Glycobiology vol. 6 no. 5 pp. 507–515, 1996

Structure of the N-linked oligosaccharide of the main diagnostic antigen of the pathogenic fungus *Paracoccidioides brasiliensis*

Igor C.Almeida, David C.A.Neville[1], Angela Mehlert[1], Achim Treumann[1], Michael A.J.Ferguson[1], José O.Previato[2] and Luiz R.Travassos[3]

Disciplina de Biologia Celular, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP 04023–062, Brazil, [1]Department of Biochemistry, University of Dundee, Dundee, Scotland, UK, [2]Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil

[3]To whom correspondence should be addressed at: Disciplina de Biologia Celular, Universidade Federal de São Paulo, Rua Botucatu, 862/8° andar, São Paulo, SP 04023–062, Brazil

The major diagnostic antigen of *Paracoccidioides brasiliensis* is the exocellularly secreted 43,000 Da glycoprotein (gp43) which contains a single N-linked oligosaccharide chain. This oligosaccharide, although poorly immunogenic in man, is responsible for the cross-reactivity of the gp43 with sera from patients with histoplasmosis, and may have a role in fungal virulence. It contains a neutral high-mannose core ($Man_7GlcNAc_2$) to which a (1→6)-linked α-D-Man*p* chain of variable length, substituted at the 2-*O* positions by single α-D-Man*p* residues, is attached. A terminal unit of β-D-galactofuranose is (1→6)-linked to one of the (1→2)-linked mannosyl residues, either in the C or in the A arm of the oligosaccharide. The heterogeneity of the oligosaccharide is determined by the different sizes of the A arm and the sites of insertion of the β-galactofuranosyl unit. The complete structure was determined by methylation analysis, ^{1}H-NMR, mass spectrometry, acetolysis and mannosidase degradation. Electrospray mass spectrometry showed that the oligosaccharide comprises several subtypes ranging from $Hex_{18}GlcNAc_2$ to $Hex_{10}GlcNAc_2$ which accounts for the diffuse migration of the gp43 in polyacrylamide gels. The average size of the most frequent subtype is $Hex_{13.6}GlcNAc_2$. Dilute acid treatment to remove β-D-Gal*f* reduced the molecular masses of the majority of the subtypes by a single sugar unit.

Key words: Paracoccidioides brasiliensis/gp43/diagnostic antigen/N-linked oligosaccharide

Introduction

Paracoccidioides brasiliensis is a dimorphic pathogenic fungus that causes paracoccidioidomycosis (PCM), an infection that starts in the lungs but has a tendency to disseminate to any organ or system. The disease is prevalent in several Latin American countries. Owing to the polymorphism of the clinical picture, it can be misdiagnosed, mainly in females and youngsters (Mendes, 1994). Recently, an exocellular antigen of the yeast phase of this fungus has been used to detect with great sensitivity and specificity the presence of antibodies in patients with PCM (Puccia and Travassos, 1991a; Taborda and Camargo, 1993, 1994). This antigen is generally described as a 43 kDa glycoprotein, based on its migration in SDS–polyacrylamide gels (Puccia *et al.*, 1986, 1991). It is secreted into the medium by yeast cells growing in the logarithmic phase (Stambuk *et al.*, 1988). Although it can be hydrolyzed by papain and pronase (Puccia and Travassos, 1991b), it is more resistant to endogenous proteases than other *P. brasiliensis* proteins (Puccia *et al.*, 1991). The epitopes recognized by patients' antibodies are peptidic in nature and are conformationally restricted (Puccia and Travassos, 1991a; Gesztesi *et al.*, unpublished data). Sensitive and specific reactions can only be obtained with the gp43 in liquid phase, whereas on solid substrates (e.g., polysterene) the reactions are less specific and the antigen is also recognized by sera from patients with histoplasmosis and Jorge Lobo's mycosis although at much lower dilutions (Puccia and Travassos, 1991a; Travassos, 1994). The poor but still relevant reactivity of the gp43 with antibodies from patients with histoplasmosis has been attributed to the carbohydrate component of the molecule, since N-deglycosylation using different methods gave rise to a protein of 38 kDa, which strongly reacted with sera from patients with PCM but not at all with sera from patients with histoplasmosis. A high mannose structure for this oligosaccharide was suggested based on its strong affinity for concanavalin A (Puccia *et al.*, 1986) and sensitivity to endoglycosidase H (Puccia and Travassos, 1991b).

In the present work we have determined the structure of the N-linked oligosaccharide of *P. brasiliensis* gp43 antigen and found it to contain a branched high-mannose core ($Man_7GlcNAc_2$) and a (1→6)-linked outer chain, with terminal α-D-mannopyranosyl units and substitution at one end by a β-D-galactofuranosyl residue. This structure can be a source of cross-reactivity with antibodies from patients with other mycoses but, more importantly, it can influence the reactivity of the gp43 with complement and cells of the immune system thus playing a role in the infection itself.

Results

Enzymatic N-deglycosylation of the gp43

N-Linked oligosaccharides were obtained from the gp43 by deglycosylation with PNGase F which also converted the gp43 into an insoluble gelatinous precipitate that was removed by centrifugation. Analysis of pellet and supernatant by SDS–PAGE showed that the former contained most (>95%) of the deglycosylated polypeptide, with an apparent molecular mass of 38 kDa as previously described by Puccia and Travassos (1991a,b), using four different methods of deglycosylation. The treatment alone of gp43 with PNGase F, for the removal of the N-linked oligosaccharide, does not render the protein water-insoluble, unless the denaturated protein is heated (100°C, 10 min) and kept at –20°C, overnight, after the enzymatic treatment.

Composition and heterogeneity of the N-linked oligosaccharide

The N-linked oligosaccharide released by PNGase F treatment was further purified by 1-butanol extraction of the lyophilized

material, ion-exchange chromatography and Sephadex G10 gel filtration. The total N-linked oligosaccharides were analysed by GC-MS of the corresponding derivatized methanolysis products, showing the presence of Gal, Man and GlcNAc (1: 13:2). The same fraction was analysed by ES-MS, before and after mild acid hydrolysis (Figure 1). The neutral oligosaccharides were observed as double-charged pseudo molecular negative ions that were adducts with trifluoroacetic acid from the mobile phase (i.e., $[M + 2CF_3CO_2^-]^{2-}$). The cluster of pseudomolecular ions in Figure 1A indicated the presence of oligosaccharides ranging from $Hex_{10-18}GlcNAc_2$, with the major species being $Hex_{12-15}GlcNAc_2$. After mild acid hydrolysis in 2 M acetic acid, under conditions suitable for the cleavage of acid-labile Gal*f* bonds, there was a significant shift in the oligosaccharide pattern towards structures of lower molecular weight so that the major structures of the acid treated sample were $Hex_{11}GlcNAc_2$ to $Hex_{14}GlcNAc_2$, suggesting removal of one galactofuranosyl unit per oligosaccharide chain. The satellite ions associated with each $[M + 2CF_3CO_2^-]^{2-}$) ion in Figure 1B are due to the formation of acetyl esters from the acetic acid hydrolysis. The percentage of each molecular species and the oligosaccharide average size (in hexose units) before and after mild hydrolysis is shown in Table I. The native oligosaccharides showed an average size of 13.6 hexose units linked to $(GlcNAc)_2$. After removal of the Gal*f* residues the oligosaccharides had an average size of 12.6 hexose units.

