EVIDENCE FOR THE PLASMA MEMBRANE LOCALIZATION OF CARBOHYDRATE-CONTAINING MACROMOLECULES FROM EPIMASTIGOTE FORMS OF *TRYPANOSOMA CRUZI*

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1. Introduction

The existence of carbohydrate-containing substances on the surface of *Trypanosoma cruzi* was demonstrated by cell agglutination with Con A [1] and by cytochemical studies in tissue culture forms [2]. A sugar-containing macromolecular complex has been extracted from *T. cruzi* epimastigote whole cells. This complex consists of 4 PAS-positive bands as judged from SDS–polyacrylamide gel electrophoresis (bands A–D) [3]. The electrophoretic pattern of bands A, B and C can be altered by pre-treatment of the complex with a proteolytic enzyme, pointing to a glycoprotein nature. Band D, however, is a carbohydrate- and lipid-rich substance which has tentatively been named LPPG [4–6]. Although both the complex and pure LPPG are effective in the inhibition of Con A-induced agglutination of epimastigotes, non-circumstantial evidence for the plasma membrane localization of these substances was lacking. Such evidence and the antigenic nature of part of the complex are presented in this communication.

2. Experimental

Epimastigote forms of *T. cruzi* (Y strain) were cultivated in LIT medium [7]. Extraction of the carbohydrate complex and LPPG was performed as in [3,4]. Bands A–C were purified from the complex by exhaustive extraction with chloroform/methanol/H_2O (10:10:3) until no LPPG could be detected in SDS–polyacrylamide gels. The glycoproteins were then recovered by precipitation with 75% ethanol.

Plasma membrane vesicles were obtained according to [8]. Labeling of surface carbohydrate was performed essentially as in [9] except that KB^3H_4 (New England Nuclear) was added to cells 90 min after galactose oxidase (Worthington). Cell washing was made 2 min after label addition with excess cold potassium borohydride in 150 mM NaCl. Before use, appropriately diluted galactose oxidase was preincubated at 50°C for 30 min [9]. Under these conditions no protease contamination could be detected as judged by the azo-albumin reaction [10]. Cells incubated with KB^3H_4 in the absence of galactose-oxidase were not labeled.

Antibodies were obtained from rabbits as follows: animals were initially inoculated with 2 mg of a mixture of bands A–C (500 μg carbohydrate) together with an equal volume of Freund's complete adjuvant. Two weeks later animals were weekly injected for 1 month with 1 mg antigen plus an equal volume of Freund's incomplete adjuvant. Rabbits were bled by cardiac puncture. Serum collected from animals before antigen injection was used as control. Indirect immunofluorescence was performed in microscope slides with formalized epimastigotes, as follows: suitable immune serum dilutions were incubated with fixed cells at 34°C for 30 min. After gentle washing the slides were reincubated with a 1/20 dilution of fluorescein-conjugated anti-rabbit IgG antibody. Immunodiffusion was made in 1% agarose plates

Abbreviations: Con A, concanavalin A; LPPG, lipopeptidophosphoglycan; SDS, sodium dedocylsulfate; BSA, bovine serum albumin; TCA, trichloroacetic acid

(7.5 X 2.5 cm) for 24 h at room temperature followed by 24 h at 4°C in a moist atmosphere. Immunoelectrophoresis was performed in 1% agarose plates with sodium barbital (pH 8.6) at 6 mA for 60 min. Microscope slides were immersed in 1% NaCl for at least 24 h, drained and stained with amidoblack. For these experiments the immune serum was 10-fold concentrated by lyophilization.

Polyacrylamide disc-gel electrophoresis was made in 15% gels in the presence of 0.1% SDS [11]. After PAS-staining gels were scanned at 560 nm [12]. Gels were transversely sliced at ~1.5 mm thickness and prepared for liquid scintillation spectrometry [13]. Total neutral sugar was determined by the phenol–sulfuric acid method [14].

3. Results

3.1. *Labeling of the cell surface with KB^3H_4*

Phenol extraction of intact cells pre-labeled with KB^3H_4 and galactose oxidase yielded a radioactive glycoprotein complex having 260, 290 and 90 cpm/μg carbohydrate in three different experiments. The electrophoretic pattern of the carbohydrate complex and the tritium incorporation profile are shown in fig.1. As can be seen band D (LPPG) was not labeled. A similar pattern was observed when the isolated complex was labeled with galactose oxidase and KB^3H_4. This means that even though LPPG is galactose rich [4], it cannot be labeled by this method. In order to further assure that label was bound to the macromolecules the isolated complex was chromatographed on a Bio Gel P-150 column in the presence of 0.1% SDS as in [3]. All of the radioactivity was eluted with glycoproteins A–C. The specificity of the reaction was assessed by paper chromatography of the labeled carbohydrate complex after hydrolysis in 2 N HCl for 2 h at 100°C. Only galactose was found to be labeled using a solvent system composed of butanol/pyridine/H_2O (3:1:1).

