Detection of sialoglycomolecules in five plant trypanosomatids and in an insect phytophagous isolate

André Luís Souza dos Santos, Celuta Sales Alviano, Rosangela Maria de Araújo Soares *

Departamento de Microbiologia Geral, Instituto de Microbiologia Prof. Paulo de Goës (IMPPG), Centro de Ciências da Saúde (CCS), Bloco I, Universidade do Brasil (UFRJ), Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil

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Abstract

The sialoglycoprotein profiles of five plant trypanosomatids (Phytomonas spp.) and of one flagellate (Herpetomonas sp.) isolated from the salivary gland of a phytophagous insect (Phthiapicta) were analyzed by Western blotting using three distinct lectins (LFA, SNA and MAA), which recognize specifically sialic acid residues in glycoconjugates. All six flagellates presented at least one polypeptide recognized by the lectins, with the exception of Phytomonas francix71ai, which did not show any reactivity with SNA agglutinin. Phytomonas serpens and P.francix71ai showed the most distinct pattern of sialoglycoproteins. Phytomonas mcgheei, Herpetomonas sp. and the two other Phytomonas spp., isolated from latex, displayed an identical sialomolecule profile. We discuss the possible role of the sialoglycoproteins in the physiology of these trypanosomatids. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Lectin; Plant trypanosomatid; Sialic acid; Sialoglycoprotein

1. Introduction

Sialic acids are a family of monosaccharides comprising several derivatives of neuraminic acid including N-glycolyl and N-acetyl groups, as well as O-acetyl, and other O-substitutions [1,2]. They are important terminal or subterminal units in oligosaccharides from complex glycoconjugates expressed at the cell surface of eukaryotic cells. Naturally occurring sialic acids are known to mediate many important biological phenomena involving cell–cell and cell–matrix interactions [1]. They have also been reported to play a relevant role in microbial pathogenicity [2,3].

Flagellates of the family Trypanosomatidae are parasites of a wide range of animals and plants [4]. The occurrence of sialic acid as constituent of the cell surface of trypanosomatids has been reported in the genera Trypanosoma [5], Crithidia [6–8], Herpetomonas [9–11] and Phytomonas [12]. Flagellate trypanosomatids of the genus Phytomonas are etiologic agents of diseases affecting fruits and plants of great economic importance including tomato, cashew, coffee, cassava, coconut and oil palm [13]. Plant flagellates are transmitted to their plant hosts by phytophagous insects, mainly piercing hemipterans [4,13].

Lectins may play a role in plant disease resistance. Lectins are non-catalytic proteins that interact specifically with carbohydrate residues, and may function as important effectors in the host defense response in plants [14]. Lectins that recognize sialic acid are valuable reagents for the detection, isolation and characterization of glycoconjugates containing this sugar [2]. Most such lectins occur in invertebrates such as crabs, lobsters and slugs [15]. However, a few sialic acid-binding lectins of plant origin have proved to be exceptionally useful. They are the elderberry bark lectin (Sambucus nigra, SNA) [16] and the seeds of the leguminous plant Maackia amurensis (MAA) [17].

In this work, we report the interaction between three peroxidase-labeled lectins (SNA, MAA and Limax flavus [LFA]), which recognize specifically sialic acid residues, with glycoconjugates of five distinct plant trypanosomatid isolates and one flagellate isolated from the salivary gland
of a phytophagous hemiptera (Phthia picta) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western blotting analysis.

2. Materials and methods

2.1. Microorganisms and in vitro culture

The five isolates of trypanosomatid flagellates of plants and one isolate from a phytophagous insect are listed in Table 1. All in vitro cultures were performed using brain heart infusion (BHI) medium supplemented with 10% fetal bovine serum (FBS) and cultures were maintained at 26°C.

2.2. Flagellate extracts

Two days after inoculation (exponential phase), the flagellates \(2 \times 10^6\) cells were harvested by centrifugation at \(1500 \times g\) for 2 min and washed three times with cold phosphate-buffered saline (PBS: 150 mM NaCl, 20 mM phosphate buffer, pH 7.2). Then, cells were resuspended in \(100\) μl of PBS and lysed at room temperature by the addition of 1% (w/v) SDS, followed by centrifugation at \(5000 \times g\) for 10 min. The supernatants obtained after centrifugation corresponded to the parasite cellular extracts [18].

