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Metabolic Labeling of Surface Neo-sialylglyconjugates Catalyzed by *Trypanosoma cruzi* trans-Sialidase

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Abstract

Trypanosoma cruzi, the protozoan agent of Chagas disease, has evolved an innovative metabolic pathway by which protective sialic acid (SA) residues are scavenged from host sialylglycoconjugates and transferred onto parasite surface mucin-like molecules (or surface glycoconjugates from host target cells) by means of a unique *trans*-sialidase (TS) enzyme. TS-induced changes in the glycoprotein sialylation profile of both parasite and host cells are crucial for the establishment of a persistent *T. cruzi* infection and for the development of Chagas disease-associated pathogenesis. In this chapter, we describe a novel metabolic labeling method developed in our labs that enables straightforward identification and molecular characterization of SA acceptors of the TS-catalyzed reaction.

Keywords

Trypanosoma cruzi; Sialic acid; *trans*-Sialidase; Sialylglycoconjugates; Mucins

1 Introduction

T. cruzi has evolved an innovative metabolic pathway by which the incorporation of sialic acid (SA) residues onto parasite molecules does not involve a conventional, Golgi-located cytidine monophosphate-SA:glycoconjugate sialyltransferase. Instead, SA residues are “scavenged” from host sialylglycoconjugates and transferred onto acceptor molecules on the parasite surface [1]. This reaction is catalyzed by a unique *trans*-sialidase (TS) enzyme and involves two steps: the cleavage of a terminal SA residue α 2–3-linked to a β -

galactopyranose group in the donor macromolecule and the subsequent formation of an equivalent linkage in the acceptor substrate [2–7].

Acquisition of SA residues onto the parasite surface coat protects *T. cruzi* bloodstream trypomastigotes against the host immune system [8] and is crucial for their interaction with target cells [9]. In addition to its role in SA scavenging, TS emerges as a major *T. cruzi* phenotypic determinant, involved in multiple phenomena underlying parasite immune evasion, virulence, and pathogenesis. As shown, exposure to parasite-shed TS leads to modifications on the surface sialylation pattern of different host target cells such as platelets, T- and B-lymphocytes, and endothelial cells [10–22]. This, in turn, changes the signaling/functional properties of these cells, allowing persistent *T. cruzi* infection and/or triggering Chagas disease-attributable symptoms [23–25].

Functional dissection of *T. cruzi* biology has been restricted by the limited flexibility of the genetic manipulation technology applicable to this parasite [26]. Besides, the recognized structural and functional heterogeneity of the *T. cruzi* surface coat poses additional methodological complications for the identification of SA acceptors of the TS-mediated reaction [4, 27, 25]. As shown, SA acceptors on the parasite surface such as heavily *O*-glycosylated mucins and mucin-associated surface proteins (MASPs) are encoded by complex and developmentally regulated gene families showing differences in their polypeptide sequence and/or post-translational processing, including *O*-glycosylation [28–32]. In this framework, metabolic labeling approaches emerge as particularly valuable tools for the identification and molecular characterization of SA acceptors of the TS-catalyzed reaction.

In the last years, novel metabolic labeling techniques that transcend the limitations of conventional strategies using radioactive or stable isotopes were developed. In these techniques, collectively known as Staudinger-Bertozzi ligation and click chemistry, the presence of the small chemical reporter used as a probe is revealed by a downstream chemical reaction with an exogenous label which is typically either a fluorophore or an affinity tag [33]. In the case of carbohydrate analogs, the most common modification is the presence of an azide group on the carbon 2, which does not interfere on their cellular uptake and/or subsequent incorporation into glycans [34]. Modified sugars are then labeled by different compounds capable of reacting with the azide group. Among azide-specific reactions, those involving phosphines and cyclooctynes (copper-free) are bio-inert and suitable to use in living cell, whereas those involving alkynes (copper-catalyzed) are very toxic and can be used to label fixed cells or cell extracts. Finally, the modularity of bioorthogonal chemical detection and ease of appending different labels to the azide-reacting compound (i.e., biotin group, affinity tag) allow multiple forms of glycoconjugate analysis (e.g., imaging and biochemical) or enrichment in a single experiment [34].

