Proteolytic Release of Cell Surface Proteins during Differentiation of *Trypanosoma brucei*†

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ABSTRACT: The surface of the bloodstream forms of *Trypanosoma brucei* is covered by the abundant glycosylphosphatidylinositol-anchored variant surface protein (mfVSG). During differentiation of bloodstream forms to the insect-stage or procyclic forms, the mfVSG is replaced by another glycoprotein, designated procyclic acidic repetitive protein (PARP) or procyclin. Shortly after differentiation is triggered in vitro, a cell-associated fragment of mfVSG can be detected which is subsequently released into the culture medium. In the case of the mfVSG of the variant clone MITat 1.4 (470 amino acid residues), fragmentation occurs close to the COOH-terminus (Gln_{433} or Thr_{434}) as shown by NH_2-terminal sequencing, metabolic labeling experiments, and molecular weight determinations by laser desorption/ionization mass spectrometry. Two invariant surface glycoproteins, which are anchored in the membrane by hydrophobic sequences close to their COOH-termini, are lost from the surface with similar kinetics as mfVSG. The data suggest that trypanosomes synthesize or activate a developmentally-regulated proteinase which degrades the glycoproteins at the surface, at the membrane lining the flagellar pocket, and/or in an early endocytic compartment.

Proteolytic processing of membrane proteins to soluble isoforms appears to be an important mechanism with a variety of functional implications in cell biology (Ehlers & Riordan, 1991). Particularly striking examples are the processing of the β-amyloid precursor of Alzheimer disease (Selkoe, 1990) or of proteins involved in transmembrane signaling such as the transforming growth factor α (Massague, 1990). The precise proteolytic cleavage sites have been identified only in a few cases, and no convincing candidates for membrane protein solubilizing proteases have been characterized (Ehlers & Riordan, 1991). Proteolysis may also be a mechanism that promotes changes in surface protein composition in differentiating cells. One such system is the subject of this paper.

The unicellular, parasitic protozoan *Trypanosoma brucei* has a multistage life cycle in the mammalian host and in an arthropod vector, the tsetse fly (Vickerman, 1985). The best characterized differentiation step is the transition of the bloodstream forms found in the mammal to the procyclic forms, which arise from the bloodstream forms upon transfer into the midgut of the biting insect. The differentiation is already initiated in the mammal by the transition of the dividing slender forms to intermediate and stumpy forms, resulting in a pleomorphic population. Under appropriate conditions, a pleomorphic population of bloodstream forms can transform synchronously to procyclic cells in vitro (Figure 1). Upon prolonged passage in rodents, trypanosomes lose the ability to form intermediate and stumpy forms. Such monomorphic strains retain the ability to differentiate to procyclic forms in vitro; however, the transition occurs much less synchronously (Overath et al., 1983; Roditi et al., 1989).

The major surface proteins of the bloodstream and procyclic forms are stage-specific. Bloodstream forms are covered by a dense coat of the membrane-form variant surface glycoprotein (mfVSG,[1] 10^7 molecules/cell) which can be changed by antigenic variation (Cross, 1990). Recently, two other much less abundant glycoproteins, designated ISG65 and ISG75 (for invariant surface glycoprotein with a molecular mass of 65 and 75 kDa, respectively), have been described (Ziegelbauer & Overath, 1992; Ziegelbauer et al., 1992). Procyclic cells carry a predominant surface glycoprotein designated procyclin (Richardson et al., 1988) or procyclic acidic repetitive protein (PARP; Clayton & Mowatt, 1989). During the transition between the two stages, mfVSG synthesis is repressed, and procyclin synthesis is rapidly turned on (Figure 1). Previous experiments have shown that in differentiating cells mfVSG is released from the surface by a specific process leading to the appearance of a fragment in the culture medium (Overath et al., 1983; Shapiro, 1986; Bülow et al., 1989). This fragment (50 kDa) has a smaller size than mfVSG (61 kDa) or its soluble counterpart (sVSG) obtained upon cell lysis by cleavage of the glycosylphosphatidylinositol (GPI) membrane anchor by the endogenous GPI-specific phospholipase C [GPI-PLC; see review by Carrington et al. (1991a)], and it lacks an immunological epitope, which is newly exposed on sVSG as a consequence of GPI-PLC cleavage. Therefore, coat release appears to involve proteolytic cleavage leading to a most likely NH_2-terminal fragment.

This report extends previous studies in two directions. First, the processing of mfVSG during differentiation is characterized as part of a more general cell surface protein processing activity, and, second, the cleavage site in one particular VSG is determined to an accuracy of about two amino acid residues.

