest that *endo*-β-galactosidase-released saccharides contain repeating lactosamine structures. The presence of poly-*N*-acetyllactosamine repeats was confirmed by MS analyses described below.

Mass spectral analyses of the saccharides released from intact LPG treated with *endo*-β-galactosidase indicated a wide range of heterogeneity and different degrees of glycan release (Figs. 3 and 4), whereas hydrolysis of LPG with mild TFA led to a diverse array of compounds, including free oligosaccharides. With either MALDI or ESI, the sequence of acid- and/or glycosidase-released glycan fractions could be clearly read from the MS/MS spectra, as demonstrated by the PSD spectrum (Fig. 3b) resulting from metastable decomposition of the $[M + Na]^+$ ions that appear at *m/z* 771 in the MALDI-TOF mass spectrum (Fig. 3a), of a saccharide released by *endo*-β-galactosidase treatment of intact LPG and in the ESI-CID QoTOF mass spectra of saccharides released by glycosidase or mild acid treatment of LPG, as shown in Figs. 4a and b, respectively. The MALDI-FTMS results (*e.g.*, Fig. 5 and Table 1) indicated that other oligosaccharides were rich in dHex (likely Rha based on monosaccharide analysis). Assignments for additional ions analyzed by MS/MS are underway.

The MALDI-TOF mass spectrum of the *endo*-β-galactosidase released glycans (Fig. 3a) had peaks at *m/z* 406, 568, 771, 933, 1,136 corresponding to the $[M + Na]^+$ of HexHexNAc, HexHexNAcHex, $(HexNAcHex)_2$, $(HexHexNAc)_2Hex$ and higher members of the series. The sequences of the glycan residues could be determined from the post-source decay spectra (*e.g.*, Fig. 3b). Post-source decay results in only glycosidic cleavages, and therefore the order of the residues can be determined, but information on the linkage sites is not available. Treatment with β-*N*-acetyl hexosaminidase changed the MALDI mass spectrum shown in the top panel to a simpler series (Fig. 3c) having peaks at 365-u intervals that correspond to HexHexNAc (likely *N*-acetyllactosamine multimers), at *m/z* 538, 933, 1,298, *etc*. The presence of glycan regions having extended *N*-acetyllactosamine repeats was later confirmed by nanospray MS/MS and capLC/MS/MS analyses of samples that had been subjected to reduction and permethylation. (The glycans were reduced before permethylation to avoid the peak splitting that would be introduced by resolution of α- and β-anomers during chromatographic separations.) Examples of the tandem mass spectra acquired during the LC/MS analysis of the reduced and permethylated glycans released by treatment of the intact LPG with *endo*-β-galactosidase are provided in Fig. 4. Figure 4a shows the CID MS/MS spectrum obtained for the glycan with $[M + Na]^+$ *m/z* 738.8 and Fig. 4b, the glycan with $[M + Na]^+$ *m/z* 1,187.6. In contrast to PSD, CID-MS/MS produces some cross-ring cleavages and thus furnishes information on the linkages [48]. In the tandem mass spectrum shown in Fig. 4, the $^{3,5}A_2$ and $^{3,5}A_4$ ions (B_1 + 88 and B_3 + 88, respectively) indicate Hex1–4HexNAc linkages, while the absence of such an indicating ion suggests the presence of 1–3 linkages between HexNAc and Hex.