The N-linked oligosaccharides were also labeled with 2-AB, before and after mild acid hydrolysis with TFA, and analysed by Bio-Gel P4 and Dionex HPAEC. The heterogeneity of the total fraction is apparent in Figure 2A (Bio-Gel P-4) and, especially, in 2B (Dionex). Two major species of the native oligosaccharides running as 16.2 and 15.2 GU, respectively, are observed in the Bio-Gel P-4 profile. When applied to Dionex, the total oligosaccharide fraction showed at least 26 species and isomers. A reduction in size of the fraction was obtained upon release by TFA of the Gal*f* residues as shown in Figure 2C and 2D. A major oligosaccharide with 14.2 GU agrees with a structure containing $Hex_{12}GlcNAc_2$. The considerable reduction in the number of molecular species in the TFA-treated oligosaccharide fraction probably resulted from the presence in the native material of different isomers, separable by Dionex chromatography, with residues of Gal*f* at different positions in the molecule. In the frequent subtype $Hex_{14}GlcNAc_2$ for instance, five isomers are possible with a single Gal*f* residue being attached to either one of the four 2-*O*-linked mannosyl side chains of the A arm, or to the (1→2)-linked mannosyl unit of the C arm. The remarkable simplification of the elution profile after removal of Gal*f* residues reflects the characteristic influence of this isomeric form on Dionex HPLC of neutral oligosaccharides. A shift of 4.0 Dionex units was observed, for instance, after mild-acid hydrolysis of a single residue of Gal*f* from a Gal*f*- (Man*p*)$_3$ oligosaccharide of *Leishmania donovani* (McConville and Blackwell, 1991).

Methylation analysis and ^{1}H- NMR spectra of the oligosaccharide

Methylation analysis of the total reduced fraction of oligosaccharide alditols (Table II) showed the presence of terminal nonreducing units of Gal*f* and Man*p*, of 2-*O*-, 6-*O*-, 3,6-di-*O*- and 2,6-di-*O*-substituted Man*p* residues, and also of 4-*O*-substituted N-acetylglucosamine and 4-*O*-substituted N-acetylglucosaminitol. Methylation analysis after mild acid treatment (and subsequent reduction to convert released Gal to Gal-ol) showed that most of the Gal*f* residues had been removed and that the proportion of 6-*O*-substituted Man units was reduced, with simultaneous increase in the number of terminal nonreducing units of Man*p*. These data indicate that the nonreducing terminal Gal*f* residues are attached to the underlying oligomannose structure via Gal*f*(1→6)-Man*p* glycosidic linkages. ^{1}H-NMR analysis of the oligosaccharide fraction confirmed the presence of α-Man (^{1}H-signals in the 5.0–5.15 ppm range) and β-GlcNAc residues (^{1}H signals at δ 4.631, and at δ 5.213 for the α-anomer of the reducing unit and at δ 2.066 and 2.090, for the N-acetamido protons; D_2O, 60°C). For comparison, the 4-*O*-substituted β-GlcNAc unit which is linked (1→3) to GalNAc in the *Axolotl mexicanum* oligosaccharide has a ^{1}H signal at δ 4.613 (D_2O, 27 °C) and that of the methylic protons at δ 2.079 (Plancke *et al.*, 1994). Signals at δ 4.898 and 4.793 confirmed the presence of 3,6-di-*O*-substituted α- and β-Man*p* units respectively, which are the main branch points of the oligosaccharides. Assignment to the ^{1}H signal of the terminal β-Gal*f* unit is more difficult because it appears in a region crowded with the mannose ^{1}H peaks (Figure 3A). Removal of this unit by partial hydrolysis, however, led to the disappearance of a signal at δ 5.086 with the concomitant increase of a signal at δ 5.136 which corresponds to the ^{1}H of nonreducing end-units of α-Man (Figure 3B). Moreover, the COSY spectrum (not shown) of the gp43 oligosaccharide showed that the H1-H2 coupling constant for the δ 5.086 signal is less than 2.5 Hz, which can only arise from a β-anomeric Gal*f* unit. The ^{1}H signal of β-Gal*f*(1→3)-linked to methyl α-mannopyranoside is at δ 5.13, $J_{1,2}$ 1.4 Hz (Vliegenthart *et al.*, 1983). The coupling constants ($J_{1,2}$) for α-anomers of Gal*f* are higher, for example, that of methyl-α-D-galactofuranoside is 3.7 Hz. That for the ^{1}H of α-Gal*f* (1→2)-linked to α-Man*p* (δ 5.21) is 4.7 Hz (Takayanagi *et al.*, 1992).

Partial acetolysis and enzyme digestion of the total oligosaccharide

The N-linked oligosaccharides were submitted to partial acetolysis and the products, digested or not by ASAM and JBAM α-mannosidases, were subsequently labeled with 2-AB and analysed by gel filtration in Bio-Gel P-4 (Figure 4). The major product of acetolysis (6.8 GU; 58%) is consistent with the Manα1–2Manα1–3Manβ1–4$GlcNAc_2$ structure. The 8.5 GU acetolysis product (31%) most likely represents a partial acetolysis product that retains one Manα1–6Man linkage [e.g., Manα1–2Manα1–3(Manα1–3Manα1–6)Manβ1–4$GlcNAc_2$–2-AB]. Support for these assignments comes from the combined acetolysis and ASAM-treatment experiment in which the 8.5 GU product gave rise to 7.6 GU and the 6.8 GU product to 5.9 GU, consistent with the removal of (1→2)-linked Man*p* units. As expected, acetolysis followed by JBAM treatment generated Manβ1–4$GlcNAc_2$ (4.9 GU) and monosaccharides (not shown). A reaction scheme with the 2-AB-labeled glycan fragments obtained by partial acetolysis and enzymatic hydrolyses of the gp43 N-linked oligosaccharides is given in Figure 5.

The probable structure of the most frequent N-linked oligosaccharide ($Hex_{14}GlcNAc_2$) of *P. brasiliensis* gp43 is depicted on Figure 6. It shows a structure with a 2-*O*-substituted (1→6)-linked mannopyranosyl outer chain of variable length (A arm) attached to the α1–3 branch of the high-mannose core as in *Saccharomyces cerevisiae* mannoproteins (Hernandez *et al.*, 1989; Ballou *et al.*, 1990). The possibility, however, that the