EXPERIMENTAL PROCEDURES

Trypanosomes and Differentiation Protocol. Differentiation of the monomorphic *T. brucei* variant clone MITat 1.4

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[1] Abbreviations: VSG, variant surface glycoprotein; mfVSG, membrane-form VSG; sVSG, soluble VSG; nVSG, NH_2-terminal fragment of VSG; ISG, invariant surface glycoprotein; GPI, glycosylphosphatidylinositol; PLC, phospholipase C; PBS, phosphate-buffered saline.

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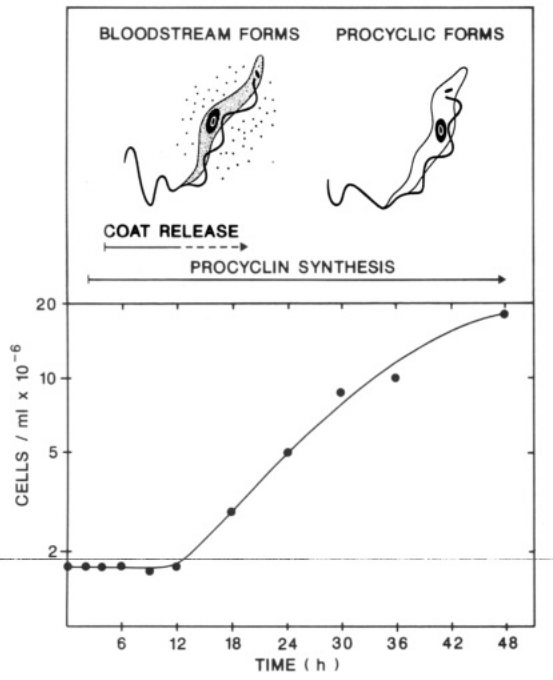

FIGURE 1: Differentiation of *T. brucei*. The lower part of the figure shows the growth curve of a differentiating population of pleomorphic trypanosomes after differentiation was triggered at $t = 0$ h. The cell count remains constant for about 12 h. Synthesis of procyclin, the main surface protein of procyclic forms, becomes detectable after a delay of 1–2 h, while coat release is initiated after a lag of about 4 h (upper panel). For about 12 h, the cells carry both antigens. These kinetics are typical for the experiments performed in this study. For further details, see Bülow et al. (1989), Roditi et al. (1989), and Ziegelbauer et al. (1990).

(Cross, 1975) was performed as described by Czichos et al. (1986) and Bülow et al. (1989) in medium B (Baltz et al., 1985) supplemented with glutamine (300 mg/L), threonine (48 mg/L), sodium pyruvate (110 mg/L), hypoxanthine (14 mg/L), adenosine (21.4 mg/L), thymidine (4 mg/L), penicillin/streptomycin (12 mL/L; Sebak, Aidenbach, FRG), and 15% inactivated fetal bovine serum without feeder layer cells. Differentiation of the pleomorphic variant clone AnTat 1.8 (Bülow et al., 1989), isolated from infected mouse blood by DEAE-cellulose chromatography (Lanham & Godfrey, 1970), was performed as described by Ziegelbauer et al. (1990) in the presence of hemin (7.5 μg/mL) but without feeder cells. The VSGs of variant clones AnTat 1.8 and MITat 1.4 are serologically identical.

Labeling Procedures. MITat 1.4 trypanosomes were labeled with either [^{35}S]cysteine (1181 Ci/mmol; Amersham, Braunschweig, FRG) or [^{35}S]methionine (1094 Ci/mmol; NEN, Dreieich, FRG) in media lacking the respective amino acid as described by Bülow et al. (1989). Labeling of variant clone MITat 1.4 with [1-^{3}H]ethan-1-ol-2-amine hydrochloride (29.8 Ci/mmol, Amersham) was performed at 37 °C, a density of 5×10^5 cells/mL, and 2.5 μCi/mL for 16–18 h in Baltz medium containing 15% inactivated bovine serum previously dialyzed against phosphate-buffered saline (PBS). The cells were washed twice and subsequently triggered for differentiation.

Cell surface proteins of variant clone AnTat 1.8 trypanosomes were biotinylated at a cell density of 1×10^8 cells for 5 min at 27 °C using 0.5 mM NHS-LC-biotin [sulfosuccinimidyl 6-(biotinamido)hexanoate; Pierce, Rockford, IL] as described by Ziegelbauer and Overath (1992) and subjected to the differentiation protocol thereafter.