In this chapter, we describe the protocol developed in our labs to study SA acceptors of the TS reaction on both *T. cruzi* parasites and different host cell types. This method is based on the use of a synthetic “SA donor” analog, i.e., a SA residue with an azide group on its carbon 5, which is α 2–3-linked to a β -galactopyranose. When added exogenously, such compound is recognized as a suitable SA donor by the *T. cruzi* TS, hence leading to the

formation of a Neu5A α 2–3Gal β signature on SA acceptor glycoconjugates. Herein described method allowed the undertaking of structural and/or functional studies on the *T. cruzi* membrane composition and dynamics, and shed new light on the complex interphase between this parasite and the mammalian host [31, 35–39].

2 Materials

2.1 Metabolic Labeling of Neo-sialylglycoconjugates Formed by *T. cruzi* trans-Sialidase

1. *T. cruzi* parasites or mammalian cells (see Note 1).
2. Refrigerated micro-centrifuge.
3. 1 $\times$ phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4. Sterilize by autoclaving.
4. 2-Deoxy-D-glucose stock solution: 1 M in ddH₂O.
5. Stock solution of the SA donor analog: Neu5A α 2–3Lac β OMe or Neu5A α 2–3Gal β OMe (both from Glycohub, Inc.): 100 mM in ddH₂O.
6. Recombinant *T. cruzi* TS (see Note 2).
7. Stock solution of the bioorthogonal probe: 1.25 mM phosphine-FLAG or 0.5 mM cyclooctyne-FLAG or cyclooctyne-biotin (see Note 3) in DMSO.
8. Sterile Eppendorf tubes and micropipette tips.

2.2 Imaging-Based Methods

1. *T. cruzi* parasites or mammalian cells.
2. Refrigerated micro-centrifuge.
3. PBS.
4. Electron microscopy grade *p*-formaldehyde (PFA) solution: 16% (w/v).
5. Primary monoclonal anti-FLAG antibody (see Note 4).
6. Alexa Fluor-conjugated secondary antibodies.
7. Stock solution of 4',6-diamidino-2-phenylindole (DAPI): 1 mg/mL in ddH₂O.
8. Coverslips (diameter: 12 mm).
9. NH₄Cl stock solution: 1 M in ddH₂O.
10. Indirect immunofluorescence (IIF) blocking buffer: 2% (w/v) BSA, 2.5% (v/v) horse serum in PBS.
11. Glass slides.
12. Poly-L-lysine stock solution: 0.1% (w/v) in ddH₂O.
13. Mounting reagent.
14. Fluorescence microscope.

15. Swinging bucket refrigerated micro-centrifuge.
16. Sterile Falcon and Eppendorf tubes and tips.
17. Flow cytometry equipment and analyzing software.

2.3 Biochemical-Based Methods

1. SDS-PAGE and Western blot device, including transfer apparatus, transfer buffer, blotting membrane (PVDF, nitrocellulose), and blotting paper.
2. Lysis/immunoprecipitation (IP) buffer: 150 mM NaCl, 0.1% (v/v) NP40, 1% (v/v) Triton X-100, 50 mM Tris-HCl pH 7.6, complete protease inhibitor cocktail, 50 μ M Tosyl-L-lysine Chloromethyl ketone hydrochloride (TLCK).
3. 1 $\times$ Tris-buffered saline (TBS): 50 mM Tris-HCl pH 7.6, 150 mM NaCl.
4. Western blot washing solution: 0.05% (v/v) Tween-20 in TBS.
5. Primary monoclonal anti-FLAG antibody.
6. Horseradish peroxidase (HRP)-conjugated streptavidin.
7. HRP-conjugated secondary antibodies.
8. Protein G-agarose slurry.
9. SDS-PAGE sample buffer: 106 mM Tris-HCl, 141 mM Tris base, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.51 mM EDTA, 0.22 mM Coomassie Brilliant Blue G250, 0.175 mM phenol red pH 8.5. Prepare at least a 5 $\times$ SDS-PAGE sample buffer solution.
10. Anti-FLAG M2 mAb magnetic beads or anti-DYKDDDDK affinity gel.
11. Magnetic separator.
12. Sterile Falcon and Eppendorf tubes and micropipette tips.
13. Refrigerated micro-centrifuge.
14. FLAG peptide.

3 Methods

3.1 Metabolic Labeling of Neo-sialylglycoconjugates Formed by *T. cruzi* trans-Sialidase

This protocol may be used to label *T. cruzi* parasites and different types of mammalian cells.