These experiments show that glycoproteins A–C are accessible from the outside pointing to their presence in the plasma membrane to epimastigote forms.

3.2. *Antibody production*

Rabbit serum prepared against the mixture of

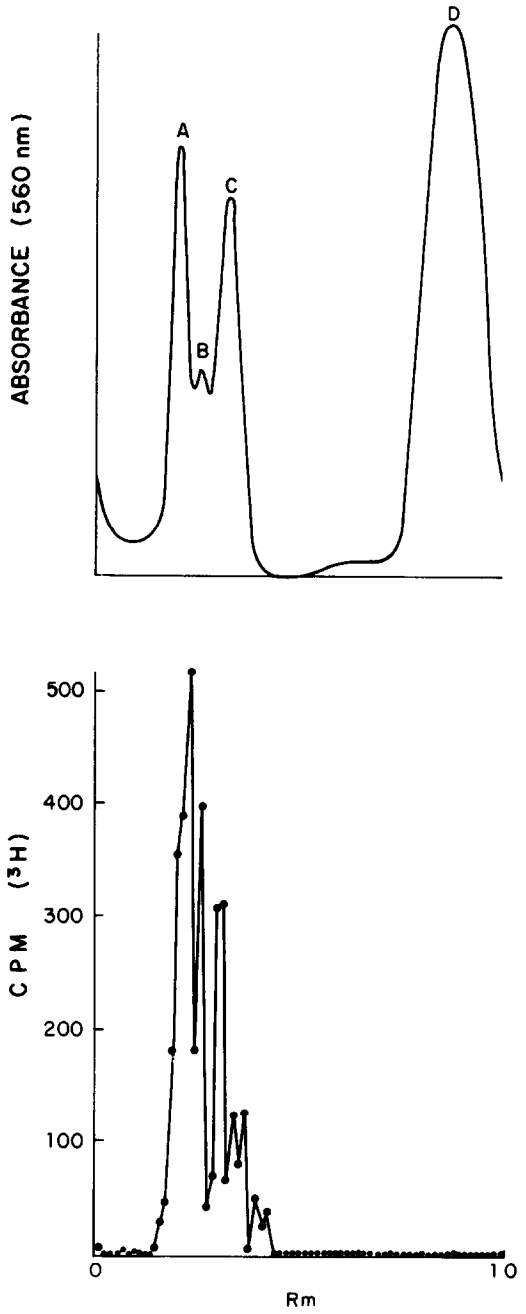

Fig.1. Radioactive profile of the *T. cruzi* carbohydrate-containing macromolecular complex subjected to 0.1% SDS–polyacrylamide gel electrophoresis. The top gel containing 50 μg total carbohydrate was stained with PAS and scanned at 560 nm. The bottom gel containing 15 μg total carbohydrate was sliced and counted as in section 2. Recovery of radioactivity was 25%.

Fig.2. Stained immunoelectrophoresis plate demonstrating reactivity between rabbit immune serum (10-fold concentrated) and antigen ABC. Anode is at left of figure.

bands A–C was able to agglutinate epimastigote forms of *T. cruzi* up to a dilution of 1/512. Serum (diluted 1/4) collected before immunization failed to agglutinate the cells. Immune serum (1/128), preadsorbed to 200 μg/ml of a mixture of bands A–C for 15 min at room temperature, failed to agglutinate a suspension of 1.3×10^8 cells/ml. No inactivation of agglutinating antibodies was detected when the adsorbents used were LPPG (300 μg/ml) or BSA (860 μg/ml).

At least three lines of precipitation could be observed by immunodiffusion or immunoelectrophoresis (fig.2). Although bands A–C were not separated from each other their mixture was free of other proteins and of LPPG as judged by polyacrylamide gel electrophoresis. The preparation, however, contained a 260 nm absorbing contaminant. Treatment of the mixture with pancreatic ribonuclease (1 mg/ml) and deoxyribonuclease (100 μg/ml) for 4 h at 37°C in the presence of 5 mM magnesium acetate and 5 mM Tris–HCl (pH 7.4) [15] followed by phenol reextraction and ethanol precipitation did not alter substantially the A_{260} of the preparation. This might be due to strong interactions between polynucleotides and glycoproteins. However polynucleotides could be removed by DEAE-cellulose column chromatography or 5% TCA precipitation. The TCA-soluble glycoproteins give essentially the same immunoelectrophoretic patterns as those in fig.2 showing that contaminating polynucleotides did not elicit antibody formation.

Indirect immunofluorescence revealed plasma membrane fluorescence after incubation of epimastigotes with immune serum. Fluorescence was observed on the cell body as well as on the flagellar membrane with a serum dilution of 1/512. These results also indicate the presence of glycoproteins A–C in the flagellar and cell plasma membranes of *T. cruzi*.