Isolation of sVSG and nVSG. Radioactively labeled VSG from the culture supernatant was immunoprecipitated as described by Czichos et al. (1986) using formalin-fixed *Staphylococcus aureus* cells (Immuno-Precipitin; BRL, Neu-Isenburg, FRG) or protein A–Sepharose 4 fast flow beads (Pharmacia-LKB, Freiburg, FRG) as solid support. For the isolation of glycosylated products, [^{35}S]methionine-labeled VSG containing culture supernatant (0.5 mL) was supplemented with 0.5 mL of lectin buffer (10 mM Tris-HCl, 140 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% bovine serum albumin, 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 0.5 mM iodoacetamide, 1 mM phenylmethanesulfonyl fluoride, and 0.02% sodium azide, pH 7.4) and 50 μL of Con A–Sepharose beads (Sigma, Deisenhofen, FRG), previously washed twice in lectin buffer. The sample was rotated for 60 min at 4 °C. Thereafter, the beads were washed for 5 min with the following buffers: lectin buffer (twice); 10 mM Tris-HCl/140 mM NaCl, pH 7.4; 10 mM Tris-HCl, pH 6.8. Bound proteins were eluted by boiling for 3 min in sample buffer and analyzed by SDS–PAGE and fluorography.

Alternatively, unlabeled VSG was isolated by affinity chromatography. Anti-MITat 1.4 sVSG IgG (70 mg, a generous gift from Dr. R. Bülow) was coupled to activated CH-Sepharose 4B (Pharmacia-LKB) as described by the manufacturer. Culture supernatant (460 mL) from differentiated MITat 1.4 trypanosomes (titrated to pH 8.0 using 1 M Na_2HPO_4) was applied to the IgG–Sepharose column (10 mL, previously washed with 0.2 M glycine hydrochloride, pH 2.4, and equilibrated to PBS, pH 8.0) at a rate of 12.5 mL/h at 4 °C. The column was washed with 26 volumes of PBS, pH 8.0, and bound proteins were eluted with 0.2 M glycine hydrochloride, pH 2.4, at a rate of 60 mL/h. Protein-containing fractions were neutralized using 1 M Tris-HCl, pH 8.0, pooled, and concentrated by ultrafiltration.

Deglycosylation. Deglycosylation of isolated VSG with either *N*-glycosidase F or endoglycosidase H was performed as suggested by the manufacturer (Boehringer, Mannheim, FRG) for 16–18 h at 37 °C.

SDS–PAGE, Fluorography, and Immunoblotting. These protocols have been described (Bülow et al., 1989; Ziegelbauer & Overath, 1992). Mouse antisera against recombinant proteins ISG65 and ISG75 were used at a dilution of 1:200 and streptavidin-conjugated alkaline phosphatase (Sigma) at a dilution of 1:1000 or 1:5000.

Protein Determination and Amino Acid Sequence Analysis. Protein determinations were performed using the bicinchoninic acid method as described by the manufacturer (Pierce). For NH_2-terminal amino acid sequence analysis, proteins were applied to a 9% SDS–PAGE and blotted onto a poly(vinylidene difluoride) membrane, stained with Coomassie Blue, excised, and sequenced by Edman degradation on an Applied Biosystems 477A protein sequencer (kindly performed by Dr. R. Halter, Max-Planck-Institut für Biologie, Tübingen).

Mass Spectrometry. Molecular weight determinations by mass spectrometry were performed as described by Hillenkamp and Karas (1990).

RESULTS

Release of Cell Surface Proteins during Differentiation. The surface proteins of a pleomorphic population of bloodstream forms of *T. brucei* variant clone AnTat 1.8 were biotinylated using sulfosuccinimidyl 6-(biotinamido)hexanoate (Ziegelbauer & Overath, 1992). The only protein that could be detected on the blot of a cell lysate using streptavidin coupled to alkaline phosphatase was mfVSG (M_r 61 000, Figure 2A, $t = 0$ h). After biotinylation, the cells were subjected to the differentiation conditions, a decrease in temperature from 37