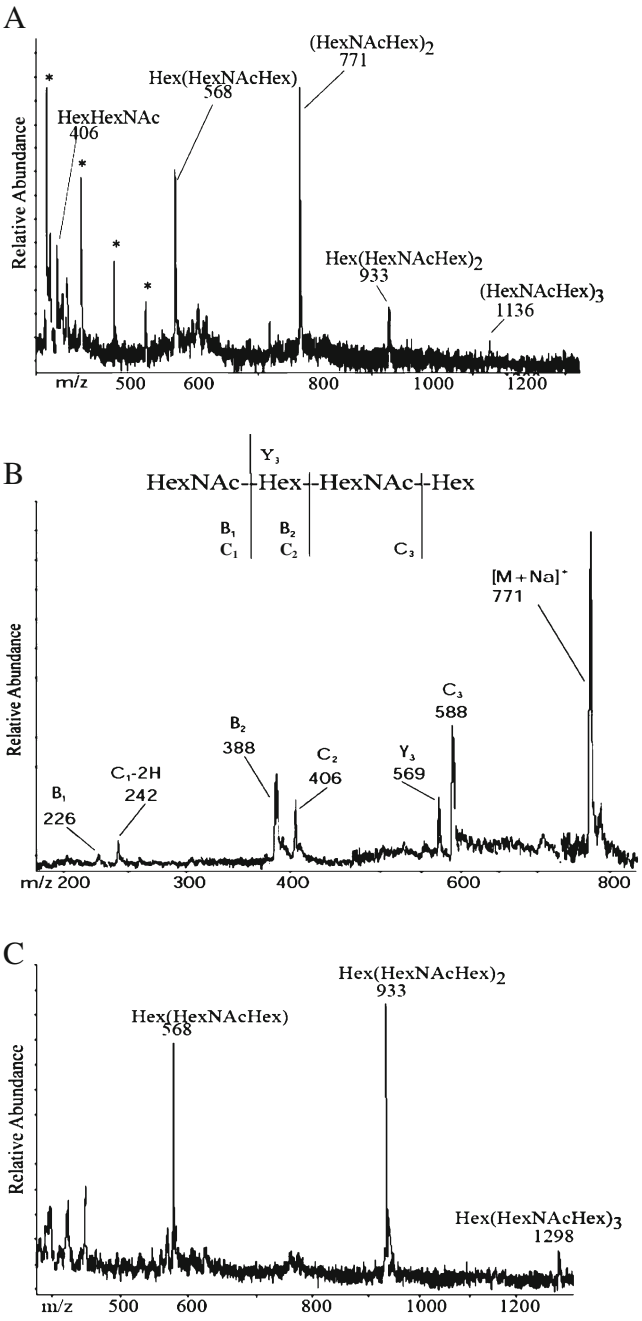

Fig. 3 **a** MALDI-TOF mass spectrum of the glycans released from TV LPG by *endo*-β-galactosidase. **b** PSD spectrum of the $[M + Na]^+$ *m/z* 771 in the spectrum shown above. All fragments except some of the peak centered at *m/z* 588 contain sodium. This peak likely contains contributions from the B_3(Na) fragment at *m/z* 587 and a C_3(H) fragment at *m/z* 591. **c** MALDI-TOF mass spectrum of the glycans released from TV-LPG by *endo*-β-galactosidase, after subsequent treatment with hexosaminidase

In addition to the poly-*N*-acetyllactosamine regions, other segments of the LPG glycan structure contain few amino-sugars but have multiple internal deoxyhexose residues (most likely rhamnose, based upon HPAE-PAD analysis). The compositions of the various components observed in the MALDI-FTMS spectrum of the native LPG glycans, present in such an acid-released fraction, are assigned in Table 1. MALDI-FTMS SORI-CID analysis of these LPG-derived oligosaccharides obtained after mild (100 mM) TFA hydrolysis indicated the presence of carbohydrates with a high content of deoxyhexose residues (Table 1). A typical tandem MS spectrum, obtained for $[M + Na]^+$ *m/z* 960, is shown in Fig. 5. This spectrum is consistent with the carbohydrate composition proposed on the basis of the accurate mass measurement given in Table 1, that assigns the peak at *m/z* 960.3578 to $HexNAc_1dHex_4Pent_1$ (calc. $[M + Na]^+$ *m/z* 960.3536). From the observed fragments (Fig. 5) it is possible to conclude that parallel losses of Pent(Xyl), HexNAc and dHex(Rha) are indicative of a branched structure and deoxyhexose residues are likely adjacent to one another. Loss of 74 u likely results from ^{3,5}X-type cleavage of a HexNAc (or similar cleavage within a Pent residue). Although there remains some ambiguity because of the redundant compositions of fragments from candidate structures, it is clear that extensive deoxyhexose residues are present in the molecule. While it is possible that rearrangements occur during fragmentation of native oligosaccharides, it seems likely that isomeric structures are present. Additional MS^n experiments, as well as NMR analyses, will be required to fully define all the potential structures.

Structural nature of the CPI-glycan core domain

Mild acid treatment also released the native CPI-GC core domain, which was isolated, treated and analysed as described in "Materials and methods". As elaborated below, the native and the *endo*-β-galactosidase treated CPI-GC moieties were largely responsible for chemokine production in human genital tract epithelial cells.

The native CPI-GC domain showed a heterogeneous band around 10–20 kDa when analyzed by SDS-PAGE analysis and PAS staining [22]. When analyzed by MALDI-TOF MS, the *m/z* 3,000–25,000 region of the mass spectrum showed only a single high mass peak centered at *m/z* 8,700 (Fig. 6). Compositional analysis of CPI-GC by HPAE-PAD showed the presence of significant amounts of Rha (25–30%), GlcN (38–43%), Gal (22–27%), Xyl (4–8%) and small amounts of GalN (~1% or less) and Glc (1–2%).

The monosaccharide compositional analysis of the entire CPI-GC fraction revealed large amounts of Gal, GlcN and Rha, further suggesting the presence of lactosamine types of saccharides in the CPI-GC molecule and the presence of Rha residues outside of the lactosamine repeats. This is consistent with the mass spectrometry data shown above. Xylose was also present in each of these fractions. In

