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The variant surface glycoproteins of *Trypanosoma equiperdum*

Identification of a phosphorylated glycopeptide as the cross-reacting antigenic determinant

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The cross-reacting antigenic determinant in the variant surface glycoproteins (VSGs) of *Trypanosoma equiperdum* was studied by testing the ability of VSG glycopeptides to bind heterologous anti-VSG sera. VSG glycopeptide purification revealed the presence of 3 oligosaccharide sidechains on the mature VSG. These consist of two sidechains containing only mannose and glucosamine and a third containing galactose and mannose (in a 5:1 ratio) as well as phosphorous and ethanolamine. This phosphorylated fragment completely blocked the binding of VSG to heterologous anti-VSG and therefore contained the cross-reacting determinants.

Immunological cross-reaction *Phosphorylated glycopeptide* *Trypanosome* *Variant surface glycoprotein*

1. INTRODUCTION

Antigenic variation enables the African trypanosomes to evade the host's immune system. The phenomenon is due to the sequential expression of alternative variable surface glycoproteins (VSGs) which coat the entire surface of the parasite [1,2]. The purified VSGs derived from the same species of trypanosomes exhibit extensive differences in their amino acid composition, isoelectric points, N-terminal amino acid sequences [3–5] and in their secondary structure [6]. Even the total carbohydrate composition varies considerably among the different VSGs from *T. brucei* [7], although in *T. equiperdum* the sugar content is more constant in total amount (7–8%) and composition (mainly mannose, galactose, *N*-acetyl glucosamine) [5]. In contrast, VSGs are characterized by the presence of common structures which are probably implicated in the transfer and attachment of the VSGs to the cell membrane. Radioimmunoassay tests reveal the presence of cross-reacting determinants [8–10] which appear to involve carbohydrate moieties [11] attached to the C-terminal amino acid through ethanolamine residues [12]. We have reported in *T. equiperdum* the presence of an unusual phosphorylated residue located in the C-terminal part of the VSGs [13]. Here, we demonstrate that this phosphorylated residue is located in a glycopeptide which contains all the cross-reacting determinants.

2. MATERIALS AND METHODS

2.1. *Trypanosome and VSG purification*

Two *T. equiperdum* (Pasteur strain) variants were used: the basic antigenic type BoTat-1 (Bordeaux-Trypanozoon-antigenic-type 1) and BoTat-28, a variant appearing late in a chronic rabbit infection. The VSGs were purified by af-

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finity chromatography on concanavalin A–Sepharose as in [14].

2.2. *Isolation of glycopeptides*

BoTat-1 VSG was labelled in vivo with ^{32}P and purified as in [13]. About 3000 cpm (by Cherenkov counting) were mixed with 20 mg BoTat-1 VSG and digested with 0.2 mg trypsin (TPCK, 224 units/mg, Worthington) in 2 ml 0.05 M ammonium bicarbonate buffer pH 8.5 at 37°C for 45 min. The reaction was terminated by the addition of 0.3 ml 1 M acetic acid. After addition of 1 g urea (Ultrapur, Schwartzman) the material was applied directly on a Bio Gel-P30 column (100–200 mesh, 2.6 × 100 cm) equilibrated with distilled water. Fractions (4 ml) were collected at 12 ml/h. The column effluent was monitored as appropriate: continuously for absorption at 206 nm using a LKB Uvicord S, in fraction aliquots for ^{32}P radioactivity counting and carbohydrate assay. The fractions containing ^{32}P activity were pooled, lyophilized and resuspended in 1 ml 0.01 M ammonium bicarbonate buffer (pH 8.5). One-half (0.5 ml) was digested with pronase (protease from *Streptomyces grinseus,* Sigma) and the rest with proteinase K (E. Merck, Darmstadt) for 26 h at 37°C (3 additions of 2 μg pronase or proteinase K at 0.15 and 20 h and addition at 15 h of 0.06 g urea). After a further addition of 0.18 g urea, both digests were fractioned on a Bio Gel-P4 column (−400 mesh 1.6 × 100 cm) equilibrated with water. Fractions (2 ml) were collected at 4 ml/h and the effluent was monitored for absorption and radioactivity as already described.

2.3. *Amino acid composition*

Samples were hydrolysed in sealed tubes for 24 h in 5.6 M HCl at 105°C under vacuum. Amino acids, hexosamines and ethanolamine were separated on a single column of DC-6A resin (Dionex Corporation, Sunnyvale, CA 94086) on a Beckman 119 CL amino acid analyzer using the buffer system in [15].

2.4. *Carbohydrate analysis*

Carbohydrate was detected in the fractions with the phenol/sulfuric acid method [16]. Gas–liquid chromatographic analysis of neutral and 2-amino-2-deoxy-hexose were performed as in [17] using a 5840A Hewlett Packard gas chromatograph equipped with a dual flame ionization detector.

