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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF PLASMA MEMBRANE POLYPEPTIDES OF *TRYPANOSOMA BRUCEI*

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A plasma membrane-enriched vesicle fraction has been prepared from *Trypanosoma brucei* by sonication and differential centrifugation on sucrose gradients. This fraction is enriched 5-fold in the plasma membrane marker enzymes adenyl cyclase (EC 4.6.1.1) and ouabain-inhibitable, ($Na^+ + K^+$)-dependent adenosine triphosphatase (EC 3.6.1.3). It is also enriched up to 14-fold in iodinated surface proteins, and up to 4-fold in [^{3}H]mannose-labeled glycoproteins, of which the major variable surface coat glycoprotein is the main constituent. Proteins of the plasma membrane fraction and other subcellular fractions have been identified by electrophoretic analysis in sodium dodecyl sulfate-polyacrylamide gradient slab gels. Several high molecular weight surface glycopeptides have been selectively investigated and partially characterized by a combination of metabolic labeling with [^{3}H]mannose, lactoperoxidase-catalyzed surface iodination, and affinity chromatography on Con A-Sepharose. In addition to the major variable surface coat glycoprotein (estimated $M_r = 58000$), there are several minor surface glycopeptides ($M_r = 76000$, 86000 and 92000–100000)which are apparent extrinsic membrane components, and two surface glycopeptides ($M_r = 42000$ and 130000) which are intrinsic membrane components.

Introduction

African trypanosomes, the etiologic agents of sleeping sickness in humans and ngana in cattle, are protozoan hemoflagellates with a complex developmental cycle involving mammalian and tsetse fly hosts. The parasite cell surface is dominated by a major variable surface coat glycoprotein [1]. Trypanosomes evade the immune response of the host by a process of antigenic variation [2] involving expression of serologically distinct surface coat glycoproteins by sub-populations of parasites which are unaffected by the trypanocidal response to the major variant antigenic type of the population. Thus, the cell surface of the trypanosome is a critical interface governing the interaction between host and parasite. Modification of this structure or its function as a result of host or parasite activity could be important in regulating intracellular metabolism in the parasite, and in determining the progress of an infection.

Although no metabolic function has been described for the variable surface coat glycoprotein it may function as a barrier between antibodies or drugs and the cell membrane proper. Other functions associated with the surface membrane of

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Abbreviations: Buffer 1, 50 mM Tris-HCl/50 mM KCl/5 mM $MgCl_2$ buffer (pH 7.4); TLCK, tosyl-lysyl chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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trypanosomes include active transport systems for glucose [3] and trypanocidal drugs [4] as well as ouabain-inhibitable, $(Na^+ + K^+)$-dependent ATP-ase activity (EC 3.6.1.3) in *Trypanosoma lewisi* [5] and *Trypanosoma brucei* 427 [6]. Variations in the distribution of intramembrane particles have been demonstrated by freeze-cleave electron microscopy during *T. brucei* infections in both immunocompetent and immunosuppressed rats [7]. In addition, the trypanosome surface has the ability to bind serum proteins, especially nonspecific host immunoglobulins [8–10], and this may be related to the presence of specific receptor protein(s) on the cell surface [11]. There is, however, little structural information about the protein components of the membrane which may be required for expression of these differentiated functions during the developmental cycle of the parasite.

Our interest in the role of trypanosome cyclic AMP in regulation of differentiation in this parasite [12], and in the structure/function interactions between the variable surface coat glycoprotein and other components of the trypanosome membrane has led us to examine the polypeptide composition of the cell membrane of *T. brucei*. Although plasma membrane fractions have been prepared from *T. brucei* [6,13], from epimastigote forms of *Trypanosoma cruzi* [14] and from *Leishmania donovani* promastigotes [15], detailed information on polypeptide composition of these fractions is lacking. We have prepared a plasma membrane-enriched fraction from long slender bloodstream trypomastigotes of *T. brucei* YTatl and have identified several high molecular weight polypeptides of this fraction with respect to their appearance on Coomassie blue stained gels. In addition, we have investigated the ability of cells incubated in [^{3}H]mannose to incorporate this isotope into the major variable surface coat glycoprotein and into other membrane glycopeptides, several of which, by their accessibility to iodination reagents in intact cells, are presumptive cell surface glycoproteins.

Methods

Trypanosomes. YTat1, a triple-cloned pleomorphic variant of *T. brucei* TREU-164 [16] was used in these experiments. Maintenance, infection and isolation of the organisms from rats was previously described [16,17].