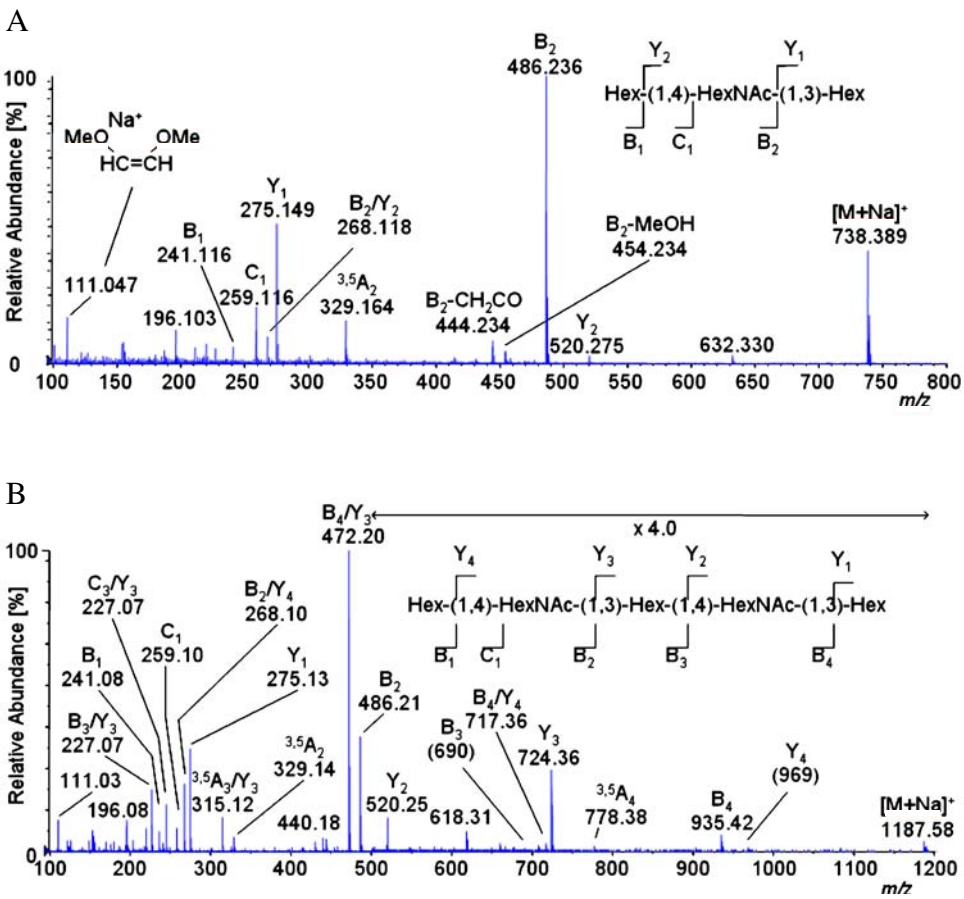

Fig. 4 Quadrupole orthogonal TOF CID MS/MS spectra obtained during **a** nanospray and **b** capLC-nanospray MS analysis of TV LPG-derived glycans with **a** $[M + Na]^+$ *m/z* 738.8, **b** $[M + Na]^+$ *m/z* 1,187.6. Putative structures of the $[M + Na]^+$ ions are shown for these major components from *T. vaginalis* LPG that were analyzed after release by *endo*-β-galactosidase or mild acid hydrolysis, reduction and permethylation. The component whose spectrum is shown in **a** was generated by both enzymatic and acid release; the component in **b** appeared in only the acid-released fractions

contrast, treatment with β-galactosidase, *N*-acetyl-β-hexosaminidase, rhamnosidase, and xylosidase failed to release any monosaccharides from native CPI-GC. After the native CPI-GC fraction was treated with *endo*-β-galactosidase, the released products were separated by C18-SepPak chromatography into a saccharide fraction and post-enzymatic CPI-GC. The released saccharide fraction was analyzed by Glyko-FACE (Fig. 7). The saccharides ranged in size from 2–6 Glc units, suggesting the presence of *N*-acetyllactosamine repeats; similar to the results obtained above (Fig. 2).

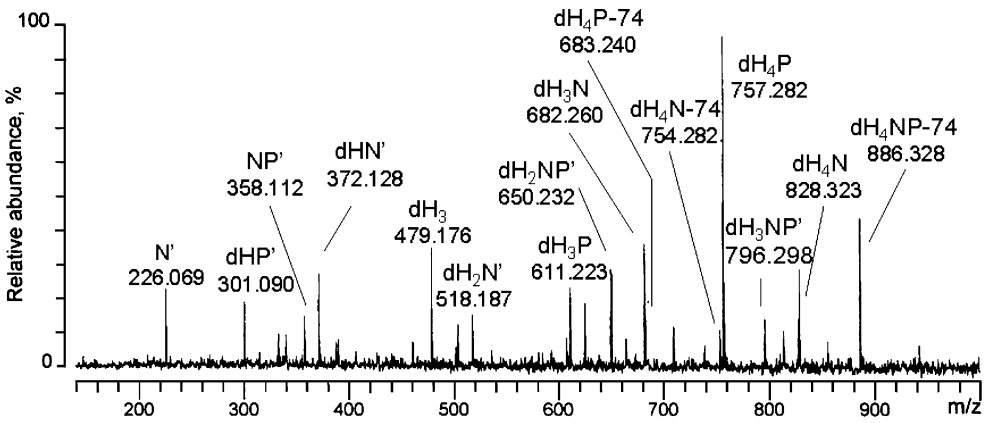

Fig. 5 SORI-CID mass spectrum obtained for the $[M + Na]^+$ *m/z* 960 in the MALDI-FTMS spectrum of oligosaccharides that were released from TV LPG by mild acid hydrolysis (outer branch saccharide). Compositions are indicated as *dH*, deoxyhexose; *N*, *N*-acetylhexosamine; *P*, pentose. Ions marked with an *apostrophe* (') are B- (or Z)-type ions, the rest are Y- (or C)-type ions; loss of 74 u most likely originates through elimination of $CHOHCHCH_2OH$ from a nonreducing HexNAc (or Pent) terminus of the intact glycan or cleavage of an internal HexNAc residue that has 1,4-linkage, to form the $^{3,5}X_n$-series. Low *m/z* ions are internal ions. All ions contain Na