2.5. *Assay of VSG cross-reacting determinants*

Heterologous inhibition radioimmunoassay was used to assay for the presence of VSG cross-reacting determinants.

The assay was performed essentially as in [10] using 1–3 ng ^{125}I-labelled antigen, hyperimmune rabbit antisera raised against purified VSGs and sheep anti-rabbit IgG serum. Unlabelled competitors (VSG or fragments) were mixed with the antiserum at 37°C, 1 h prior to the addition of the labelled antigen. Heterologous antisera were used at the concentration sufficient to precipitate ~70% of the antigen in the absence of competitor.

3. RESULTS

In order to characterize the phosphorylated fragment and to identify the cross-reacting antigenic determinants, BoTat-1 VSG glycopeptides were purified and analysed for chemical composition and cross reaction.

3.1. *Purification of BoTat-1 VSG C-terminal glycopeptides*

As reported [13], limited trypsinisation of BoTat-1 ^{32}P-labelled VSG (M_r 58 000) results in a large N-terminal fragment of M_r 40 000 which is no longer labelled as determined by SDS gel electrophoresis and autoradiography. Thus 20 mg BoTat-1 VSG mixed with labelled variant glycoprotein were digested with trypsin and the resultant peptides were subjected to gel filtration on a Bio Gel-P30 column. Several peaks were detected (fig.1) in particular a large peak (A) eluting in the excluded

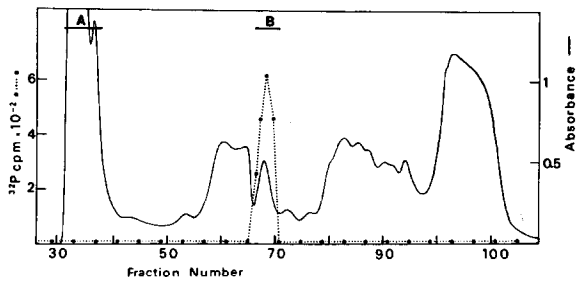

Fig.1. Purification of a tryptic phosphorylated fragment from BoTat-1 VSG on Bio-Gel P30: (——) A_{206}; (●···●) ^{32}P radioactivity.

volume of the column and corresponding to the M_r 40000 peptide as determined by SDS gel electrophoresis, and a homogeneous peak (B) of app. M_r 10000. All of the ^{32}P radioactivity was located in peak B which is also characterized by the presence of carbohydrates.

Peak B was further digested either with pronase or with proteinase K and subjected to gel filtration on a Bio Gel-P4 column (fig.2). There were two peaks (1 and 2) which always eluted in the same position on P4 regardless of which protease was used, as well as other peaks containing higher M_r peptides. The ^{32}P radioactivity was only detected in peak 2, although some label was also present in the higher-M_r pronase fragments and probably represents partial digestion products.

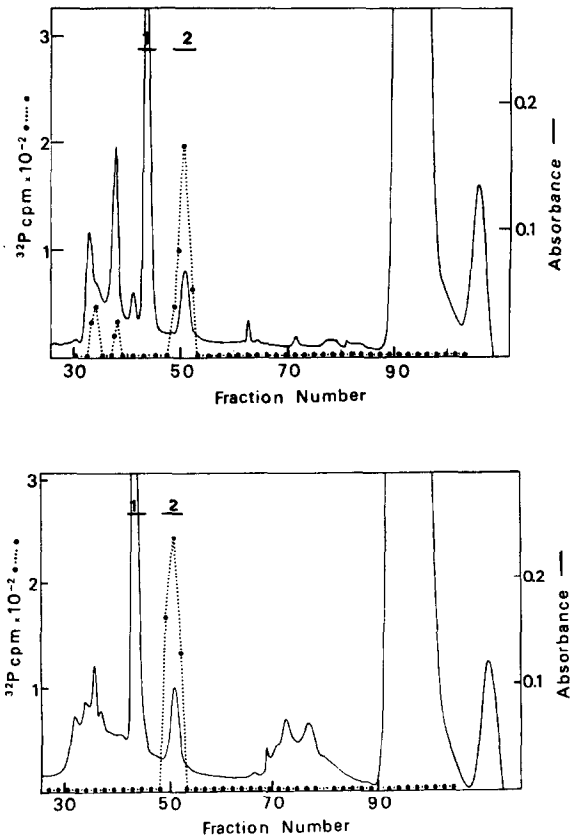

Fig.2. Fractionation on Bio-Gel P4 of the tryptic phosphorylated fragment digested with pronase (A) or proteinase K (B): (——) A_{206}; (●···●) ^{32}P radioactivity.

3.2. *Chemical composition of the glycopeptides*

The chemical composition (table 1) shows the presence of 2 types of glycopeptides in BoTat-1 VSG.