1. Wash the cell suspension three times with chill cold PBS (*see* Note 5).
2. Resuspend in PBS supplemented with 1 mM Neu5Aza2-3Lac β OME or 1 mM Neu5Aza2-3Gal β OME at 1×10^8 cells/100 μ L.
3. Add 100 ng of recombinant *T. cruzi* TS (this step is not necessary when working with *T. cruzi* trypomastigotes) [2] (*see* Note 6).
4. Allow the reaction to proceed for 15–30 min at room temperature (RT) with gentle agitation (*see* Note 7).

5. Wash the cells at least twice with chill cold PBS.
6. Resuspend the cells to a concentration of $1 \times 10^8/200 \mu\text{L}$ with the bioorthogonal probe (phosphine-FLAG, 250 μM ; cyclooctyne-FLAG or cyclooctyne-biotin, 50 μM) in PBS for 30 min at RT (Table 1).

3.2 Imaging-Based Methods

3.2.1 Non-permeabilising Indirect Immunofluorescence Assays

1. Harvest at least 1×10^7 cells, and wash them three times with 1 mL of PBS.
2. Fix samples for 30 min in PBS 4% (v/v) PFA at RT.
3. Dilute to 1% (v/v) PFA with PBS, and incubate overnight at 4 °C.
4. Wash at least twice with PBS.
5. Adhere 1×10^6 cells to poly-L-lysine-coated coverslips for 30 min at RT.
6. Wash cells with PBS, and incubate them with 100 μL of PBS containing 25 mM NH_4Cl for 30 min at RT (to quench surface aldehydes).
7. Block with 100 μL of IIF blocking buffer for 30–60 min.
8. Incubate with primary antibody in 100 μL of IIF blocking buffer for 1 h.
9. Immerse the coverslip three times (10 s each) in a 50 mL-beaker with PBS.
10. Incubate with secondary antibody in 100 μL of blocking solution for 1 h, in the dark.
11. Immerse the coverslip three times (10 s each) in a 50 mL beaker with PBS.
12. Incubate with DAPI (0.5 $\mu\text{g/mL}$ in PBS) for 15 min.
13. Wash once with PBS.
14. Mount samples, and let dry overnight at RT, in the dark.
15. Observe slides under a fluoresce microscope (Fig. 1).

3.2.2 Non-permeabilizing Flow Cytometry Assays

1. Harvest cells, and wash them twice with filtered PBS. Remember to consider one extra tube for isotype control (e.g., control without primary antibody).
2. Fix samples for 30 min in PBS 4% (v/v) PFA at RT. Then dilute to 1% (v/v) PFA with PBS, and incubate overnight at 4 °C.
3. Wash at least twice with PBS.
4. Incubate 1×10^7 with 100 μL of PBS containing 25 mM NH_4Cl for 30 min at RT (to quench surface aldehydes).
5. Wash once with PBS (1 mL), and block with 100 μL of IIF blocking buffer for 30–60 min.
6. Incubate with primary antibody in 100 μL of IIF blocking buffer for 1 h.

7. Wash three times with 1 mL of PBS.
8. Incubate with secondary antibody in 100 μ L of blocking solution for 1 h, in the dark.
9. Wash three times with 1 mL of PBS.
10. Analyze using FACS (Fig. 2).

3.3 Biochemical-Based Methods

3.3.1 Western Blot

1. Label cells with the FLAG epitope or biotin as described in Subheading 3.1.
2. Resuspend parasites or mammalian cells in 1 $\times$ SDS-PAGE sample buffer, and boil for 5 min.
3. Resolve extracts corresponding to 1.5×10^7 cells into SDS-PAGE, and transfer onto PVDF or nitrocellulose membrane.
4. Block the membrane for 1 h with TBS supplemented with 5% low-fat dried milk or 2% BSA for Western blot development using anti-FLAG primary antibody or streptavidin-HRP, respectively.
5. Incubate for 1 h with anti-FLAG primary antibody or streptavidin-HRP (in case your sample is labeled with a biotin group) in blocking solution.
6. Wash three times with Western blot washing solution (10 min each).
7. Incubate with 2 μ g/mL of secondary antibody in Western blot blocking solution.
8. Wash twice with Western blot washing solution (10 min each).
9. Wash once with TBS for 10 min. Avoid **steps 7 and 8** when working with HRP-streptavidin.
10. Proceed with chemiluminescent detection.

