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Original article

Immunocharacterization of the mucin-type proteins from the intracellular stage of *Trypanosoma cruzi*

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Abstract

The surface of *Trypanosoma cruzi* is covered by different groups of mucins that are differentially expressed during the parasite life cycle. We have previously identified the major mucins from the bloodstream trypomastigote stage. Here, we present additional evidence that together with our previous observations allows for the identification of a second mucin group also expressed in the mammal-dwelling stages, but predominant in the intracellular amastigote. These mucins are encoded by many genes, are mostly composed of tandem repeats and are highly conserved except for an exposed hypervariable (HV) N-terminal peptide. Antibodies against HV-peptides are restricted to ~50% of the chronically infected human population, are monospecific (i.e. directed towards a single HV), and display low-avidity. In contrast, immunization with a single HV-peptide triggers high-avidity, cross-reacting humoral responses against multiple HV sequences, but not against other *T. cruzi* surface antigens. The diversity present in the HV regions and the characteristics of the antibody response against them suggest a role of these molecules in eluding and/or modulating the mammalian host immune system.

Keywords: *Trypanosoma cruzi*; Amastigotes; Mucins; Hypervariability; Cross-reactivity

1. Introduction

The surface of *Trypanosoma cruzi*, the agent of Chagas disease, is covered by a thick coat of mucin molecules [1,2]. They are the major acceptors of sialic acid, which is transferred from host glycoconjugates by a specific *trans*-sialidase (TS) also present on the parasite surface [3]. Sialylated mucins are involved in adhesion to the host cells [4,5], and in protection against lytic antibodies [6]. Additional immunomodulatory roles have been proposed for these molecules [7–9]. *T. cruzi* has a complex life cycle involving different developmental stages within the insect vector (epimastigotes and metacyclic trypomastigotes) and the vertebrate host (bloodstream trypomastigotes and amastigotes). While transforming from one stage to another, the parasite undergoes profound morphological changes including a complete remodeling of its surface coat [10,11]. This correlates with a change in the expressed mucin molecules, as indicated by amino acid composition analyses, SDS-PAGE mobility and differential reactivity to monoclonal antibodies [1,2]. In the last years, a complex mucin-gene repertoire has been described in *T. cruzi* and partially correlated with the major biochemically defined groups of parasite mucins [2]. Briefly, mucins present in the insect stages are encoded by a homogeneous family of ~50–70 genes termed TcSMUG [12] whereas those from the mammal-dwelling stages are encoded by a different family of ~700 genes, named TcMUC [13,14]. TcMUC members have conserved N- and C-termini that code for an endoplasmic reticulum import signal and a glycosylphosphatidyl inositol (GPI) anchor signal, respectively. The central domain is composed either by tandem repeats with

Abbreviations: GPI, glycosylphosphatidyl inositol; HV, hypervariable; IIF, indirect immunofluorescence; TS, trans-sialidase; TSSA, trypomastigote small surface antigen.

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the consensus sequence T_8KP_2 (TcMUC I) or by variable, non-repetitive sequences (TcMUC II) [15]. Both TcMUC I and TcMUC II are structurally and phylogenetically related groups [16], although they also show enough differences as to potentially have different functions.

In a recent work we identified the TcMUC II products as the major mucin molecules in bloodstream trypomastigotes, being also but less expressed in amastigote forms [17]. The TcMUC I mature products are highly *O*-glycosylated in the tandem repeats region, and are predicted to harbor an N-terminal 7–18 amino acids-long hypervariable (HV) peptide well exposed to the extracellular milieu [14,18]. By analogy to other protozoan parasites [19–22], the exposure of related variant antigens on the surface of mammal-dwelling stages would suggest a role for TcMUC I molecules in immune evasion and/or host cell adhesion. However, the expression of the TcMUC I molecules is uncertain, mostly inferred from serologic studies [18]. The only direct evidence was provided by our mass spectrometry survey of purified trypomastigote mucins, which yielded a single match corresponding to a TcMUC I HV-peptide [17]. In the present study, we analyze the expression of the TcMUC I molecules and the antigenic properties of their HV regions.