Table 1 FTMS analysis of native TV-LPG glycans from an acid-released fraction

m/z obs	*m/z* cal	Composition				
		HexNAc	Hex	DeoxyHex	Pent	Na
668.2396	668.2378	1		2	1	1
682.2558	682.2534	1		3		1
814.2988	814.2957	1		3	1	1
828.3138	828.3113	1		4		1
960.3578[a]	960.3536	1		4	1	1
974.3745	974.3693	1		5		1
1,031.3954	1,031.3907	2		4		1
1,106.4177	1,106.4115	1		5	1	1
1,163.4399	1,163.4330	2		4	1	1
1,177.4550	1,177.4486	2		5		1
1,238.4660	1,238.4538	1		5	2	1
1,252.4742	1,252.4694	1		6	1	1
1,268.4706	1,268.4643	1	1	5	1	1
1,295.4867	1,295.4752	2		4	2	1
1,309.4997	1,309.4909	2		5	1	1
1,323.5140	1,323.5065	2		6		1
1,325.4916	1,325.4858	2	1	4	1	1
1,366.5244	1,366.5123	3		4	1	1
1,398.5367	1,398.5273	1		7	1	1
1,441.5433	1,441.5331	2		5	2	1
1,455.5592	1,455.5488	2		6	1	1
1,469.5767	1,469.5644	2		7		1
1,471.5509	1,471.5437	2	1	5	1	1
1,512.5827	1,512.5703	3		5	1	1
1,601.6171	1,601.6067	2		7	1	1
1,644.6239	1,644.6125	3		5	2	1
1,658.6481	1,658.6282	3		6	1	1
1,733.6598	1,733.6490	2		7	2	1
1,747.6743	1,747.6646	2		8	1	1
1,804.7018	1,804.6861	3		7	1	1
1,806.7003	1,806.6653	3	1	5	2	1
1,865.6970	1,865.6912	2		7	3	1

[a] The composition of *m/z* 960.3578 was confirmed by the SORI-CID MS/MS analysis shown in Fig. 5

Functional analysis of LPG fragments and CPI-glycan core domains

To determine the biological role(s) and relative importance of the outer branch saccharides and the CPI-GC domains in the pathogenic epithelial responses to trichomoniasis, we compared LPG and fractions released by periodate, mild acid (TFA), *endo*-β-galactosidase and PI-PLC (see Fig. 1) to a nontoxic biologically active dose of the proinflammatory cytokine IL-1β (25 ng/mL) and to infection with live trichomonads.

The effects on epithelial cell viability following 24-h exposure to various LPG sub-domains and intact LPG were assessed as percent survival rates versus untreated cell cultures (Fig. 8). IL-8 production was measured in the cell culture supernatants in parallel to the MTT viability assays (Fig. 9). Intact LPG was nontoxic to both vaginal and endocervical epithelial cells at doses <250 μg/mL and at the same time significantly increased IL-8 production in a

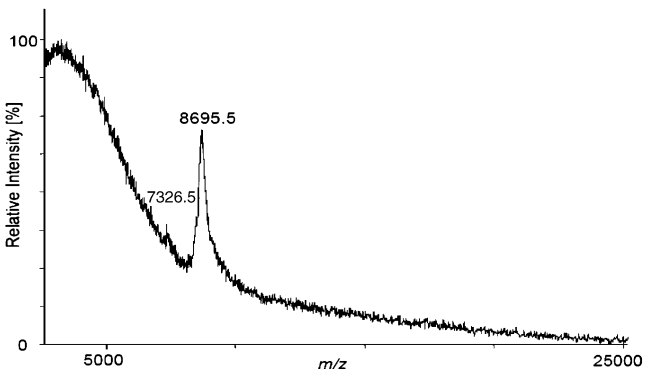

Fig. 6 MALDI-TOF mass spectrum of the CPI-GC released by mild acid treatment of the TV-LPG, plotted over the range *m/z* 3,000–25,000