2.3. Electrophoresis

Samples containing cellular extracts (equivalent to \(5 \times 10^6\) cells) were added to SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.002% bromophenol blue) and mixed with 10% (v/v) \(\beta\)-mercaptoethanol, followed by heating at 100°C for 5 min. Proteins were analyzed in 12% SDS-PAGE by the method described by Laemmli[19]. Electrophoresis was carried out at 4°C, at 100 V, for 2h. Molecular mass of sample polypeptides was calculated from mobility of Gibco BRL (Grand Island, NY, USA) molecular mass standards.

2.4. Western blotting analysis

Total protein extracts from flagellates were separated in SDS-PAGE and the polypeptides electrophoretically transferred at 4°C at 100 V/300 mA for 2 h to nitrocellulose membranes. The membranes were blocked in a blocking solution (150 mM NaCl, 10 mM Tris, pH 7.5, 10% Tween 20) for 2 h at room temperature. Membranes were incubated separately with the following peroxidase-labeled lectins (EY Laboratory): LFA at 1:5000 dilution, MAA at 1:500 dilution and SNA at a final dilution of 1:400 in blocking solution for 1 h, in the absence or in the presence of 0.2 M sugar inhibitors (fetuin plus sialylactose, fetuin and sialylactose, respectively), and then washed five times in the same solution. Bands were visualized by reaction with 0.5 mg ml\(^{-1}\) diaminobenzidine in 1.5 M Tris-HCl buffer (pH 7.4) and 0.01% H\(_2\)O\(_2\). The color development was stopped by immersing the membrane sheets in distilled water [20].

3. Results

The phytophagous isolate and all the plant trypanosomatids examined, with the exception of Phytomonas franc\(\times 71\)[-ai, contain glycoproteins that bind with the three lectins used in this comparative study: LFA, SNA or MAA, which specifically recognize sialic acid residues in glycoconjugates (Fig. 1). To simplify the analysis of the results obtained in the Western blotting, we chose to separate the different reactivity profiles according to their lectin specificity and the trypanosomatid isolates (Table 2).

The LFA agglutinin recognizes all sialic acid linkages, with a major predilection for \(N\)-acetylneuraminic acid rather than \(N\)-glycolylneuraminic acid residues. Three distinct patterns of lectin reactivity were observed when LFA was used (Fig. 1A). Pattern 1 (composed of at least seven polypeptides with molecular masses ranging from 15 to 36 kDa) was detected only in Phytomonas serpens, while pattern 2 (composed of five polypeptides with 36, 40, 44, 100 and 200 kDa) was seen only in P. franc\(\times 71\) and pattern 3

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**Table 1**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Code</th>
<th>Host Family</th>
<th>Species</th>
<th>Source</th>
<th>CT-IOC code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytomonas serpens (isolate 9T)</td>
<td>Pser</td>
<td>Plantae: Solanaceae</td>
<td>Lycopersicon esculentum</td>
<td>tomato</td>
<td>189(^a)</td>
</tr>
<tr>
<td>Phytomonas megcheei (isolate 163M, clone A)</td>
<td>Pmac</td>
<td>Plantae: Gramineae</td>
<td>Zea mays</td>
<td>maize</td>
<td>181(^a)</td>
</tr>
<tr>
<td>Phytomonas franc(\times 71)[-ai</td>
<td>Pfra</td>
<td>Plantae: Euphorbiaceae</td>
<td>Manihot esculenta</td>
<td>cassava</td>
<td>011(^b)</td>
</tr>
<tr>
<td>Phytomonas sp.</td>
<td>Pjam</td>
<td>Plantae: Euphorbiaceae</td>
<td>Iatrophila macrana</td>
<td>latex</td>
<td>083(^b)</td>
</tr>
<tr>
<td>Phytomonas sp.</td>
<td>Pehy</td>
<td>Plantae: Euphorbiaceae</td>
<td>Euphorbia hyslopfolia</td>
<td>latex</td>
<td>salivary glands</td>
</tr>
</tbody>
</table>

\(^a\)The trypanosomatids were kindly provided by Dr. Maria Auxiliadora de Sousa from CT-IOC (Coleção de Tripanosomatídeos, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil).