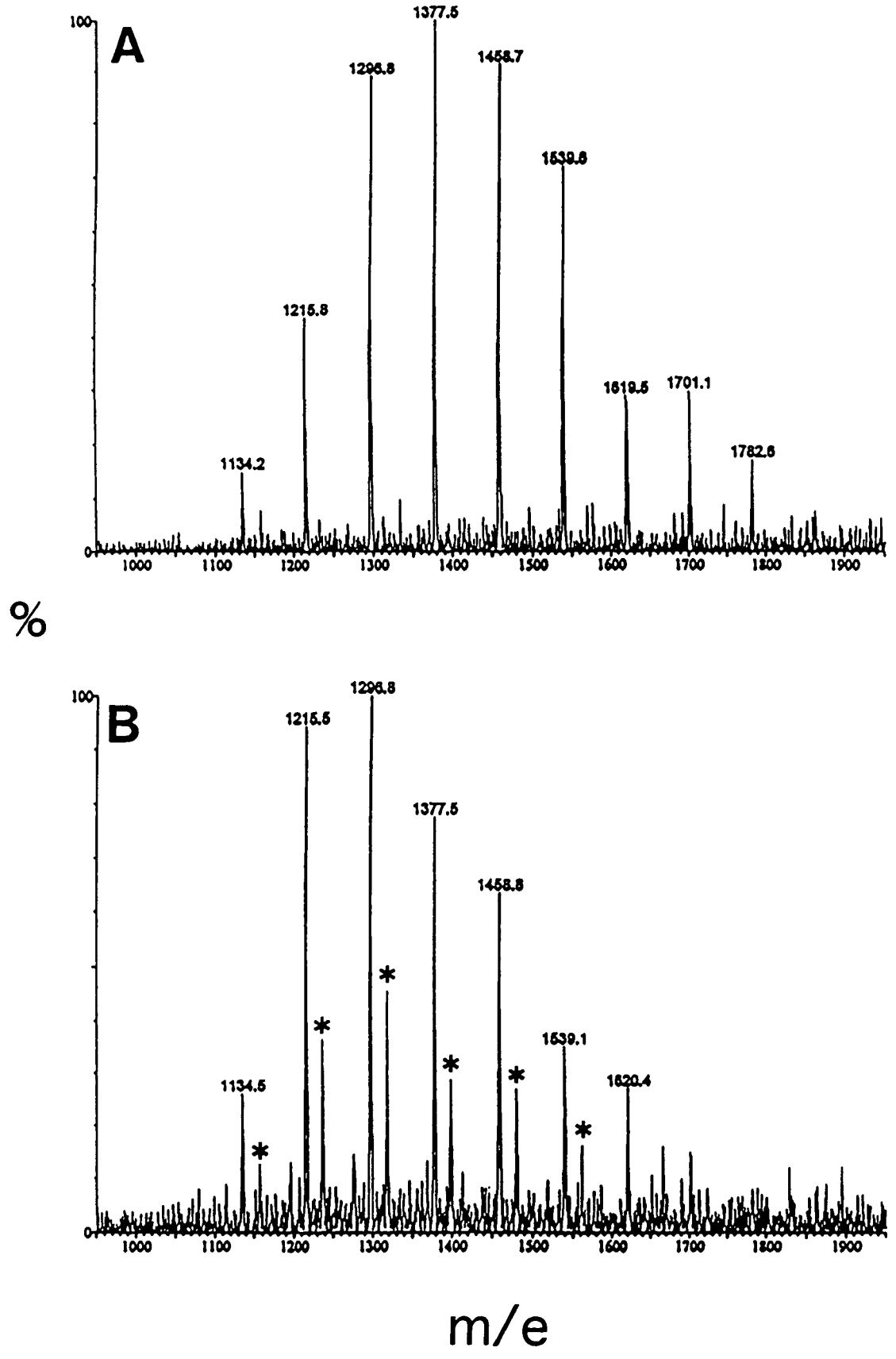

Fig. 1. ES-MS of the total N-linked oligosaccharides from the gp43 antigen, before (**A**) and after mild acid hydrolysis (**B**). Double-charged pseudo molecular negative ions are adducts with trifluoroacetic acid from the mobile phase. Oligosaccharides ranged from $Hex_{10}GlcNAc_2$ to $Hex_{18}GlcNAc_2$. In **B**, satellite ions (*) are acetyl esters arising from acetic acid hydrolysis.

Table I. ES-MS pseudomolecular ion species of the gp43 N-linked oligosaccharides before and after mild acid hydrolysis

Molecular species	$[M + 2CF_3CO_2]^{2-}$ Pseudomolecular ion (Da)	Molecular mass (Da)	Native		Acid-treated	
			%	(Hex U[a] × %)/100	%	(Hex U × %)/100
$Hex_{10}GlcNAc_2$	1134	2044	3.1	0.31	6.1	0.61
$Hex_{11}GlcNAc_2$	1216	2206	8.9	0.98	21.8	2.40
$Hex_{12}GlcNAc_2$	1297	2368	18.3	2.20	24.1	2.89
$Hex_{13}GlcNAc_2$	1378	2530	20.7	2.69	17.7	2.30
$Hex_{14}GlcNAc_2$	1459	2692	18.8	2.63	15.0	2.10
$Hex_{15}GlcNAc_2$	1540	2854	14.7	2.20	8.3	1.24
$Hex_{16}GlcNAc_2$	1621	3016	6.0	0.96	4.4	0.70
$Hex_{17}GlcNAc_2$	1702	3178	6.0	1.02	2.3	0.39
$Hex_{18}GlcNAc_2$	1783	3340	3.4	0.61	—	—
				13.6[b]		12.6[b]

[a]Hex U, number of hexose units of each molecular species.
[b]These values represent the average number of hexose units per oligosaccharide, before and after acid treatment, respectively.

outer chain is on the C arm cannot be excluded with the present data since the partial acetolysis products would be the same. The various positions where a terminal (1→6)-linked residue of β-Gal*f* can be attached are also shown.

Discussion

The N-linked oligosaccharide of *P. brasiliensis* gp43 contains the core structure of a high-mannose type ($Man_7GlcNAc_2$)

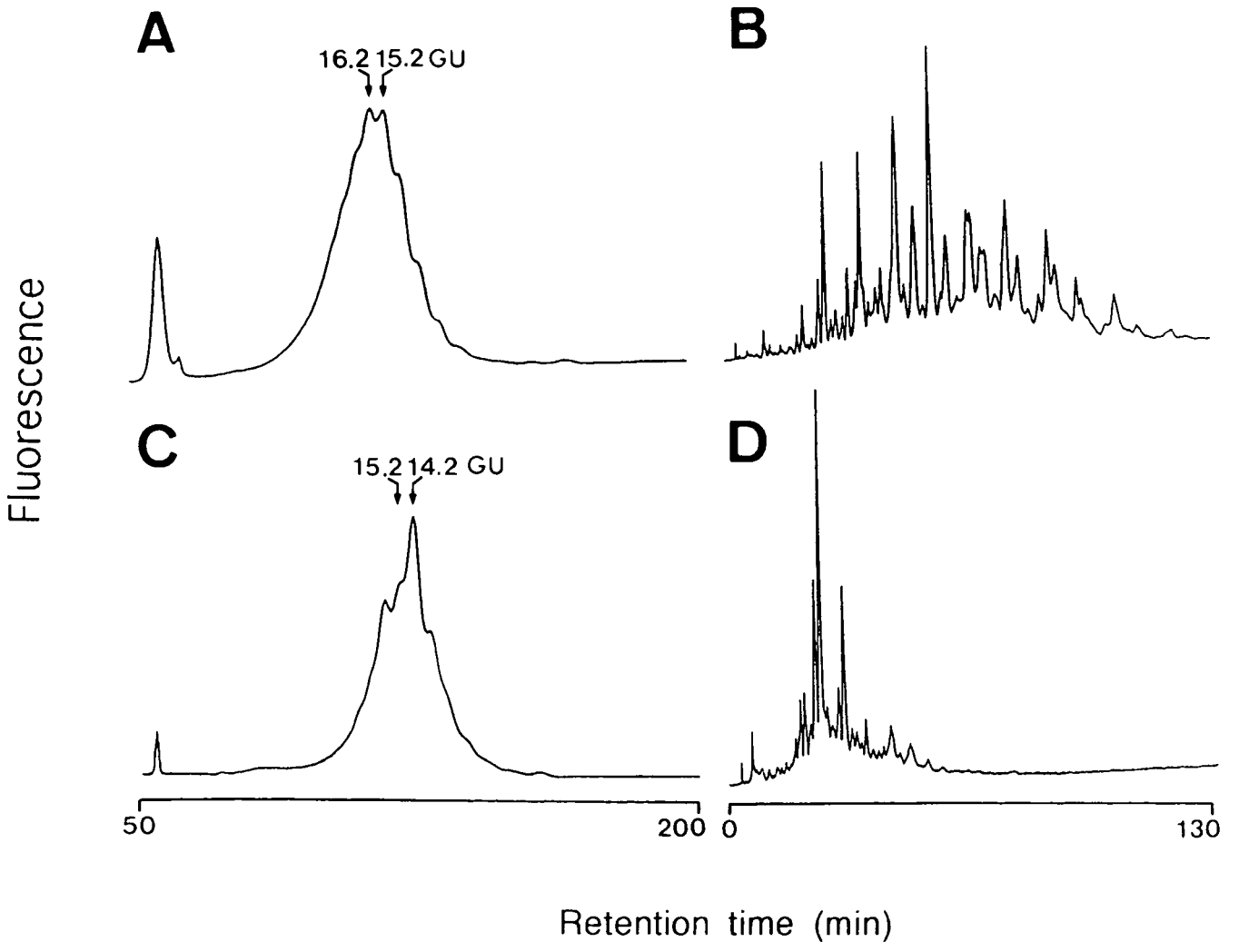

Fig. 2. Separation by gel-filtration in Bio-Gel P4 (**A** and **C**) and Dionex ion exchange chromatography (**B** and **D**), of the 2-AB labeled N-linked oligosaccharides before (**A** and **B**) and after partial acid hydrolysis to remove Gal*f* residues (**C** and **D**).