Isolation of the plasma membrane-enriched vesicle fraction. Approximately $50 \cdot 10^9$ DEAE-purified trypanosomes [18] were used for each membrane isolation. The cells were washed by suspension in phosphate-buffered saline with glucose (pH 8.0) [16] and centrifugation at $10000 \times g$ for 1 min. The cell pellet was resuspended at $(1–5) \cdot 10^9$ cells/ml in 50 mM Tris-HCl, 5 mM $MgCl_2$, 50 mM KCl (pH 7.4) buffer (buffer 1) containing 1 mM tosyl-lysyl chloromethyl ketone (TLCK), a serine protease inhibitor, and sonicated by 4–7 15-s bursts at minimum setting in a Bronwill 'Biosonik II' sonicator. In experiments where subcellular fractions were assayed for enzymatic activity, the protease inhibitor was omitted from the buffer since it inhibits adenyl cyclase (EC 4.6.1.1) activity [19]. The sonicate was centrifuged for 1 min at $12000 \times g$ to remove nuclei and intact cells, and the pellet was washed once. The supernatants were pooled, and treated with deoxyribonuclease I (final concentration 50 μg/ml) for 5 min at 37°C. The DNAase-treated supernatant was centrifuged at $30000 \times g$ for 60 min to obtain a crude particulate fraction which was suspended in buffer and layered over a cushion of 1.3 M sucrose then centrifuged at $82000 \times g$ for 20 min in the SW 27.1 rotor of a Beckman L5-50B ultracentrifuge. The $82000 \times g$ pellet containing microsomal material and flagellar components was saved and processed for electrophoretic analysis. The material sedimenting to the sample/sucrose interface was removed by aspiration, diluted with buffer 1 and layered over a preformed discontinuous sucrose gradient consisting of layers of 1.12, 1.14, 1.16, 1.18 and 1.20 g/cm^3 sucrose in buffer. The gradient was centrifuged to equilibrium at $60000 \times g$ for 18 h. Membrane material was collected by aspiration at the interfaces of the sucrose layers as follows: 1.12/1.14 interface, band 1; 1.14/1.16 interface, band 2; 1.16/1.18 interface, band 3; and 1.18/1.20 interface, band 4. Membrane fractions were diluted in glass-distilled water or buffer 1 and centrifuged at $82000 \times g$ for 1 h. Membrane pellets were then suspended in the appropriate buffer for immediate analysis or frozen in liquid N_2 until used.

Gel electrophoresis. Subcellular fractions were

diluted with 1/2 volume of solubilization buffer (9% sodium dodecyl sulfate, 3 mM EDTA, 6% β-mercaptoethanol, and 15% glycerol in 30 mM Tris-HCl (pH 6.8)), and immersed for 3 min in a boiling water bath. Samples were analyzed by electrophoresis in sodium dodecyl sulfate 6–20% gradient polyacrylamide slab gels [20]. Gels were stained with Coomassie blue and those containing ^{3}H- or ^{125}I-labeled proteins were dried for fluorography [21] or autoradiography, respectively. Gel lanes containing ^{3}H-labeled polypeptides were also excised from the slab, sliced into 1 mm pieces, dissolved overnight at 37°C in 3% (v/v) Protosol in a cocktail consisting of 4 g of 2,5-diphenyloxazole and 0.1 g of *p*-bis(2-(5-phenyloxazolyl)) benzene per liter of toluene, and analyzed by scintillation spectrometry in a Beckman LS7500 liquid scintillation system.

Iodination. Trypanosomes were iodinated by a modification of the procedure of Marchalonis [22]. Briefly, $1 \cdot 10^9$ trypanosomes isolated from a log-phase infection were suspended in 200 μl of phosphate-buffered saline containing 5 mM KCl, 1 mM $MgCl_2$ and 10 mM glucose (pH 7.4) [12] on ice, and 50 μl of lactoperoxidase (1 mg/ml, 2 U/ml) and 1 mCi of carrier-free Na ^{125}I were added. At zero time, the reaction was initiated by addition of 30 μl of 0.001% H_2O_2 in buffer. An additional 10 μl of H_2O_2 was added 1 min and 2 min after the start of iodination. The reaction was terminated at 3 min by dilution of cells in the cold buffer. Cells were washed four times by centrifugation and resuspension in the buffer containing 10 μM KI. For subcellular fractionation experiments, iodinated cells were mixed with $(5–10) \cdot 10^9$ unlabeled carrier cells and fractions prepared as described.

In reconstruction experiments, lentil lectin-purified variable surface coat glycoprotein was prepared and iodinated by the chloramine-T method as previously described [16].

Metabolic labeling of glycoproteins with [^{3}H]-mannose. Freshly isolated trypanosomes were incubated for 90 min at 37°C at $(5–10) \cdot 10^7$ cells/ml in Dulbecco's modified Eagle medium containing 1000 mg/l glucose, 25 mM Hepes, 0.1% bovine serum albumin and 15 μCi/ml [^{3}H]mannose (15–20 Ci/mmol, New England Nuclear, Cambridge, MA) as previously described [23]. Subcellular fractions were prepared as described above and glycopeptides analyzed by electrophoresis.