\(^b\)These flagellates were isolated and kindly provided by Dr. Márcia Attias (Instituto de Biofísica Carlos Chagas Filho, Universidade do Brasil-UFRJ).
composed of at least five polypeptides ranging from 15 to 25 kDa) was the most common protein profile detected in the other four isolates. The lectin binding was specifically inhibited in the presence of 0.2 M fetuin plus 0.2 M sialylactose (Fig. 1A, lane i).

SNA and MAA recognize sialic acids in α2,6 and α2,3 linkages, respectively. For SNA binding, galactose or N-acetyl-D-galactosamine is the required sugar unit, whereas for MAA the necessary underlying sequence is Galβ1-4N-acetyl-D-glucosamine. When we used the SNA agglutinin, we also observed three distinct profiles: the first one detected in P. serpens, composed of at least five major bands (15, 20, 22, 25 and 32 kDa), another composed of approximately 11 low molecular mass polypeptides ranging from 15 to 32 kDa and observed in all other flagellates (pattern 3), with the exception of P. francix71-ai (pattern 2), which displayed no detectable reactivity (Fig. 1C). The binding was completely inhibited by 0.2 M sialylactose (Fig. 1C, lane i). The MAA agglutinin generated three distinct patterns of reactivity: the first one only detected in P. serpens (pattern 1: 15, 16, 19, 20, 21 and 23 kDa), another only detected in P. francix (pattern 2: with a single polypeptide with approximately 200 kDa) and a third profile composed of eight polypeptides, ranging from 15 to 60 kDa (15, 16, 19, 20, 21, 23, 44 and 65 kDa) observed in the other four flagellates (Fig. 1B). The binding was totally inhibited by 0.2 M fetuin (Fig. 1B, lane i).

4. Discussion

Lectins have been used in Trypanosomatidae flagellates to identify the surface carbohydrate structures in Leishmania, Trypanosoma, Crithidia, Phytomonas and Herpetomonas, to trigger cell differentiation in Herpetomonas samuelpessoai, to identify the developmental stages of Trypanosoma, to study the symbiont–host interaction in Crithidia, to discriminate between pathogenic and non-pathogenic forms of Trypanosoma [21] and to differentiate Phytomonas sp. from Herpetomonas and Crithidia [22]. In the present work, an analysis of the content of sialic acid-containing glycoproteins was available in some Phytomonas species and one flagellate isolated from the salivary glands of a phytophagous hemiptera by Western blotting using specific lectins. Some sialic acid-binding lectins have been successfully used for in situ location of glycoconjugates. The most frequently used are the agglutinins from wheat germ, Limus polyphemus, LFA, SNA and MAA. With the latter two agglutinins, sialic acid α2,3 and α2,6, linkages to galactose can be distinguished [23].

P. francix and P. serpens showed the most heterogeneous pattern of sialoglycoproteins when compared with the other flagellate isolates. Therefore, this class of sugar-binding proteins could also be used as an additional criterion for the identification of phytotflagellate isolates. Moreover, the distinct pattern of sialoglycoproteins might be directly involved in some parasite host cell (insect and/or plant) interaction, such as preferential host site colonization and the mode of attachment of the flagellates to the host tissue. It is known that host specificity may depend in

Table 2

<table>
<thead>
<tr>
<th>Flagellate (code)</th>
<th>Protein profile*</th>
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<tbody>
<tr>
<td></td>
<td>LFA</td>
</tr>
<tr>
<td>Pser</td>
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</tr>
<tr>
<td>Pfra</td>
<td>2</td>
</tr>
<tr>
<td>Pmai</td>
<td>3</td>
</tr>
<tr>
<td>Ppya</td>
<td>3</td>
</tr>
<tr>
<td>Pekt</td>
<td>3</td>
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<tr>
<td>Herp</td>
<td>3</td>
</tr>
</tbody>
</table>

*Sialoglycoprotein profiles are shown in Fig. 1, and designated by us as 1, 2 and 3 for the three lectins used in this study: LFA, SNA and MAA.
part on cellular recognition based on carbohydrate-containing surface components that act as complementary molecules to protein or glycoprotein receptors. Compatible associations between host plants and microorganisms can be mediated by specific lectins [14]. In this context, in a study of our laboratory, we showed that some of the glycoprotein with sialic acid residues demonstrated in these plant flagellates was localized on the cell surface (data not shown). Moreover, in some microorganisms, a direct correlation between surface saccharides and pathophysiological responses is suggested. For instance, fungal and bacterial glycopeptides stimulate the disease caused by the pathogen itself [14].