Table II. Partially methylated alditol acetates (PMAAs) derived from gp43 N-linked oligosaccharide alditols[a]

PMAA derivative	Origin	Total oligosaccharides	
		Native	Acid-treated
Galactitol			
2,3,5,6-tetra-*O*-methyl	Terminal Gal*f*	1.1	0.2
Mannitol			
2,3,4,6-tetra-*O*-methyl	Terminal Man*p*	4.5	6.2
3,4,6-tri-*O*-methyl	2-*O*-Substituted Man*p*	2.3	2.2
2,3,4-tri-*O*-methyl	6-*O*-Substituted Man*p*	1.8	0.8
3,4-di-*O*-methyl	2,6-di-*O*-Substituted Man*p*	2.9	3.2
2,4-di-*O*-methyl	3,6-di-*O*-Substituted Man*p*	2.1	2.3
NAc-Glucosaminitol[2]			
3,6-di-*O*-methyl	4-*O*-Substituted GlcNAc	0.7	0.8
1,3,5,6-tetra-*O*-methyl	4-*O*-Substituted GlcNAc-ol	0.4	0.4

[a]Values are molar ratios to permethylated *scyllo*-inositol (1.0).
[b]Partially methylated GlcNAc-ol derivatives are underestimated in the method used.

which derives from the conserved $Man_9GlcNAc_2$ precursor through the action of two α1–2 mannosidases (Figure 7). One is equivalent to the yeast specific mannosidase (Jelinek-Kelly and Herscovics, 1988) which removes the terminal (1→2)-

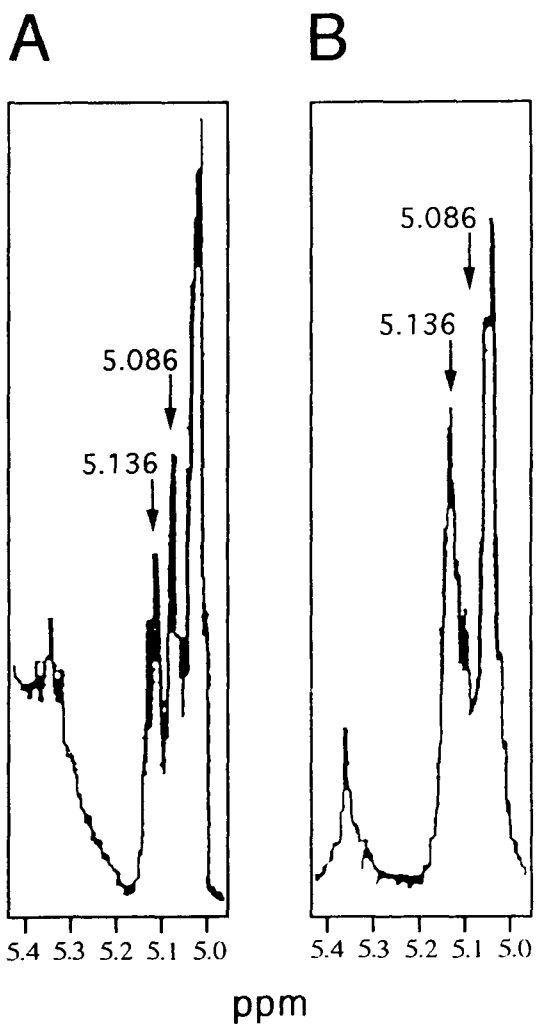

Fig. 3. ^{1}H-NMR spectra (H-1 region) of the N-linked oligosaccharide before (**A**) and after partial acid hydrolysis (**B**).

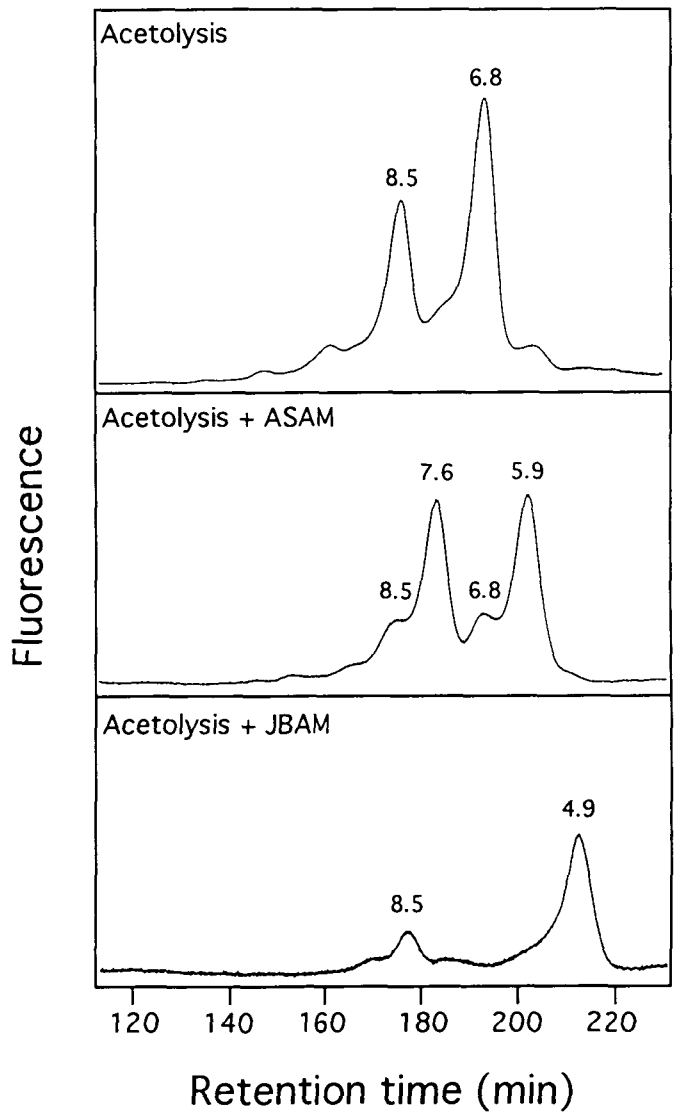

Fig. 4. Bio-Gel P-4 elution profiles of the glycan derivatives of the gp43 N-linked oligosaccharides obtained by partial acetolysis, enzymatic hydrolysis and 2-AB labeling. Numbers above the peaks represent the size of the glycans in glucose units (**GU**). Minor peaks represent residual undegraded oligosaccharides after enzymatic treatment. **ASAM,** *Aspergillus saitoi* α-1,2-mannosidase; **JBAM,** jack bean α-mannosidase.