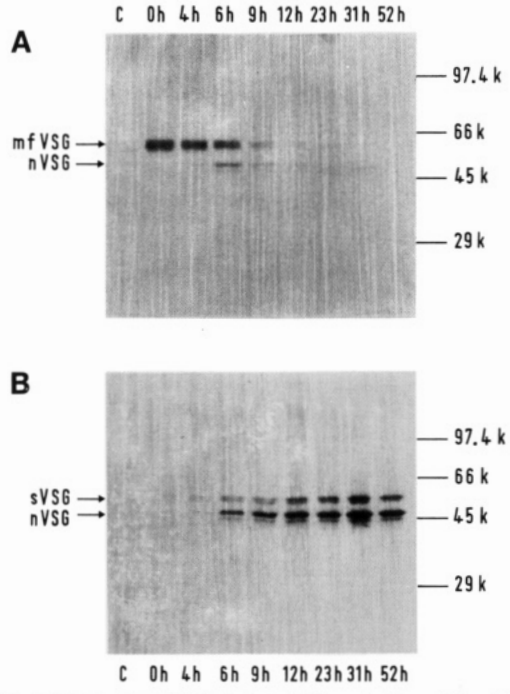

FIGURE 2: Release of mfVSG during differentiation. Bloodstream forms of variant clone AnTat 1.8 were labeled by NHS-LC-biotin and then subjected to the differentiation procedure. At the times indicated, cells and supernatant were separated by centrifugation. SDS lysates from 2×10^6 cells (panel A) or proteins immunoprecipitated from 1 mL of supernatant using anti-VSG antibody (panel B) were separated by SDS–PAGE, blotted onto a poly(vinylidene difluoride) membrane, and probed with streptavidin-conjugated alkaline phosphatase. C refers to a control of 2×10^6 unlabeled bloodstream forms (panel A) or of proteins precipitated from the supernatant 49 h after differentiation was triggered (panel B).

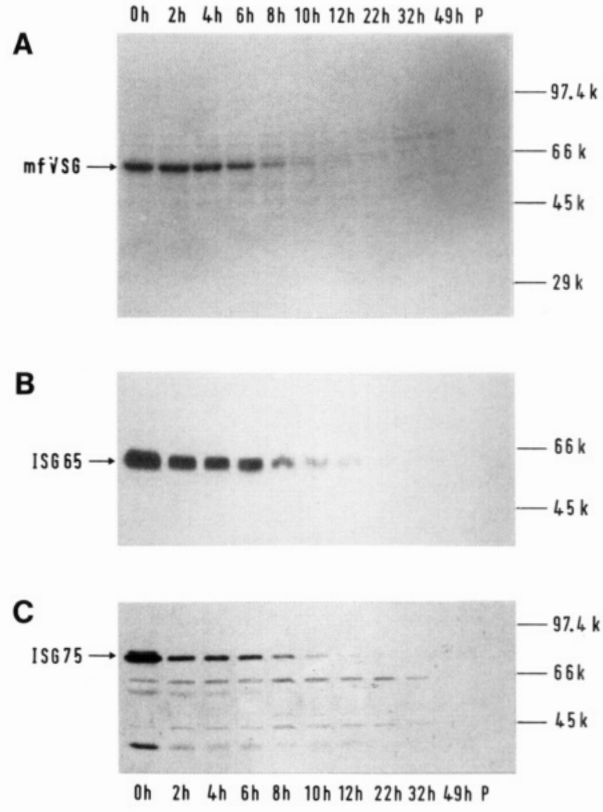

FIGURE 3: Release of invariant surface glycoproteins. Bloodstream forms of variant clone AnTat 1.8 were subjected to differentiation in vitro. After the times indicated, cells and supernatant were prepared by centrifugation. The SDS lysates from 5×10^6 cells were separated by SDS–PAGE, blotted onto a poly(vinylidene difluoride) membrane, and stained with Ponceau S (panel A) or probed with antisera against ISG65 (panel B) or ISG75 (panel C).

to 27 °C and the addition of *cis*-aconitate and citrate. Transformation to procyclic cells occurred synchronously and with similar kinetics as described before for unbiotinylated cells (Ziegelbauer et al., 1990); also, the cells did not divide during the first 12 h and, thereafter, grew exponentially as procyclic cells (cf. Figure 1). The amount of cell-associated mfVSG remained constant for about 4 h and decreased to near-undetectable levels by 12 h. Biotinylation enabled the detection of a cell-associated fragment of VSG (M_r 53 000, cf. Figure 2A, t = 4, 6, and 9 h), henceforth referred to as nVSG (NH_2-terminal fragment of VSG). The abundance of this fragment reached a maximum at 6 h, i.e., at the same time when a similar sized fragment appeared in the culture supernatant (panel B). The amount of nVSG in the supernatant increased until 12 h and then remained constant. In summary, nVSG was initially formed in association with the cells and was subsequently released into the culture supernatant. As observed before (Bülow et al., 1989), coat release appeared to be a specific process triggered with a delay after subjecting the trypanosomes to the differentiation conditions.