2. Materials and methods

2.1. Parasites

The CL-Brener genome project reference clone [35] was used throughout this study. Distinct developmental stages were obtained as described [36,15].

2.2. Peptides and antisera

Peptides were custom synthesized by Sigma-Genosys and coupled through their C-terminal cysteine residue to either maleimide-activated keyhole limpet hemocyanin (KLH) or maleimide-activated bovine serum albumin (BSA, both from Pierce) following manufacturer's guidelines. KLH-coupled peptides were injected subcutaneously into rabbits and mice as described [23]. Immunoglobulins were affinity-purified from rabbit sera using protein A columns (HiTrap, Amersham Biosciences). Loc5 peptide-specific antibodies were purified from a mouse immunized with KLH-Loc5 by using the Loc5 peptide coupled to a Sulfolink column (Pierce) following the manufacturer indications. Specific antibodies were eluted with 0.1 M Glycine pH 3, neutralized, desalted by NAP10 column (Amersham Bioscience) filtration and concentrated by speedvac.

2.3. Serum survey

Sera from untreated chronic chagasic patients were purchased from the Instituto Nacional de Parasitología "Dr. M. Fatala Chaben" (Buenos Aires, Argentina).

2.4. Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microplates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 200 ng of BSA-coupled peptides in PBS and processed as described [23]. For the avidity assays, serum samples were washed twice (5 min each) with PBS supplemented with 0.1% Tween 20 and 6 M urea before the addition of the secondary antibody. Duplicate samples processed in parallel were washed in the same buffer without urea. The avidity index (AI) was calculated as the mean OD values for the wells washed with urea buffer/mean OD values for wells washed with control buffer. The cut-off value was determined using BSA-coated wells assayed in parallel. In order to work within the linear range of absorbance, every serum was diluted 1:20 for testing the anti-HV-peptide antibodies and 1:500 for the anti-TSSA peptide antibodies. For the Chemiluminescence-ELISA (CL-ELISA) assays polystyrene microplates (FluoroNunc Plate, Nunc) were coated as described above. Infection sera were preadsorbed against BSA 1 h, and diluted 1:100, 1:500 and 1:1,000, incubated for 2 h at room temperature and washed four times with Tris-buffered saline (TBS) 0.2% Tween 20. Secondary antibodies conjugated to HRPO were diluted 1:10,000, incubated 1 h at room temperature, washed as before and revealed by chemiluminescence detection.

2.5. Dot blot assays

A drop with 10 ng of each peptide coupled to BSA was applied to a nitrocellulose filter, blocked with TBS supplemented with 5% non-fat milk and incubated 2 h with specific antibodies purified from immunized mouse diluted 1/100. Washes were performed four times with TBS 0.2% Tween 20. Anti-mouse antibodies conjugated to HRPO were diluted 1:10,000, incubated and revealed as described above.

2.6. Indirect immunofluorescence (IIF)

A drop of parasites (5×10^6 per ml in PBS) was layered onto a Poly-L-Lysine (Sigma–Aldrich) coated coverslips and let stand for 20 min at room temperature. Parasites were fixed with 4% paraformaldehyde for 20 min and washed with NH_4Cl 25 mM for another 20 min. Blocking and antibody solutions were prepared in PBS containing 2% BSA and 5% normal goat serum. Anti KLH-HV-peptides antibodies diluted 1:20 were allowed to react for 16 h at 4 °C followed by PBS washings. Alexa 488-conjugated goat anti-rabbit immunoglobulins G (H + L) (1:10,000, Molecular Probes) were added for 60 min at room temperature and washed as before. Coverslips were mounted in 5 μl of FluorSave Reagent (Calbiochem) and observed and photographed using a Nikon Y-FL fluorescence microscope. To evaluate the reactivity of intracellular stages 10,000 Vero cells were plated onto round coverslips 96 h after infection with 1×10^6 trypomastigotes per coverslip. The infected cells were washed with PBS, fixed and processed as described above, with the addition of 0.5%