3.3.2 Affinity Purification

1. Label cells with FLAG epitope as described in Subheading 3.1.
2. Lyse labeled cells or parasites with lysis/IP buffer for 15 min.
3. Centrifuge at 14,000 rpm for 30 min at 4 $^{\circ}$ C.
4. Incubate the supernatant fraction for 16 h at 4 $^{\circ}$ C with 100 μ L of either anti-FLAG mAb magnetic beads or anti-DYKDDDDK affinity gel.
5. Wash four times with IP buffer (1 mL, 5 min each).
6. Elute with two column volumes of 1.5 mg/mL of FLAG peptide in IP buffer for 3 h or with 2 $\times$ SDS-PAGE sample buffer without reducing agents for 10 min (see Note 8).

4 Notes

1. Cultures of *T. cruzi* should be performed according to local regulations about biosafety and laboratory best practices, taking into consideration that parasites can be accidentally transmitted to humans through the mucosae or wounded skin. *T. cruzi*-infected animals and their organs should be manipulated as containing live parasites, thus possibly infective for humans. For details on how to obtain, culture, and purify major *T. cruzi* developmental forms (i.e., epimastigotes, metacyclic trypomastigotes, amastigotes, and cell-derived trypomastigotes), see [39].
2. We are currently using an engineered TS molecule (accession number: L26499) bearing seven amino acid substitutions (Asn58Phe, Ser495Lys, Val496Gly, Glu520Lys, Asp593Gly, Ile597Asp, and His599Arg) as compared to the wild-type enzyme [2].
3. We evaluated the performance of three bioorthogonal probes: phosphine-FLAG (Sigma, discontinued), dibenzylcyclooctyne-FLAG, and dibenzylcyclooctyne-biotin (both from Jena Bioscience). Their features and applications are detailed in Table 1.
4. We recommend either anti-FLAG M2 clone (Cat: F3165, Sigma) or anti-DYKDDDDK Tag Antibody clone L5 (Cat: 637303, Biolegend).
5. If you are working with adherent cells, avoid the use of proteolytic enzymes (like trypsin or collagenase) for cell de-attachment. In this case, we recommended the use of PBS supplemented with 2 mM EDTA and a cell scraper as de-attachment method.
6. When labeling mammalian cells or *T. cruzi* developmental forms that do not express a significant amount of TS (such as epimastigotes) it is required the addition of recombinant *T. cruzi* TS. In the presence of *T. cruzi* TS, lack of labeling may be attributed to steric hindrance phenomena or, more likely, to the absence of SA acceptors (i.e. glycoconjugates bearing terminal β -galactopyranoses in the correct configuration) on the surface of the target cell. In this regard, it is noteworthy that *T. cruzi* amastigote forms, in spite of expressing certain mucin-like genes [40] are completely refractory to TS-catalyzed sialylation (see Fig. 2).
7. This incubation step may also be performed on ice, with a concomitant decrease in labeling efficiency [39].
8. Avoid the use of low pH solutions (i.e. 0.1 M Glycine HCl, pH 3.0) for elution because the SA linkage is labile under these conditions.

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Fig. 1. SA residues are not incorporated onto the surface of *T. cruzi* amastigote forms. IF analysis of a representative example of differential SA acquisition by a mixed population of *T. cruzi* cell-derived trypomastigotes (yellow arrowheads) and extracellular amastigotes (white arrowheads). Incorporation of Neu5Az onto the parasite surface coat was revealed by labeling the neo-sialylglycoconjugates with phosphine-FLAG and anti-FLAG antibody as described in text

Fig. 2.

Kinetics of SA incorporation onto the surface of *T. cruzi* trypomastigotes. Flow cytometry-based determination of Neu5Az transfer to live cell-derived trypomastigotes using Neu5Aza2-3LacβOMe compound as SA donor. The amount of Neu5Az transferred was revealed by dibenzyl cyclooctene-FLAG and anti-FLAG antibody. (a) Typical cytogram obtained at the end of the Neu5Az transfer protocol. (b) Correlation between the concentration of Neu5Aza2-3LacβOMe compound used in the assay and the amount of cell-derived trypomastigote labeling

Table 1

Features and recommended applications of different bioorthogonal probes

	Copper-catalyzed reaction	Copper-free reactions
Azide-reactive compounds structure	Alkyne	Phosphine
Suppliers	Thermo-Fisher Jena Bioscience Click Chemistry Tools	Cyclooctyne Thermo-Fischer Cayman Chemical Jena Bioscience Click Chemistry Tools
Western blot	✓	✓
Immunoprecipitation		✓
Flow cytometry/immunofluorescence (fixed cells)	✓	✓
Flow cytometry/immunofluorescence (live cells)		✓