Glycopeptide 1 (Gp1) contains 2 amino acids: threonine and asparagine and/or aspartic acid. The carbohydrate moiety was found to be composed of mannose and *N*-acetyl glucosamine in a molar ratio of 5:2, respectively.

Glycopeptide 2 (Gp2) contains two amino acids, serine and glycine which were detected in a molar ratio of 5:1, respectively. This glycopeptide was also characterized by the presence of phosphorus and ethanolamine (EtN).

3.3. *Localisation of the cross-reacting determinants within the phospho glycopeptide Gp2*

Previous competitive radioimmunoassay tests [10] have shown the presence of cross-reacting determinants in 2 VSGs of *T. equiperdum*, BoTat-1 and BoTat-28. The different BoTat-1 VSG fragments were tested for their ability to inhibit the cross-reaction of ^{125}I-BoTat-1 VSG *vs* anti-BoTat-28. The tryptic fraction corresponding to peak B contained the cross-reacting determinants (fig.3). After pronase or proteinase K treatment,

Table 1

Chemical composition of the proteinase K glycopeptides from BoTat-1 VSG

	Gp1	Gp2
Asx	1	–
Thr	1.5	–
Ser	–	0.8
Gly	–	1
Etn	–	+
^{32}P	–	+
Man	5	1
Gal	–	5
GlcNac	2[a]	–

[a] The initial value was corrected for the coefficient of release of GlcNac in the conditions employed

Results are expressed in molar ratio relative to Asx and GlcNac for Gp1 and to Gly and Man for Gp2

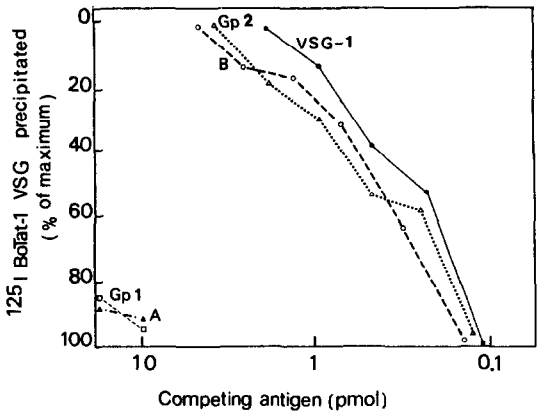

Fig.3. Inhibition of the cross-reaction ^{125}I-BoTat-1 VSG versus anti-BoTat-28 VSG by native BoTat-1 VSG, tryptic fragment A (▲•—▲) and B (○--○) and proteinase K glycopeptides Gp1 (□--□) and Gp2 (△--△).

only the phosphoglycopeptide Gp2 was able to inhibit the heterologous reaction. Inhibition reached 100% and the slope of the inhibition curve produced by the glycopeptide was identical to that of the intact VSG.

4. DISCUSSION

We report here an isolation procedure which allows the purification of 2 BoTat-1 VSG glycopeptides. Chemical composition revealed the presence of 2 types of structures.

Glycopeptide Gp1 contains a classical oligosaccharide sidechain composed of mannose and *N*-acetyl glucosamine in a respective molar ratio of 5:2. This composition differs from the glycosyl sidechain in [18] which is located in the N-terminal part of BoTat-1 VSG on asparagine at position 57 within the sequence Ala–Asn*–Ser–Thr. The latter oligosaccharide also contains only mannose and *N*-acetyl glucosamine, but in a respective molar ratio of 9:2. This N-terminal mannose rich sidechain is probably involved in the agglutination of BoTat variants in the presence of concanavalin A [14].

Glycopeptide Gp2 is characterized by an unusual structure containing phosphorus and ethanolamine. According to the results reported by Holder [12], Gp2 probably corresponds to the G-terminal glycopeptide. Indeed, in *T. brucei* VSGs, an oligosaccharide is linked through ethanolamine in an amide bond to the α-carboxyl group of the C-terminal amino acid which is either aspartic acid or serine (which is also present in Gp2). As described in [13], phosphorus is present in *T. equiperdum* VSGs to the extent of 1 mol phosphate/mol glycoprotein, and the linkage is resistent to alkaline phosphatase. Since ^{32}P could only be detected in Gp2, this glycopeptide appears to contain all the phosphorus. The phosphate group probably links, through a phosphodiester bond, either an ethanolamine group to a sugar residue or 2 sugar residues.

This phosphoglycopeptide also contains the cross-reacting determinants and shows the same affinity for cross-reacting antibodies as intact VSG. However, this cross-reacting determinant does not seem to be unique since all VSGs studied did not cross react with each other (unpublished).

This unusual structure is probably implicated in the transfer and attachment of VSGs since it has been recently reported that *T. brucei* VSGs exist in a membrane form, which is modified or degraded during the phosphoglycoprotein purification [19].

Work is in progress to characterize further these structures.

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