On the other hand, LPPG failed to induce formation of antibodies in rabbits when injected alone (≤ 10 mg), or mixed with lecithin and cholesterol in the form of a sonicated water suspension, or complexed with Con A.

3.3. *Carbohydrate complex enrichment in plasma membrane vesicles*

Vesicles shed by epimastigote cells upon incubation with 200 mM acetate (pH 4.0) consist of plasma membrane fragments as can be demonstrated by conventional electron microscopy, particle distribution in freeze-fracture studies and enrichment of cell bound iodine during vesicle purification [8]. Figure 3 shows a comparison between cell homogenate and vesicle

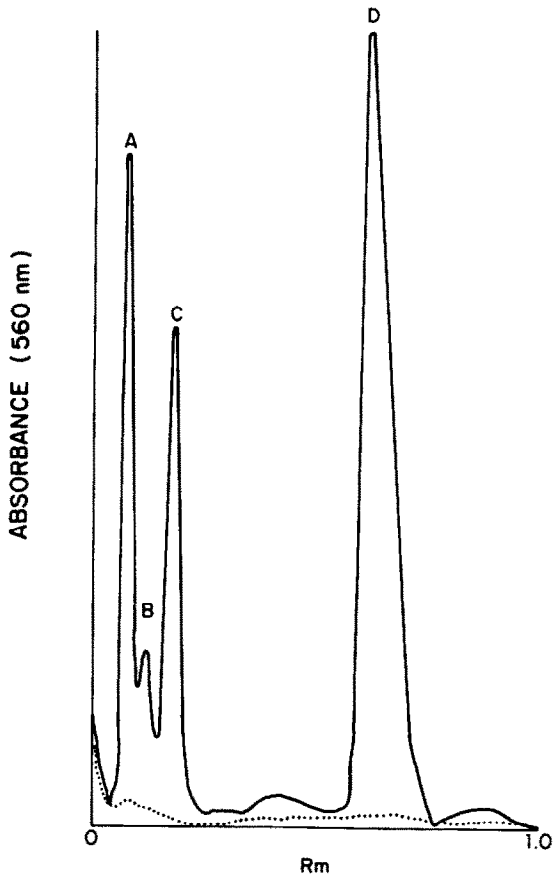

Fig.3. Polyacrylamide gel spectrophotometric profiles of membrane vesicles (——) and whole-cell homogenate (– – –) stained with PAS and scanned at 560 nm. Each gel contained 4.2 μg protein.

contents of bands A–C and LPPG when comparable amounts of protein from both preparations were subjected to SDS–polyacrylamide gel electrophoresis.

It was possible to show a linear relationship between developed color, determined through the areas under the curves obtained by densitometry, and carbohydrate content of each PAS-positive band (5–50 μg carbohydrate). Therefore, polyacrylamide gel electrophoresis of suitable amounts of whole cell homogenate and purified vesicles made it possible to estimate the relative enrichment of the sugar-rich macromolecules in these membrane preparations. In three different experiments, bands A, C and LPPG were on the average 30–50-fold more represented in vesicles than in whole cell homogenates. Band B, much less represented, was not quantitated due to the overlapping of bands A and C. Due to a partial overlap of LPPG and unknown compounds running with the marker dye samples were extracted with chloroform–methanol (2:1) before electrophoresis. These experiments demonstrate that bands A, C and LPPG are plasma membrane bound.

4. Discussion

The fact that plasma membrane vesicles from *T. cruzi* epimastigote forms are enriched in the sugar-containing macromolecules [3] is, per se, sufficient evidence for the plasma membrane localization of these substances. This evidence is reinforced by the external labeling of bands A–C by KB^3H_4 through the galactose oxidase catalyzed reaction. This reaction shows a strict specificity for galactose and *N*-acetyl galactosamine [16] and has been applied to the external labeling of glycoproteins and glycolipids belonging to cell surfaces [9,17,18]. The fact that LPPG, although membrane bound and galactose rich, could not be labeled either in vivo or in vitro suggests that galactose residues in LPPG are not accessible to enzyme attack probably due to a steric hindrance.

Antibodies produced against a mixture of bands A–C revealed at least three different precipitin lines suggesting that all three substances are antigenic. However, this conclusion can only be definitely drawn after the isolation of each substance is achieved. In any case, the immunofluorescence and the cell agglutination studies with antibodies against the mixture of bands A–C reinforce the conclusion for a plasma membrane localization of these glycoproteins. LPPG failed to induce antibody formation in rabbits under the conditions described. This result does not agree with speculations [15] attributing the identity of a circulating antigen found in infected animals to LPPG. However, the interesting possibility that this substance has different antigenic properties depending whether or not it is associated to its natural milieu, should be tested.

Since the four PAS-positive bands are the only ones to appear in SDS–polyacrylamide gels even if total cell homogenates are screened, one cannot escape from the conclusion that they constitute, at least quantitatively, the most important group of plasma membrane bound sugar-containing macromolecules in epimastigote forms of *T. cruzi*.

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