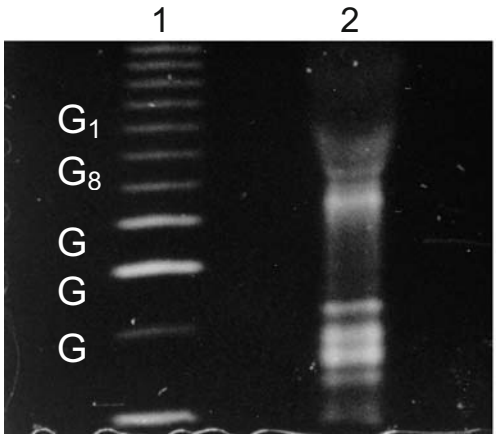

Fig. 7 FACE Analysis of *endo*-β-galactosidase treated CPI-GC. The CPI-GC was treated with *endo*-β-galactosidase for 18 h and the released saccharides were purified by C18 Sep-Pak and derivatized with ANTS for FACE analysis. Lane *1*—standard glucose oligomers; lane *2*—CPI-GC digest

dose-dependent manner (Figs. 8a–b and 9a). Periodate treated LPG was nontoxic even at higher doses (>500 μg/mL) (Fig. 8a–b) and completely lost its proinflammatory properties (Fig. 9a). The native CPI-GC fraction was as active as intact LPG. IL-8 production was induced by nontoxic doses of LPG and CPI-GC estimated to be the equivalent of ~4–10 parasites per epithelial cell (Fig. 9a–b). In contrast, the outer branch fraction, and the PI-PLC-released saccharide-I-P (see Fig. 1) were biologically inert (Figs. 8a–b and 9a). Also, inositol-P-ceramide (CPI) isolated from *T. vaginalis* (24) failed to show any biological activity (data not shown). The saccharide fraction released from native CPI-GC by *endo*-β-galactosidase had no biological activity but the remaining post-enzymatic CPI-GC fraction had strong proinflammatory activity (Figs. 8c and 9b). The biological specificity of this reaction was confirmed by the lack of IL-8 induction by equivalent doses of native CPI-GC from the bovine trichomonad and by the ethanol diluent (EtOH). Intact LPG and the CPI-GC fractions induced IL-8 production commensurate with that induced by a high dose recombinant IL-1β (Fig. 9a–b).

The intact LPG molecule and the native CPI-GC fraction induced NF-κB activation comparable in magnitude to that induced by estimated equivalent load of live *T. vaginalis* (~5–10 parasites per epithelial cell) (Fig. 10a) and rapid (30–120 min) phosphorylation of NF-κB p65 subunit at Ser536 (Fig. 10b). Within 30 min of stimulation, the intact LPG and CPI-GC induced phosphorylation of MEK1/2 kinase (Fig. 10c) and within 1 h—pERK1/2 (Fig. 10d). Levels of total ERK and MEK1/2 remained unchanged (data not shown). Neither the saccharide fractions nor the bovine CPI-GC induced MEK1/2, NF-κB or ERK activation (data not shown).

Discussion

This study is the first to demonstrate that *T. vaginalis* LPG contains poly-*N*-acetyllactosamine (Galβ1–4 GlcNAcβ1–3 Galβ1–4GlcNAc) repeats and the first to reveal the relative biological significance of various LPG domains in triggering inflammatory cascades in vaginal epithelial cells.