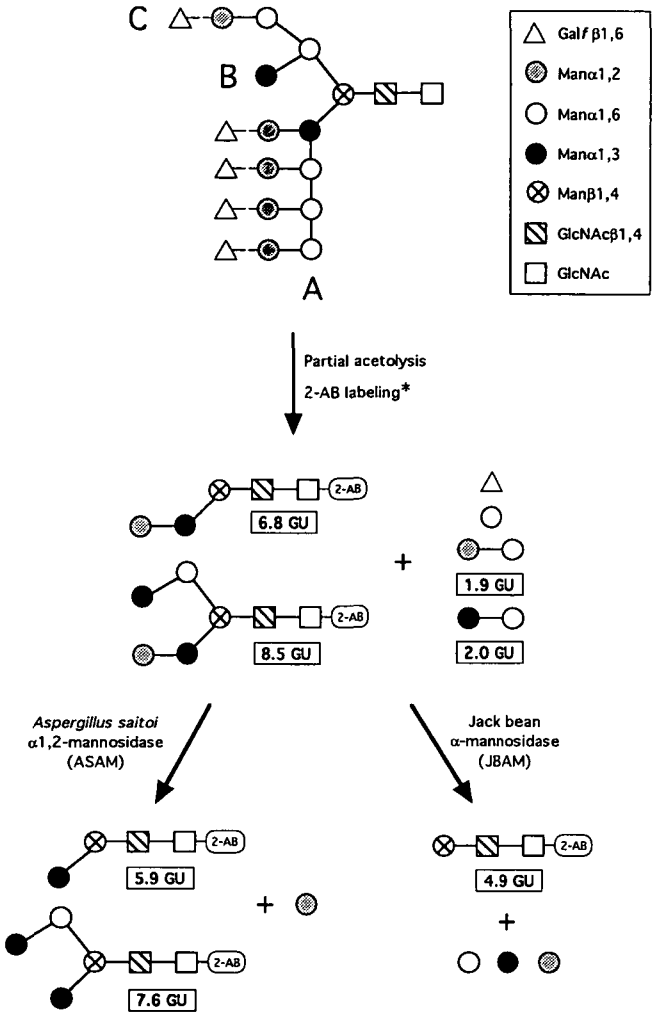

Fig. 5. Reaction scheme of partial acetolysis and enzymatic hydrolysis of the gp43 N-linked oligosaccharide. The GU values of the acetolysis products correspond to the following assignments: β-Gal*f*, 1.0 GU; α-Man*p* (1→6)-linked, 0.7 GU; α-Man*p* (1→2)-linked, 0.9 GU; α-Man*p* (1→3)-linked, 1 GU; β-Man*p* (1→4)-β-GlcNac (1→4)-GlcNAc, 4.9 GU. A single unit of β-Gal*f* (1→6)-linked to Man*p* is present per oligosaccharide molecule at one of the sites indicated (**dashed lines**). (*) Only the final products of acetolysis and acetolysis + enzymatic hydrolysis were labeled with 2-AB. 2-AB-Labeled mono and disaccharides were separately detected by paper-chromatography and analyzed by Bio-Gel P-4. Fragments of 1.0 GU (corresponding to free Gal*f* and Man*p* residues),1.9 GU (Manα1,2Man) and 2.0 GU (Manα1,3Man) were found (data not shown).

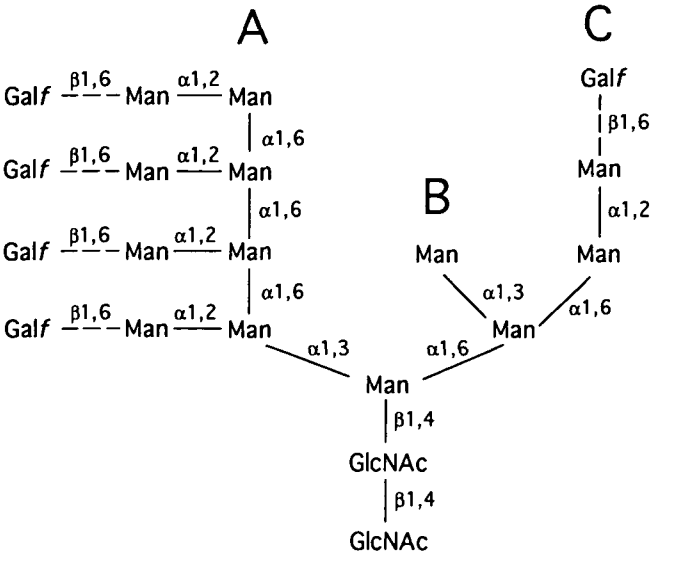

Fig. 6. Structure of the most frequent N-linked oligosaccharide ($Hex_{14}GlcNAc_2$) of the gp43. The possible sites of insertion of one β-galactofuranosyl unit per oligosaccharide molecule are indicated (dashed lines).

linked α-D-Man*p* residue from the B branch, giving rise to $Man_8GlcNAc_2$. The second mannosidase removes the terminal α-(1→2)-linked Man*p* unit from the α1–3 branch [Manα1–2Manα1–2Manα1–3Manβ1–4] giving rise to the $Man_7GlcNAc_2$ core, to which a (1→6)-linked Man*p* outer chain (A arm) is added (Figure 6). Addition of mannose to the α1–3 mannose residue for outgrowth of the outer chain, is catalyzed by an α1–6 mannosyltransferase which has been characterized in *S. cerevisiae* (Reason *et al.*, 1991) and can use different mannosyl core substrates. The second α1–2 mannosidase of *P. brasiliensis* (2 in Figure 7) seems specific for the Manα1–2Manα1–2Man sequence since it does not remove terminal Man*p* from Manα1–2Manα1–6Man or Manα1–2Manα1–3Man sequences. The outer chain (A arm) of the gp43 oligosaccharide is substituted at *O*-2 by single Man*p* units. Further substitution by single unit β-galactofuranose linked (1→6) to either one of the mannosyl side chains is a source of heterogeneity in this oligosaccharide. The C branch containing the α-Man*p* (1→2)-α-Man*p* (1→6)-Man*p* sequence may also be further substituted at 6-*O* of the nonreducing end by β-galactofuranose. For the frequent $Hex_{14}GlcNAc_2$ subtype five isomers are possible, based on the position of the β-Gal*f* residue. The actual presence of these isomers in oligosaccharides of varying A arm length is reflected in the heterogeneity of the subtypes separated by Dionex ion-exchange chromatography. A single β-Gal*f* substitution per oligosaccharide is suggested by methylation analysis and electron-spray mass spectrometry of the partial hydrolysis products of the native molecule. The assignment of only one PMR signal (δ 5.086) to H^1 of possible β-Gal*f* substituents is not surprising since all structures consist of the sequence β-Gal*f* (1→6)-α-Man*p* (1→2)-α-Man*p*.

Terminal substitution of high mannose N-linked oligosaccharides by β-galactofuranosyl units has also been described in glycoproteins from a few species of trypanosomatids, *Crithidia fasciculata* and *C. harmosa* (Mendelzon and Parodi, 1986), *Leptomonas samueli* (Moraes *et al.*, 1989) and *Herpetomonas samuelpessoai* (Mendelzon *et al.*, 1986). A single report on this kind of substitution in glycoproteins from the human pathogen *Trypanosoma cruzi* (Golgher *et al.*, 1993) may not be a common feature of glycoproteins of this species (Parodi *et al.*, 1983; Engel and Parodi, 1985), but β-Gal*f* units are, nevertheless, frequent constituents of mannose-containing glycolipids of this parasite (Lederkremer and Colli, 1995). In all cases mentioned above the single β-galactofuranosyl end-unit is (1→3)-linked to α-Man*p*. The (1→6) linkage observed in the gp43 oligosaccharide is more characteristic of fungal polysaccharides. A ^{13}C-NMR chemical shift of δ 109.5, which can arise from the C-1 of β-Gal*f* (1→6)-linked either to α-Man*p* or to another β-Gal*f* unit was observed in spectra of galactomannans of *Sporothrix schenckii* (Travassos, 1985), and *Aspergillus fumigatus* (Barreto-Bergter *et al.*, 1980). The β-configuration of Gal*f* is also more frequent than the α-configuration and is usually associated to the antigenic properties of cross-reacting galactose-containing structures in fungi. In the case of