Starting from the earliest sampling time (4 h), a variable amount of the total VSG (10–30%) was continuously released into the medium in the form of a 61-kDa component, most likely sVSG [Figure 2B; compare Overath et al. (1983)]. The appearance of this component did not show the typical lag observed for nVSG, and it occurred under conditions where the trypanosomes did not transform to procyclic cells (Bülow et al., 1989). Finally, for both nVSG and sVSG, minor amounts of additional biotinylated components with a molecular mass about 2 kDa smaller could be precipitated by anti-VSG antibodies (Figure 2B). It is possible that these are unglycosylated homologues of nVSG and sVSG, respectively.

In addition to mfVSG, the recently described transmembrane proteins ISG65 and ISG75 vanished from the parasite's surface during the differentiation to procyclic forms. As shown by the immunoblots in Figure 3, ISG65 decreased with similar kinetics as mfVSG (compare panels B and A), while the processing of ISG75 appeared to be faster (panel C). Attempts to demonstrate cleavage products in the culture supernatant were unsuccessful, most likely because these proteins are more than 100-fold less abundant than VSG.

Effect of Protease Inhibitors on VSG Release. Several protease inhibitors were tested and shown to be unable to inhibit nVSG formation during differentiation [leupeptin, 100 nM; benzamidin, 100 μM; N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 10 μM; E-64, 10 μM; pepstatin A, 100 nM; amastin, 10 μM; and phosphoramidon, 10 μM]. Because these inhibitors can be expected to have access to a protease located at the surface or in an early endocytic compartment, the experiments may suggest the existence of a novel hydrolase resistant to commonly used inhibitors.

Characterization of nVSG. These experiments were performed with the monomorphic variant clone MITat 1.4, because its mfVSG has been extensively analyzed in terms of amino acid sequence (Allen et al., 1982), GPI anchor (Ferguson et al., 1988), and N-linked carbohydrate structure (Zamze et al., 1990). The VSGs from variant AnTat 1.8 used so far and from MITat 1.4 are serologically identical and are encoded by closely related genes (Matthyssens et al., 1981). nVSG was purified from the culture supernatant of differentiated cells by affinity chromatography of anti-MITat 1.4 sVSG IgG coupled to Sepharose. From 460 mL of culture

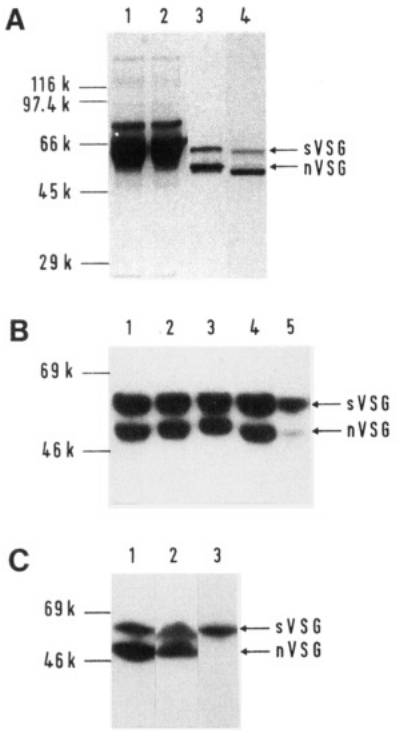

FIGURE 4: Purification and characterization of nVSG. Panel A: analysis of nVSG purification by SDS–PAGE. Lane 1, supernatant (30 μg of protein) obtained from MITat 1.4 cells about 48 h after differentiation was triggered; lane 2, flow-through of the anti-VSG affinity column; lane 3, proteins eluted from the anti-VSG affinity column; lane 4, eluted proteins after treatment with *N*-glycanase F. Proteins were visualized by staining with Coomassie Blue R-250. Panel B: glycosylation of nVSG. Cells from variant clone MITat 1.4 were labeled with [^{35}S]methionine and subjected to the differentiation procedure. Culture supernatant prepared after 36 h was subjected to precipitation by anti-VSG antibody using *Staphylococcus aureus* cells or Con A–Sepharose, and bound proteins were analyzed by SDS–PAGE and fluorography. Lane 1, proteins precipitated using anti-VSG antibody; lane 3, proteins precipitated using Con A–Sepharose; lane 2, a mixture of proteins eluted from *S. aureus* cells or Con A–Sepharose was applied. The electrophoretic mobility of nVSG is slightly faster in lane 1, intermediate in lane 2, and slower in lane 3. This artifactual behavior is caused by interference with the large amount of the unlabeled IgG heavy chain (about 55 kDa) present in the sample. Lane 4, proteins precipitated from the supernatant of a previous Con A–Sepharose precipitation performed in the presence of 100 mM methyl α-mannoside by anti-VSG antibody; lane 5, proteins precipitated by anti-VSG antibody from the supernatant of a previous Con A–Sepharose precipitation. Panel C: metabolic labeling of nVSG. Bloodstream forms from the variant clone MITat 1.4 were labeled with [^{35}S]methionine (lane 1), [^{35}S]-cysteine (lane 2), or [^{3}H]ethanolamine (lane 3) and triggered for differentiation to procyclic cells. After 36 h, proteins were precipitated from the respective supernatant using anti-VSG antibody and analyzed by SDS–PAGE and fluorography.