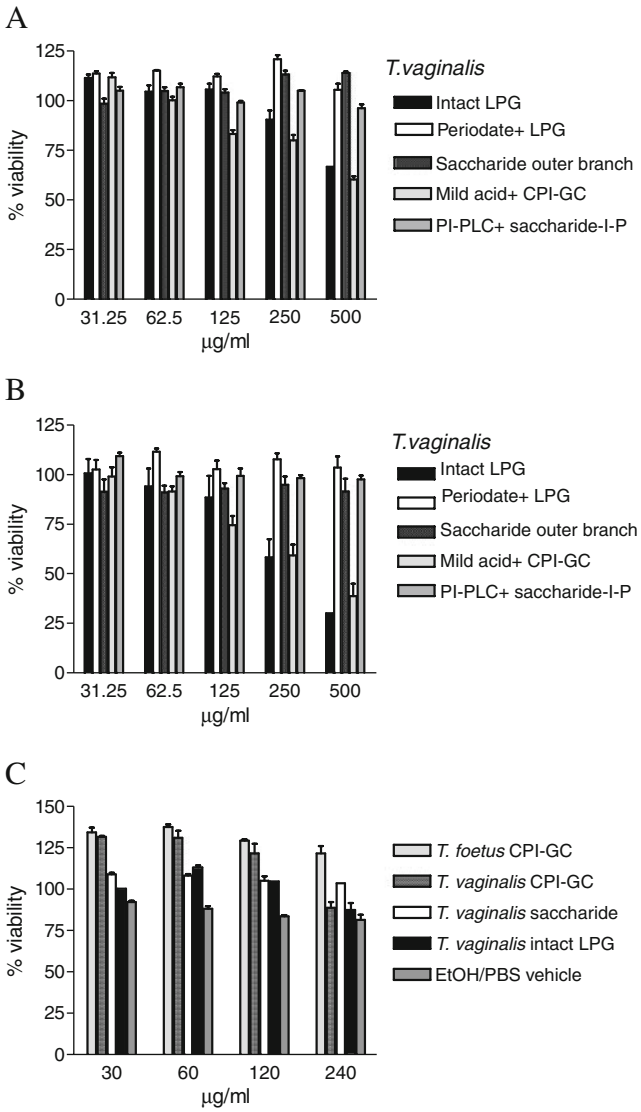

Fig. 8 Effects of LPG fractions on epithelial cell viability measured by MTT assay. Endocervical (**a**) and vaginal (**b** and **c**) epithelial cells were exposed for 24 h to intact LPG or various LPG fragments and CPI-GC obtained after treatment with periodate, mild acid (TFA) or CPI-PLC (**a** and **b**). The CPI-GC and saccharide fractions shown in **c** were obtained after *endo*-β-galactosidase treatment of the native CPI-GC. Stock solutions of lyophilized lipid fractions were prepared in PBS/ethanol (EtOH) and of saccharide fractions in PBS. Recombinant human (rh) IL-1β served as positive control. *Horizontal axes* present two-fold serial dilutions of LPG and fractions at equivalent concentrations. *Bars* represent means and SEM from triplicate cultures in one of three independent experiments with each cell line

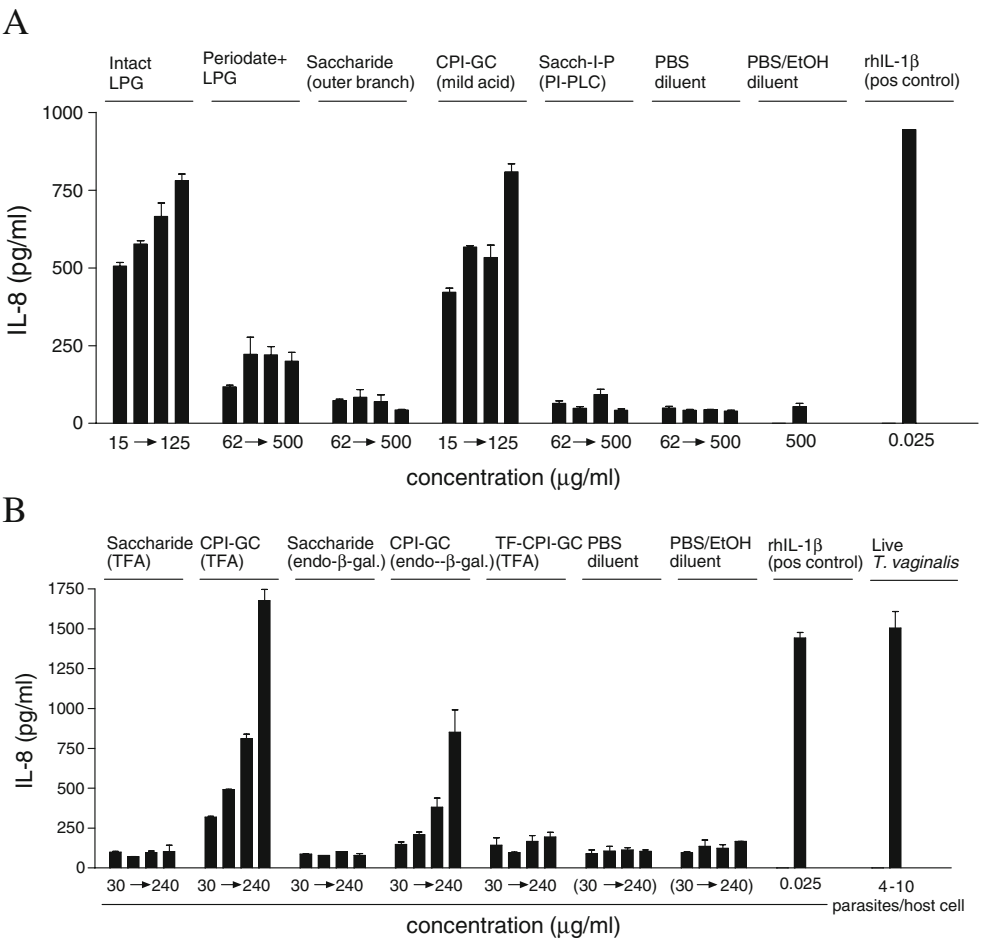

Fig. 9 Dose-dependent upregulation of IL-8 after 24 h stimulation of vaginal epithelial cells with intact *T. vaginalis* LPG and various LPG fractions in two-fold serial dilutions. Controls included the diluents PBS and PBS/ethanol (EtOH), *T. foetus* (TF) LPG CPI-GC, live *T. vaginalis* (four to ten parasite per epithelial cell) and recombinant human IL-1β. *Bars* represent the mean and SEM of duplicate cultures in one of two experiments performed with each set of fractions (**a** and **b**)

Several bioanalytical techniques such as enzymatic digestion, FACE, MS and tandem MS analyses were employed to derive the lactosamine structures in LPG and its CPI-GC core. The sensitivity of LPG and native CPI-GC to *endo*-β-galactosidase digestion, with the production of oligosaccharides that could be further digested with β-*N*-acetylhexosaminidase and β-galactosidase, was consistent with the presence of lactosamine repeats in the outer saccharide branch. Furthermore, the fragmentation patterns obtained in mass spectral analyses confirmed the results of the enzymatic digestions. The identity of terminal Gal and GlcN was derived from enzymatic, HPAE-PAD, and FACE analyses. In addition, the CPI-GC domain derived from native CPI-GC by *endo*-β-galactosidase treatment (see Fig. 7) may retain some of the poly-*N*-acetyllactosamine-type structures.