Binding to the same component by lectins MAA and SNA probably indicates a carbohydrate moiety composed of α2,3- and α2,6-linked sialic acid residues, respectively. However, the 200-kDa sialoglycoprotein exclusively detected in P. franzai contained just galactose-linked α2,3 sialic acid. Interestingly, in the flagellates used in this study, the interaction of SNA with polypeptides was stronger than that observed with LFA agglutinin. This may reflect the presence of 9-O-acetyl groups in N-acetyl-neuraminic acid, which preclude reactivity with LFA, but not with SNA [3]. The 9-O-acetylated sialic acid derivative has been demonstrated in the insect trypanosomatid Crithidia fasciculata using gas chromatography-mass spectrometry [8]. In some infections, O-acetylated sialic acids can affect the immunogenicity and pathogenicity of the infectious agent [1–3].

Sialic acids comprise several derivatives of neuraminic acid, including N-glycolyl, N-acetyl, and N,O-acetyl derivatives [1,2]. The few previous studies available about the nature of sialic acid residues in Trypanosomatidae showed that an O-acetyl substituent to the sialic acid was only suggested in H. samuelpessoa, which was developmentally regulated [10], and both N-acetyl and N-glycolyl derivatives were reported in Trypanosoma cruzi [5], Herpetomonas megaseliaca [9] and Crithidia deanei [6], whereas N-acetylenearaminic acid was the only derivative detected on the plasma membrane of Herpetomonas muscarum muscarum [11].

The nature of the sialic acid-carrying molecules of Phytophthorabruceleu is unknown; this is therefore a focus of future studies. In some microorganisms, including T. cruzi and Trypanosoma brucei, the acquisition of sialic acid is catalyzed by a parasite trans-sialidase that transfers sialic acid residues from glycoconjugates available in the environment to protozoon acceptor molecules [24]. In C. fasciculata [7,8] and H. samuelpessoa [10] cells growing in a sialic acid-free chemically defined medium it is suggested that these residues are synthesized de novo and transferred to glycoconjugate terminal galactose residues toward cytidine S′-monophosphate-sialic acid as the donor molecule [1] and are not scavenged from the medium in the form of sialylglycoconjugates [24]. Notwithstanding, the attachment of sialic acid to nascent glycoconjugates in Phytophthora may be catalyzed by specific sialyltransferases that display binding specificity for α2,3 and α2,6 linkages [2], with exception for P. franzai, since only molecules with α2,3 sialyl-galactosyl sequences were detected.

The function of these sialic acid residues is completely unknown in Phytophthora. However, general functions of sialic acids are attributed to their physicochemical properties such as electronegativity, hydrophilicity and the relatively large size of the hydrated molecules. Their exposure on cells and their presence on secreted sialoglycolipids and sialoglycoproteins, including mucus, determine and modulate cell interactions and carbohydrate-dependent physiological and pathophysiological responses. They can either mask recognition sites or serve as recognition determinants [1–3].

Members of the families Solanaceae, Gramineae and Euphorbiaceae are able to synthesize a variety of lectins, with distinct sugar-binding specificity. Lectins were classically defined as carbohydrate-binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates. So, lectins may play a relevant function in plant disease resistance, because they are able to agglutinate microorganism pathogens [15]. For instance, soybean lectin (SBL) has been implicated in the resistance response to infection by Phytophthora megasperma var. sojae [25]. The quantity of SBL is greater in cultivars resistant to P. megasperma than in susceptible cultivars. Furthermore, seed lectin is released sooner and at higher concentration into the surrounding media during germination from resistant cultivars than from susceptible cultivars. Following release, the lectin retains its hemagglutinating activity and binds to the mycelial cell wall of P. megasperma, inhibiting mycelial growth [25].

Additionally, binding of the pathogen to a lectin located in the plant may be important in the induction of the hypersensitive response, including phytoalexin and proteinase inhibitor synthesis that is an important factor in plant disease resistance [14,15].

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