supernatant, about 0.2 mg of a mixture of sVSG and nVSG was recovered (Figure 4A, lanes 1–3). An aliquot of the mixture was separated by SDS–PAGE and blotted onto a poly(vinylidene difluoride) membrane, and the N-terminal sequence of both nVSG and sVSG was determined. For both proteins, the sequence AKEALETKT was found, which is identical to the published NH_2-terminal sequence of MITat 1.4 VSG (Allen et al., 1982). This result implied that nVSG and sVSG differ at their COOH-termini.

MITat 1.4 VSG is known to be modified by an oligomannose-type oligosaccharide at residue Asn_{420} (Allen et al., 1982; Zamze et al., 1990). The presence of this oligosaccharide and, therefore, Asn_{420} in nVSG was established by precipitating proteins from the culture supernatant of [^{35}S]methionine-labeled MITat 1.4 trypanosomes by Con A–Sepharose. As

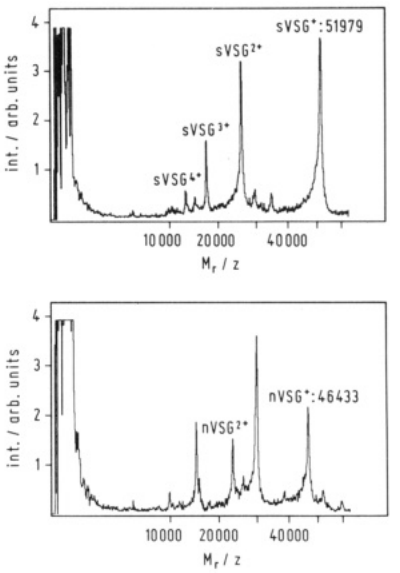

FIGURE 5: Laser desorption/ionization mass spectra of VSGs. Top, sVSG; bottom, nVSG. sVSG and nVSG were isolated by affinity chromatography, deglycosylated using endoglycosidase H, concentrated, and equilibrated to ddH_2O by ultrafiltration. As shown by SDS–PAGE, the nVSG sample contained only small amounts of sVSG, which was preferentially lost during the ultrafiltration step. Ion peaks of sVSG and nVSG carrying one or more positive charges are indicated. The unlabeled peaks of the lower panel correspond to singly or doubly charged endoglycosidase H (29 806 Da).

shown in Figure 4B, lane 3, both sVSG and nVSG could be precipitated. Moreover, methyl α-mannoside inhibited binding of nVSG and sVSG to the Con A–Sepharose, because larger amounts of both proteins could be precipitated by anti-VSG antibody after Con A–Sepharose precipitation in the presence (lane 4) rather than in the absence of 100 mM methyl α-mannoside (lane 5). The presence of an oligosaccharide in nVSG was also consistent with the reduction in the apparent molecular mass of 2 kDa after *N*-glycanase F treatment (panel A, lane 4).

The GPI anchor of MITat 1.4 mfVSG is linked via an ethanolamine residue to the COOH-terminal amino acid Asp_{470} (Ferguson et al., 1988). Previous investigators (Rifkin & Fairlamb, 1985) have found that mfVSG is essentially the only protein that can be metabolically labeled in bloodstream forms by [^{3}H]ethanolamine. It is evident from Figure 4C that nVSG could be labeled with [^{35}S]methionine (lane 1) and [^{35}S]cysteine (lane 2) but not with [^{3}H]ethanolamine (lane 3). Therefore, the COOH-terminus of MITat 1.4 nVSG must be localized between residues Asn_{420} and Asp_{470}.