The poly-*N*-acetyllactosamine structures are unique in a lipid-linked phosphoinositol-anchored molecule. It is of interest to note that all reported GPI-molecules have a glycan core containing the sequence Manα1–4GlcNα1-Ino [49] but *T. vaginalis* LPG contains no Man residues. In the past, Dionex chromatographic program was used to separate monosaccharides by the PA1 column which did not adequately separate Man from Xyl and the observed peak was attributed to the presence of Man (not Xyl), since this monosaccharide would have been expected for a molecule containing a GPI anchor. However, we later determined by two different methods (GC/MS and HPAE-PAD) that LPG does not contain Man, but rather it contains Xyl [22, 27]. *T. vaginalis* LPG and its CPI-GC appear to be unique surface glycolipids in that they express certain features of a prokaryotic glycoconjugate, *e.g.* large amounts of rhamnose. Even though *T. vaginalis* is a eukaryote, LPG is not anchored to the parasite surface by a conserved eukaryotic GPI-anchored molecule. Another important discovery is the presence of significant amounts of Rha, Gal and GlcN along with Xyl in the LPG and the CPI-GC core.

The presence of Rha and Xyl in each fraction (mild acid derived saccharides and CPI-GC) hindered the acquisition of glycosidase-based oligosaccharide sequence information. Rhamnosidase and xylosidase were unable to release Rha/Xyl, which suggests that the Rha and Xyl residues are not terminal and/or they do not have linkages that are susceptible to the available glycosidase enzymes. The limited ability of β-galactosidase and β-*N*-acetylhexosaminidase to release monosaccharides may be due to the presence of Rha and Xyl in each fraction and the presence of branches. This conclusion is supported by MS analysis, which confirmed the presence of various deoxyhexose

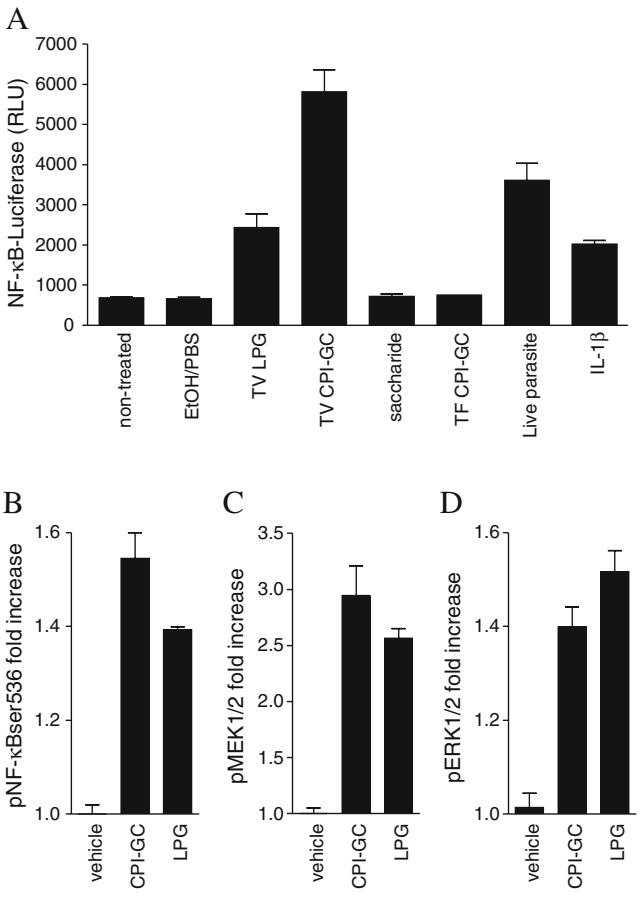

Fig. 10 Activation of NF-κB and ERK1/2 in endocervical (**a**) and vaginal (**b**–**c**) epithelial cells. Endocervical epithelial cell line stably transfected with pNF-κB lucifearse reporter (**a**) was exposed to intact TV and TF LPG, equivalent dose of native TV CPI-GC fraction and live *T. vaginalis* (four to ten parasite per epithelial cell), and rhIL-1β (25 ng/mL) for 24 h. *Bars* represent the means and SEM of luciferase activity in relative luminescent units (RLU) measured in triplicate cultures in two experiments. Vaginal epithelial cells were assessed for pNF-κB ser536 **b** at 2 h, pMEK1/2 **c** at 30 min and pERK1/2 **d** at 1 h post stimulation with TV LPG or equivalent dose (60 μg/mL) of CPI-GC. *Bars* represent means + SD from triplicate cultures in two experiments

residues (Rha based on monosaccharide analysis) in the backbone of the LPG molecule and pentose branches. These residues appear to be located outside of the regions containing *N*-acetyllactosamine repeats and may block β-galactosidase and β-*N*-acetylhexosaminidase digestion. Determination of the detailed structures of these branched regions will require higher order MS analyses, in combination with NMR studies.