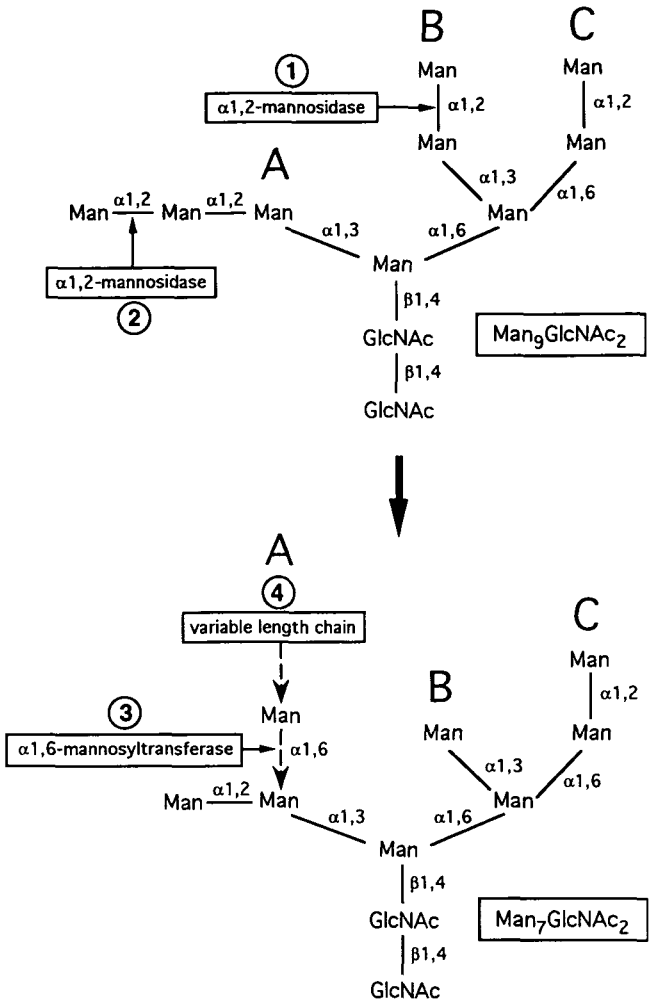

Fig. 7. Probable biosynthetic steps of the gp43 N-linked oligosaccharides using the conserved $Man_9GlcNAc_2$ precursor. (**1**) α-1,2-mannosidase hydrolysis equivalent to the yeast specific mannosidase; (**2**) α-1,2-mannosidase hydrolysis specific for the Manα1–2Manα1–2Man sequence; (**3**) α-1,6-mannosyltransferase; (**4**) sequential addition of Man residues to form the (1→6)-linked outer chain of variable length. Terminal substitutions of the core and outer chain by (1→2)-linked Man*p* and (1→6)-linked Gal*f* residues are not indicated.

the gp43, the cross-reactivity with sera from patients with histoplasmosis, observed at low dilution, can be eliminated by galactose and mannose (Puccia and Travassos, 1991a) suggesting that both the β-Gal*f* and α-Man*p* units are constituents of reactive epitopes.

The N-linked high-mannose oligosaccharide of the gp43 might seem important for the solubility of the glycoprotein, since N-deglycosylation of this antigen yielded an insoluble gelatinous product. However, enzymatic deglycosylation usually requires previous denaturation in detergent at pH 5.5, whereas the unglycosylated molecule obtained by treatment with tunicamycin or with endoglycosidase H in the absence of detergent, is water soluble (Puccia and Travassos, 1991b). In fact, secretion of the unglycosylated antigen (p38) in tunicamycin growing cultures follows the same kinetics of secretion of the native gp43. The molecular configuration of a glycoprotein, such that the oligosaccharide is not readily accessible to external ligands, was suggested by Parodi (1994) to explain the inability of glucosylation of untreated thyroglobulin as opposed to the denatured molecule (Trombetta *et al.*, 1989). In the case of the gp43, clearly the carbohydrate epitopes containing β-Gal*f* and α-Man*p* are not accessible to cross-reacting antibodies from sera of patients with histoplasmosis and Jorge Lobo's mycosis when the native gp43 is tested in solution. In contrast, when the antigen is fixed on a plastic substrate, the oligosaccharide is exposed and recognized by heterologous antibodies (Puccia and Travassos, 1991a). Such properties of the gp43 restrict the specificity of the antigen for PCM diagnosis unless the serological reactions are carried out in liquid phase.

The gp43 is constantly secreted *in vitro* and *in vivo* by growing yeast cells of *P. brasiliensis* (Stambuk *et al.*, 1988; Mendes-Giannini *et al.*, 1989). Apparently the high-mannose oligosaccharide of the gp43 is poorly immunogenic in man since most of the antibody clones from patients with PCM are directed to immunodominant peptide epitopes of the glycoprotein. Although the gp43 contains a single N-linked oligosaccharide (Travassos *et al.*, 1995), its subtypes with longer branched mannose chains, $Hex_{16-18}GlcNAc_2$, are good candidates for interaction with complement fractions in liquid phase similarly to fungal peptidomannan and peptidorhamnomannan (Travassos, 1989), but this has not yet been investigated. Such interaction could lessen the direct activation of complement by *P. brasiliensis* cells which results in increased phagocytosis (Munk *et al.*, 1992). Also, as a high-mannose structure, the N-linked oligosaccharide and other secreted D-mannose-containing antigens may act as metabolic inhibitors or cause a negative regulation of NK (natural killer) cell cytotoxicity (Peraçoli *et al.*, 1991). These effects would imply a role of the oligosaccharide in the pathogenicity of *P. brasiliensis* in addition to the properties of the gp43 as a whole, which can bind laminin and influence fungal invasiveness (Vicentini *et al.*, 1994), as well as form immunocomplexes that cause depression of cell-mediated immunity (Camargo and Cano, 1994).

Materials and methods

Purification of the gp43 antigen

The gp43 was purified from culture supernatants of *Paracoccidioides brasiliensis*, strain B339 (originally provided by Dr. Angela Restrepo-Moreno, Colombia), grown in the yeast phase at 37 °C, according to a previously described procedure (Puccia *et al.*, 1991), modified as follows. Supernatant fluids from 7-day-old cultures in yeast extract, casein peptone, D-glucose medium (YPD) were inactivated with 0.02% thimerosal for 2 h at 35°C, and then overnight at 4°C. Supernatants (10 liters) were paper-filtered, concentrated 10-fold in vacuo at 45°C, and exhaustively dialysed against phosphate-buffered saline, pH 7.2, 4°C. The gp43 was purified by immunoaffinity chromatography with monoclonal antibody 17C (Puccia and Travassos, 1991b) immobilized on Affi-Gel 10 (Bio-Rad), and elution with 50 mM citric acid, pH 2.8. The eluate was dialysed against deionised water and concentrated in YM10 Diaflo ultrafiltration membranes (Amicon, Inc., USA). Protein concentration was estimated by the method of Bradford (1976). The purity of the preparation (single band at 43 kDa) was analyzed by SDS–PAGE (Laemmli, 1970).

Isolation of N-linked oligosaccharides from the gp43

N-Linked oligosaccharides were isolated from the gp43 after treatment with recombinant peptide: N-glycanase F (PNGase F, New England Biolabs). The gp43 (60 mg) was previously denatured with 0.5% SDS, 1% β-mercaptoethanol, at 100°C for 10 min, and then incubated with 10,000 units (1,000 U/μl) of PNGase F in 50 mM phosphate buffer, pH 7.5, 0.1% NP40, at 37°C, for 48 h, in toluene atmosphere. The reaction was terminated by heating at 100°C, 3 min. The incubation mixture was maintained for 24 h at −20°C. In this condition, most (>95%, as inferred from polyacrylamide-gel silver staining) of the denatured, deglycosylated gp43 formed, after thawing, a gelatinous-insoluble product. The insoluble material was separated by centrifugation (10,000 g, 15

min., 4°C) and washed four times with deionized water. The supernatant and subsequent washes containing most of the enzymatically released N-linked oligosaccharides, were pooled and lyophilised. The dry residue was redissolved in 4 ml deionised water and extracted three times with 8 ml water saturated 1-butanol for detergent removal. The aqueous phases were grouped and lyophilised for 48 h and redissolved in 1 ml deionised water. The solution was desalted in a column of 0.5 ml of AG 50W-X12 (H^+), over 0.5 ml of AG 3-X4 (OH^-), over 0.25 ml of quaternary aminoethyl (QAE)-Sephadex A-25 (OH^-), washed with 10 ml deionised water. The N-linked oligosaccharides were uncharged as indicated by the complete recovery, in the flow-through volume of the column, of the entire carbohydrate content of the starting material. The N-linked oligosaccharides were further purified on a Sephadex G-10 column (50 cm × 1 cm), at a flow rate of 9 ml/h. The nonincluded material contained the majority (>95%) of the carbohydrate content (approximately 3 mg), as estimated by orcinol-H_2SO_4 staining of a 2 μl aliquot of each column fraction.