The recently developed laser desorption/ionization mass spectrometry (Hillenkamp & Karas, 1990) enables the exact determination of the molecular mass of proteins. Therefore, about 10 μg of MITat 1.4 sVSG, purified from bloodstream forms by affinity chromatography, and about 4 μg of the mixture of sVSG and nVSG, purified from the culture supernatant, were deglycosylated using endoglycosidase H, desalted, concentrated by ultrafiltration, and subjected to molecular weight analysis. Respective laser desorption/ionization spectra are shown in Figure 5. For sVSG, the ion peak was observed at a molecular weight of 51 979 (upper panel). In addition multiple charged ions were detected. The molecular weight obtained for sVSG was in good agreement with the calculated weight (51 900.15 or 52 062.35; see Table I for a detailed calculation). For nVSG, a molecular weight of 46 433 was determined (lower panel). A second protein with a molecular weight of 29 806 corresponded to endoglycosidase H used for deglycosylation. Comparison of the

Table I: Molecular Weight Determination of Deglycosylated MITat 1.4 sVSG and nVSG by Mass Spectrometry

	calcd M_r from protein portion[a]	calcd M_r of GPI moiety[b]	N-acetylglucosamine M_r	total calcd M_r	M_r determined by mass spectrometry
sVSG: aa Ala_1–Asp_{470}	50217.26	1479.67 (1641.87)[c]	203.22	51900.15 (52062.35)	51979 ± 50
nVSG: aa Ala_1–Thr_{432}	46069.73		203.22	46272.83	
nVSG: aa Ala_1–Gln_{433}	46197.87		203.22	46400.97	46433 ± 50
nVSG: aa Ala_1–Thr_{434}	46298.97		203.22	46502.07	
nVSG: aa Ala_1–Gln_{435}	46247.11		203.22	46630.21	

[a] See Allen et al. (1982). [b] See Ferguson et al. (1988). [c] The glycosylphosphatidylinositol moiety of MITat 1.4 is heterogeneously galactosylated [see Ferguson et al. (1988)]. The structures used for molecular weight calculations represent 55% of all structures present in MITat 1.4 VSG.

molecular weight obtained for nVSG (46 433) with the calculated molecular weight of VSG fragments differing in single amino acids (see Table I) suggested that either Gln_{433} or Thr_{434} is the COOH-terminal amino acid of nVSG.

DISCUSSION

The surface coat of trypanosomes is formed by the tight packing of rod-shaped mfVSG dimers. Early proteolysis experiments by Johnson and Cross (1979) demonstrated that VSGs are organized into an NH_2-terminal domain, including about 60% of the protein, and a COOH-terminal domain. The domain boundary has been located between amino acid residues 320 and 380 [see summary in Carrington et al. (1991b)]. Dimeric NH_2-terminal domains have been crystallized for several VSGs (Metcalf et al., 1988), and the crystal structure has been solved for two of them (Freyman et al., 1984, 1990; Metcalf et al., 1987). Comparatively little is known of the structure of the COOH-terminal domain, which forms the link to the platelike carbohydrate moiety of the GPI membrane anchor (Homans et al., 1989). On the basis of the number and spacing of conserved cysteinyl residues, COOH-terminal domains of different VSGs can be divided into several classes (Cross, 1984; Carrington et al., 1991b). The VSG of variant MITat 1.4 belongs to domain type 1, which is characterized by eight cysteinyl residues forming four disulfide bridges (cf. Figure 6; Allen & Gurnett, 1983). Since trypsin readily cleaves at Lys_{381}/Lys_{383} and Lys_{425}/Lys_{427} but not at neighboring residues, the disulfide-bridged regions (Cys_{389}–Cys_{417} and Cys_{447}–Cys_{468}) are believed to be organized in subdomains. Therefore, the polypeptide region between these subdomains may form a loop which enables proteolytic attack at residues 433 (Gln) or 434 (Thr) during the differentiation of bloodstream to procyclic forms. Our knowledge on the location of the cleavage site is limited by the accuracy of the molecular weight determination of nVSG by mass spectrometry (Table I).

Formation of a VSG fragment in the culture medium has also been demonstrated during differentiation of variant clones MITat 1.2, AnTat 1.1 (Bülow et al., 1989), and ILTat 1.3 (Shapiro, 1986). The VSGs of the latter two variants could be cleaved in the same region of the polypeptide because they likewise belong to domain type 1 (Figure 6); however, only ILTat 1.3 VSG contains consecutive Gln-Thr residues. It is possible that the hypothetical proteinase involved in the cleavage of VSG is also responsible for the processing of two invariant surface proteins (Figure 3) which have been proposed to be anchored in the membrane by α-helices (Ziegelbauer et al., 1992). In contrast, the GPI-anchored procyclin which is inserted into the surface during transformation (Figure 1; Roditi et al., 1989; Ziegelbauer et al., 1990) is clearly resistant to this activity, most likely because the proteinase cannot cleave the long Glu-Pro repeat which connects the GPI anchor and the NH_2-terminal domain of the polypeptide. Further work is required to characterize the putative proteinase which appears to be resistant to several common inhibitors.