The discovery of poly-*N*-acetyllactosamine repeats in LPG and CPI-GC opens interesting possibilities for novel LPG–host interactions. For example, LPG and its CPI-GC core may serve as ligands for β-galactoside-binding lectins such as various members of the galectin family. Galectins are expressed in several cell types, including epithelial cells, and are known to play vital roles in host-pathogen interactions and immune recognition [50–52]. In fact, our recent data suggest that galectin-1 and -3 are expressed in the human ectocervical, endocervical and vaginal epithelial cells used in our experimental systems [53].

We showed earlier that *T. vaginalis* LPG is the predominant molecule anchored to the parasite surface [23, 24]. It is highly glycosylated, contains a lipid-linked phosphoinositol-type anchor [22, 23] and that it plays a significant role in the parasite adhesion and proinflammatory activation of epithelial cells originating from the three anatomic regions of the human lower female genital tract—endocervical, ectocervical and vaginal [22]. We showed that LPG induced a marked dose-dependent upregulation of the chemokines IL-8 and MIP-3α and a moderate increase of the cytokine IL-6 in a dose-dependent and species-specific manner, whereas IL-1β and TNFα levels in the same cultures remained at baseline. It is important to note that these cells are deficient in the molecular machinery for bacterial endotoxin responses [37], which, along with the lack of bacterial contamination confirmed by endotoxin-negative testing of the purified LPG and fractions, suggests specific novel pathways of LPG signaling different from that utilized by bacterial LPS.

The results presented here clearly demonstrate a key role for the CPI-GC core of LPG in triggering inflammatory cascades in cervicovaginal epithelial cells. Importantly, LPG from the bovine parasite *T. foetus* showed no effect on cytokine production; this result demonstrates the species specificity of the cytokine and chemokine triggering mechanisms. Clearly, both the intact and the *endo*-β-galactosidase treated CPI-GC moiety from *T. vaginalis* are capable of activation of the major proinflammatory nuclear transcription factor NF-κB and production of IL-8. No other saccharide fraction had this effect. Multiple proinflammatory stimuli, *e.g.* endogenous cytokines, bacterial, viral and eukaryotic pathogens, activate NF-κB *via* a series of phosphorylation events. These events include its dissociation from the cytoplasmic inhibitor IκB and translocation to the nucleus, where NF-kB cooperates with other factors to transactivate a myriad of proinflammatory genes including IL-8 [54]. IL-8 is the most powerful chemoattractant for neutrophils and macrophages and its presence in the vaginal discharge is indicative of acute and chronic inflammatory conditions of the lower female genital tract [55–57]. In addition, IL-8 may be directly linked to increased HIV-1 replication and transmission in the genital tract mucosa [58, 59] and thus represents one possible mechanism for the increased HIV transmission incidence in women with trichomoniasis.

LPG and CPI-GC elicited phosphorylation of the NF-κB p65 subunit at Ser536. This phosphorylation event occurs after NF-κB dissociation from its cytoplasmic inhibitor IκB

and serves to slow down its export from the nucleus back to the cytosol, thus increasing the amplitude of NF-κB-induced gene transactivation. Ser536 phosphorylation also promotes NF-κB mediated transcription *via* recruitment of other transcription coactivators such as p300, enlarging the scope of proinflammatory activities [60]. LPG and CPI-GC also induced phosphorylation of ERK1/2, which is activated in macrophages upon trichomonad infection and linked to host cell apoptosis [61]. This is consistent with our findings that CPI-GC core (and not the outer branch saccharides) is responsible for the reduction of human vaginal and cervical cell viability.

Here we also demonstrate for the first time that *T. vaginalis* LPG signaling may be mediated by MEK1/2 phosphorylation. MEK1/2 has been implicated in the activation of the ERK family of protein kinases [62] and thus these data are in agreement with the downstream activation of ERK1/2 observed in response to LPG and CPI-GC.

Conclusions

The results presented in this paper suggest that, similar to the lipid A of LPS from Gram negative bacteria, the CPI-GC moiety of *T. vaginalis* LPG is important in regulating the host inflammatory response. In the context of normal human cervicovaginal epithelial cells, the immunoinflammatory activation appears to occur in the absence of the dominant LPS-responsive molecular machinery, including TLR4, MD2 and CD14. The loss of proinflammatory activity following periodate and PI-PLC treatments of LPG suggests the importance of both the lipid and glycan components of the CPI-GC in epithelial cell receptor binding initiating signaling cascades and downstream pathogenic responses. While the host receptors for any of the *T. vaginalis* surface structures are still unknown, the discovery of poly-*N*-acetyllactosamine repeats in LPG and CPI-GC opens the possibilities for interaction with the host galactoside binding lectins. Studies on pattern recognition receptors for LPG and its CPI-GC are underway to further define the role of *T. vaginalis* in mucosal immune regulation.

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