Electrospray-mass spectrometry (ES-MS)

Negative ion ES-MS spectra were recorded using a VG Quattro instrument (VG-Organic, Altrincham, UK). Oligosaccharide samples (10 μg) were introduced into the electrospray source at 10 μl/min via a Vydac C18 reverse-phase microbore HPLC column (100 × 1 mm) in 20% acetonitrile, 0.1% trifluoroacetic acid. The capillary, high-voltage lens and cone voltages were 2.4 kV, 50 V and 30 V, respectively. The cone/skimmer offset was 5 V. Multiple scans were averaged and processed using MassLynx software.

^{1}H-NMR spectroscopy

One dimensional and two dimensional COSY ^{1}H-NMR spectra of oligosaccharides were obtained using a Bruker AM 500 MHz Spectrometer equipped with a 5 mm triple resonance probe. The samples were dissolved in 0.5 ml of 100% D_2O (Aldrich), after repeated exchange in 0.5 ml of 100% D_2O. All experiments were performed at 300 K and chemical shifts were referenced externally to acetone (2.225 ppm). Correlated spectroscopy (COSY) experiments were performed using a sweep width of 2200 Hz; 512 increments were collected in f1 using 4,000 data points per increment.

Total sugar composition and methylation-fragmentation analysis

Analysis of monosaccharides after methanolysis, re-N-acetylation and trimethylsilyl derivatization was carried out as described previously (Ferguson, 1992). Methylation analysis was performed according to a modified version of the method of Ciucanu and Kerek (1984) as described in Ferguson (1992). All sugar derivatives were analysed by gas chromatography-mass spectrometry (GC-MS).

2-Aminobenzamide fluorescence labeling of oligosaccharides

The PNGase F released oligosaccharides were labeled at their reducing terminus with the fluorescent reagent 2-aminobenzamide (2-AB) following the manufacturer's protocol (Signal™ labeling kit, Oxford GlycoSystems). The fluorescent oligosaccharides were further purified from the free reagent by ascending paper chromatography using as solvent 1-butanol/ethanol/water (4:1:1, v/v/v). In this condition, 2-AB labeled oligosaccharides with three or more sugar units remain at the chromatogram origin, whereas the free reagent and 2-AB labeled mono- and disaccharides migrate with the solvent. The 2-AB labeled oligosaccharides were analyzed by Bio-Gel P-4 chromatography and Dionex high-performance anion-exchange chromatography and were detected using an on-line fluorometer (Gilson), at 420 nm ($\lambda_{exc.}$ = 330 nm). Bio-Gel P-4 chromatography was carried out using the Oxford GlycoSystems Glycosequencer in high resolution flow profile mode. Samples were co-injected with a dextran partial acid hydrolysate (GlycoSequencer calibration standard, Oxford GlycoSystems). The elution positions of the 2-AB-labeled neutral oligosaccharides were expressed as 'Glucose Units' (GU) (Yamashita *et al.*, 1982). Since 2-AB-labeled oligosaccharides show different retention times when compared to free reducing-end oligosaccharides, a correction factor was applied.

Dionex HPLC was carried out according to McConville and Blackwell (1991). Elution from the CarboPac column (4 × 250 mm) was started at 95% buffer A (0.15 M NaOH), 5% buffer B (0.15 M NaOH, 0.25 M NaOAc) followed by a linear increase of buffer B to 29% at 80 min (McConville and Blackwell, 1991).

Enzymatic and chemical cleavages

Digestions with jack bean α-mannosidase (JBAM, Boehringer Mannheim) and *Aspergillus saitoi* Manα1–2Man specific α-mannosidase (ASAM, Oxford GlycoSystems) were carried out with 0.75 U of JBAM and 0.01 U of ASAM, in 30 μl of 0.1 M sodium acetate buffer, pH 5.0, for 16 h at 37°C. The reactions with both mannosidases were terminated by heating (100°C, 3 min) and the products desalted by passage through 0.2 ml of AG 50-X12(H^+) over 0.2 ml of AG 3-X4 (OH^-) over 0.1 ml QAE-Sephadex A-25.

Partial acetolysis of oligosaccharides was carried out as described previously (Natsuka *et al.*, 1987). Mild acid hydrolysis to remove galactofuranose residues from oligosaccharides was carried out with either 100 mM trifluoroacetic acid at 80°C for 1h or 2 M glacial acetic acid, at 100°C for 4 h. The products of enzymatic digestion and of partial acetolysis were separated by Bio-Gel P-4 chromatography as described.

Acknowledgments

We are grateful to Antonio L. Furlanete for the excellent technical assistance. This work was supported by CNPq-PADCT and FAPESP Brazilian agencies.

References

Ballou,L., Hernandez,L.M., Alvarado,E. and Ballou,C.E. (1990) Revision of the oligosaccharide structures of yeast carboxypeptidase Y. *Proc. Natl. Acad. Sci. USA*, **87,** 3368–3372.

Barreto-Bergter,E., Gorin,P.A.J. and Travassos,L.R. (1980) Chemical structure of the D-galacto-D-mannan component from hyphae of *Aspergillus niger* and other *Aspergillus* spp. *Carbohydr. Res.*, **86,** 273–285.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utillizing the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248–254.

Camargo,Z.P. and Cano,L.E. (1994) Humoral immunity. In Franco,M., Lacaz,C.S., Restrepo-Moreno,A. and Del Negro,G. (eds), *Paracoccidioidomycosis.* CRC Press, Boca Raton, FL, pp. 187–201.

Ciucanu,I. and Kerek,F. (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.*, **131,** 209–217.

Engel,J.C. and Parodi,A.J. (1985) *Trypanosoma cruzi* cells undergo an alteration in protein *N*-glycosylation upon differentiation. *J. Biol. Chem.*, **260,** 10105–10110.

Ferguson,M.A.J. (1992) The chemical and enzymatic analysis of GPI fine structure. In Hooper,N.M. and Turner,A.J. (eds), *Lipid modifications of proteins: a practical approach.* IRL Press, Oxford, pp. 191–230.

Golgher,D., Colli,W., Souto-Padron,T. and Zingales,B. (1993) Galactofuranose- containing glycoconjugates of epimastigote and trypomastigote forms of *Trypanosoma cruzi. Mol. Biochem. Parasitol.*, **60,** 249–264.

Hernandez,L.M., Ballou,L., Alvarado,E., Gillece-Castro,B.L., Burlingame,A.L. and Ballou,C.E. (1989) A new *Saccharomyces cerevisiae mnn* mutant *N*-linked oligosaccharide structure. *J. Biol. Chem.*, **264,** 11849–11856.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227,** 680–685.

Lederkremer,R.M. and Colli,W. (1995) Galactofuranose-containing glycoconjugates in trypanosomatids. *Glycobiology*, **5,** 547–552.