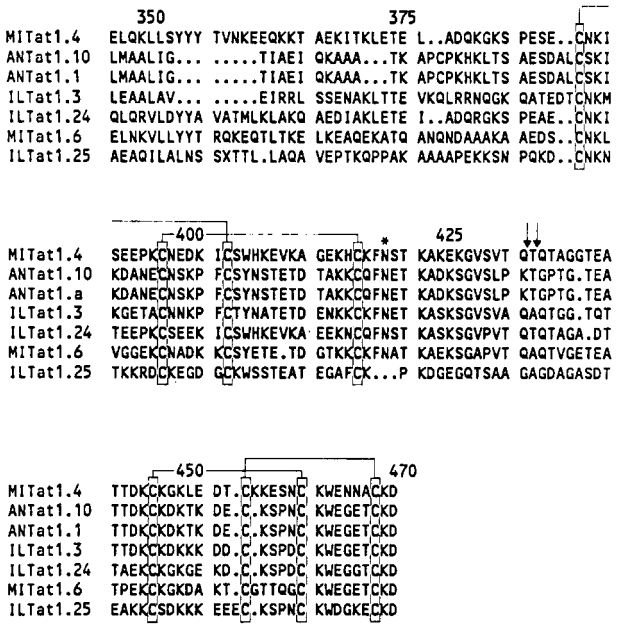

FIGURE 6: Amino acid sequences of the COOH-termini of type 1 VSGs [cf. Carrington et al. (1991b)] were aligned using the UWGCG program PILEUP (Devereux et al., 1984). Numbering refers to the MITat 1.4 sequence. Conserved cysteine residues are boxed, and disulfide linkages are shown for MITat 1.4 VSG (Allen & Gurnett, 1983). The arrows indicate the suggested cleavage sites in MITat 1.4 VSG; the asterisk indicates the N-glycosylation site.

Proteolytic processing of mfVSG is expected to lead to a COOH-terminal peptide of about 4 kDa connected to the GPI anchor. Attempts to demonstrate such a product, i.e., in cells labeled with radioactive ethanolamine or cysteine, were unsuccessful.

Figure 2 demonstrates that nVSG can be detected in association with the parasite before it is released into the culture medium. Since mfVSG is a dimer, random cleavage by a protease may initially lead to molecules where only one of the monomers is hydrolyzed; the nVSG subunit will remain bound to the other, GPI-anchored subunit. Release ensues only after cleavage of both subunits. The proteinase involved in the cleavage may either be activated or newly synthesized after triggering differentiation and may act at the cell surface, the membrane lining the flagellar pocket formed by an invagination of the plasma membrane around the arising flagellum [see review by Balber (1990)], and/or in endocytotic vesicles, which are in a rapid equilibrium with the flagellar pocket membrane (Seyfang et al., 1990). Nearly all the mfVSG of bloodstream forms is found as nVSG or sVSG in the culture medium [Figure 2 and see Overath et al. (1983) and Bülow et al. (1989)]; therefore, we consider it unlikely that processing and recycling take place from a late endosomal or lysosomal compartment because a large part of the VSG would be expected to be completely degraded in the cell. We tried to investigate the cleavage process by electron microscopy

(unpublished results). Surface-biotinylated trypanosomes were subjected to the differentiation protocol and embedded in Lowicryl, and ultrathin resin sections were probed with anti-biotin antibody and protein A–gold. Bloodstream forms showed a strong labeling at the cell surface, the membrane lining the flagellar pocket, and components [i.e., medium-derived transferrin; cf. Overath et al. (1992)] in the lumen of the flagellar pocket, which declined after 6 h. Furthermore, the membrane of intracellular vesicles close to the flagellar pocket was labeled, showing that VSG was taken up by endocytosis. However, the small size of these vesicles (100-nm diameter) did not allow us to decide if their *lumen* became filled with labeled material at the time (6 h, cf. Figure 2) when the concentration of cell-associated nVSG reached a maximum. Definitive experiments regarding the site of nVSG formation may require the development of more selective reagents, i.e., specific antibodies against the COOH-terminus of nVSG.

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