Jelinek-Kelly,S. and Herscovics,A. (1988) Glycoprotein biosynthesis in *Saccharomyces cerevisiae.* Purification of the α-mannosidase which removes one specific mannose residue from $Man_9GlcNAc$. *J. Biol. Chem.*, **263,** 14757–14763.

McConville,M.J. and Blackwell,J. M. (1991) Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani. J. Biol. Chem.*, **266,** 15170–15179.

Mendelzon,D.H. and Parodi,A.J. (1986) *N*-Linked, high mannose-type oligosaccharides in the protozoa *Crithidia fasciculata* and *Crithidia harmosa* contain galactofuranose residues. *J. Biol.Chem.*, **261,** 2129–2133.

Mendelzon,D.H., Previato,J.O. and Parodi,A.J. (1986) Characterization of protein-linked oligosaccharides in trypanosomatid flagellates. *Mol. Biochem. Parasitol.*, **18,** 355–367.

Mendes,R.P. (1994) The gamut of clinical manifestations. In Franco,M., Lacaz,C.S., Restrepo-Moreno,A. and Del Negro,G. (eds), *Paracoccidioidomycosis.* CRC Press, Boca Raton, FL, pp. 233–258.

Mendes-Giannini,M.J.S., Bueno,J.P., Shikanai-Yasuda,M.A., Ferreira,A.W. and Masuda,A. (1989) Detection of the 43,000-molecular weight glycoprotein in sera of patients with paracoccidioidomycosis. *J. Clin. Microbiol.*, **27,** 2842–2845.

Moraes,C.T., Bosch,M. and Parodi,A.J. (1989) Structural characterization of several galactofuranose-containing high-mannose-type oligosaccharides present in glycoproteins of the trypanosomatid *Leptomonas samueli. Biochemistry*, **27,** 1543–1549.

Munk,M.E., Kajdacsy-Balla,A., Del Negro,G., Cuce,L.C. and Silva,W.D.

(1992) Activation of human complement system in paracoccidioidomycosis. *J. Med. Vet. Mycol.*, **30,** 317–321.

Natsuka,S., Hase,S. and Ikenaka,T. (1987) Fluorescence method for the structural of oligomannose-type sugar chains by partial acetolysis. *Anal. Biochem.*, **167,** 154–159.

Parodi,A.J. (1994) Serendipity, or how working with glycoproteins from Trypanosomatids changed my life (with a little help from my friends). *Ci. Cult. J. Braz. Assoc. Adv. Sci.*, **46,** 249–254.

Parodi,A.J., Lederkremer,G.Z. and Mendelzon,D.H. (1983) Protein glycosylation in *Trypanosoma cruzi*. The mechanism of glycosylation and structure of protein-bound oligosaccharides. *J. Biol. Chem.*, **258,** 5588–5591.

Peracoli,M.T.S., Soares,A.M.V.C., Mendes,R.P., Marques,S.A., Pereira,P.C.M. and Rezkallah-Iwasso,M.T. (1991) Studies of natural killer cells in patients with paracoccidioidomycosis. *J. Med. Vet. Mycol.*, **29,** 373–380.

Plancke,Y., Wieruszeski,J.-M., Boilly,B. and Strecker,G. (1994) Primary structure of seven new acidic oligosaccharide-alditols from the egg jelly coats of *Axolotl mexicanum* and *Pleurodeles waltl. Ci. Cult. J. Braz. Assoc. Adv. Sci.*, **46,** 273–279.

Puccia,R., Schenkman,S., Gorin, P.A.J. and Travassos, L.R. (1986) Exocellular components of *Paracoccidioides brasiliensis*. Identification of a specific antigen. *Infect. Immun.*, **53,** 199–206.

Puccia,R. and Travassos,L.R. (1991a) 43-Kilodalton glycoprotein from *Paracoccidioides brasiliensis:* immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, and Jorge Lobo's disease. *J. Clin. Microbiol.*, **29,** 1610–1615.

Puccia,R. and Travassos,L.R. (1991b) The 43-kDa glycoprotein from the human pathogen *Paracoccidioides brasiliensis* and its deglycosylated form: excretion and susceptibility to proteolysis. *Arch. Biochem. Biophys.*, **289,** 298–302.

Puccia,R., Takaoka,D.T. and Travassos,L.R. (1991) Purification of the 43 kDa glycoprotein from exocellular components excreted by *Paracoccidioides brasiliensis* in liquid culture (TOM medium). *J. Med. Vet. Mycol.*, **29,** 57–60.

Reason,A.J., Dell,A., Romero,P.A. and Herscovics,A. (1991) Specificity of the mannosyltransferase which initiates outer chain formation in *Saccharomyces cerevisiae. Glycobiology,* **1,** 387–391.

Stambuk,B.U., Puccia,R., Almeida,M.L.C., Travassos,L.R. and Schenkman,S. (1988) Secretion of the 43 kDa glycoprotein antigen by *Paracoccidioides brasiliensis. J. Med.Vet. Mycol.*, **26,** 367–373.

Taborda,C.P. and Camargo,Z.P. (1993) Diagnosis of paracoccidioidomycosis by passive haemagglutination assay of antibody using a purified and specific antigen-gp43. *J. Med. Vet. Mycol.*, **31,** 155–160.

Taborda,C.P. and Camargo,Z.P. (1994) Diagnosis of paracoccidioidomycosis by dot immunobinding assay for antibody detection using the purified and specific antigen gp43. *J. Clin. Microbiol.*, **32,** 554–556.

Takayanagi,T., Kushida,K., Idonuma,K. and Ajisaka,K. (1992) Novel *N*-linked oligo-mannose type oligosaccharides containing an α-D-galactosidase from *Aspergillus niger. Glycoconj. J.*, **9,** 229–234

Travassos,L.R. (1985) *Sporothrix schenckii.* In Szaniszlo,P.J. and Harris,J.L. (eds), *Fungal dimorphism, with emphasis on fungi pathogenic for humans.* Plenum Press, New York, pp. 121–156.

Travassos,L.R. (1989) Antigenic structures of *Sporothrix schenckii.* In Kurstak,E. (ed), *Immunology of fungal diseases.* Marcel Dekker, New York., pp. 193–221.

Travassos,L.R. (1994) Immunochemistry of *Paracoccidioides brasiliensis* antigens. In Franco,M., Lacaz,C.S., Restrepo- Moreno,A., Del Negro,G. (eds), *Paracoccidioidomycosis.* CRC Press, Boca Raton, FL, pp. 67–86.

Travassos,L.R., Puccia,R., Cisalpino,P., Taborda,C., Rodrigues,E.G., Rodrigues,M., Silveira,J.F. and Almeida,I.C. (1995) Biochemistry and molecular biology of the main diagnostic antigen of *Paracoccidioides brasiliensis. Arch. Med. Res.*, **26,** 297–304.

Trombetta,S.E., Bosch,M. and Parodi,A.J. (1989) Glucosylation of glycoproteins by mammalian, plant, fungal and trypanosomatid protozoa microsomal membranes. *Biochemistry,* **28,** 8108–8116.

Vicentini,A.P., Gesztesi,J.-L., Franco,M.F., Souza,W., Moraes,J.Z., Travassos, L.R. and Lopes,J.D. (1994) Binding of *Paracoccidioides brasiliensis* to laminin through surface glycoprotein gp43 leads to enhancement of fungal pathogenesis. *Infect. Immun.* **62,** 1465–1469.

Vliegenthart,J.F.G., Dorland,L. and van Halbeek,H. (1983) High-resolution ^{1}H- nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrate related to glycoproteins. *Adv. Carbohydr. Chem. Biochem.*, **41,** 209–374.

Yamashita,K., Mizuochi,T. and Kobata,A. (1982) Analysis of oligosaccharides by gel filtration. *Meth. Enzymol.*, **83,** 105–126.

Received on February 12, 1996; revised on April 10, 1996; accepted on April 15, 1996.