Electron microscopy. Samples for analysis by transmission electron microscopy were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), postfixed in 2% osmium tetroxide, Epon-embedded, and sectioned on a Sorval MT-2 ultratome. Sections were stained in 1% uranyl acetate and 0.5% lead citrate and examined in a Philips EM-201 electron microscope.

Enzyme analyses. Adenylate cyclase was assayed at 37°C for 10 min in a reaction mixture consisting of 50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM theophylline, 2 mM ATP, and 25–100 μg of cell or membrane protein. The cyclic AMP formed during the reaction was detected by radioimmune assay as previously described [12]. Ouabain-inhibitable, $(Na^+ + K^+)$-dependent ATPase was measured by a modification of the method of Skou [5]. 3′- and 5′-nucleotidase activities (EC 3.1.3.6 and EC 3.1.3.5) were measured by a modification of the method of Glastris and Pfeiffer [24] with 10 mM NaF and 50 mM sodium potassium tartrate included in the reaction mixtures as inhibitors of non-specific acid phosphatase activity [25]. Inorganic phosphorus was determined by the method of Fiske and SubbaRow [26].

Binding of trypanosomes membrane glycoproteins to con A-Sepharose 4B beads. Identification of trypanosome surface glycoproteins by affinity binding to con A-Sepharose beads was performed by a modification of the method of Poliquin and Shore [27]. Briefly, $1 \cdot 10^9$ iodinated trypanosomes were mixed with $(5–10) \cdot 10^9$ unlabeled carrier cells and sonicated in the presence of 1 mM TLCK and 0.8 mM phenylmethylsulfonyl fluoride (PMSF). A sonicate fraction and a partially purified membrane fraction ($82000 \times g$ velocity gradient interface material) were prepared as described above. These subcellular fractions were then extracted with sodium deoxycholate at 1% final concentration for 1 h at 37°C, and insoluble material was removed by centrifugation at $30000 \times g$ for 15 min. The detergent supernates were adjusted to 0.5% detergent by dilution with 5 mM Tris-HCl (pH 7.4). Approx. 500 μl of packed Con A-Sepharose 4B beads, pre-equilibrated with 0.5% deoxycholate in Tris-HCl buffer, were mixed with

500 μl of detergent-extracted protein by end-over-end rotation for 1 h at room temperature in a 1.5 ml Eppendorf tube. The beads were then separated from unbound protein by centrifugation, and the supernatant removed and mixed with 1/2 volume of solubilization buffer for gel analysis. The beads were washed twice with buffer (15 min, with rotation) and washed once with buffer containing 0.1% sodium dodecyl sulfate to remove non-specifically retained protein. Bound glycoproteins were then eluted with 0.75 M α-methylmannoside or by boiling the beads for 3 min in solubilization buffer. Samples were then analyzed by electrophoresis as described.

Macromolecular analyses. Residual DNA in subcellular fractions was determined by the diphenylamine reaction [28]. Residual RNA was assayed by labeling trypanosomes by incubation with [^{14}C]uridine for 1 h in vitro and monitoring the decrease in trichloroacetic acid-insoluble cpm throughout the isolation. Protein was measured by the method of Lowry et al. [29].

Reagents. All chemicals were reagent grade. All reagents for polyacrylamide gel electrophoresis were electrophoresis purity (BioRad). Lactoperoxidase, tosyl-lysyl chloromethyl ketone, phenylmethylsulfonyl fluoride, adenosine 5′-monophosphoric acid, adenosine 3′-monophosphoric acid and DNAase I were purchased from Sigma Chemical Co. [^{3}H]Mannose, cyclic AMP [^{125}I] RIA kit, [^{14}C]uridine, Protosol, 2,5-diphenyloxazole, *p*-bis(2-(5-phenyloxazolyl))benzene and carrier-free Na^{125}I were purchased from New England Nuclear. Dulbecco's modified Eagle medium containing 1000 mg/l glucose and 25 mM Hepes were purchased from Gibco. Con A-Sepharose 4B was purchased from Pharmacia.

Results

I. Isolation of a plasma membrane-enriched fraction from T. brucei YTat1

Preparation of membrane fractions

Several procedures for cell homogenization were initially attempted. We, as well as others [6], have found that hypotonic swelling of the cells followed by homogenization with a Dounce with a tight-fitting pestle is unsatisfactory because the cell ghosts formed contain trapped cellular organelles and attached flagella, thus making further purification of the membrane impossible. We have used sonication to produce a vesicle population from which a plasma membrane fraction is obtained by differential centrifugation and identified by enrichment of the following surface markers: (a) adenyl cyclase; (b) ouabain-sensitive, ($Na^+ + K^+$)-dependent ATPase; (c) iodinatable surface proteins; (d) ^{3}H-labeled glycoproteins and (e) variable surface coat glycoprotein, some of which is retained on the membrane and can be identified by its electrophoretic profile.

Following sonication, the initial $12000 \times g$ centrifugation yields a pellet which consists of a small number of unbroken cells, nuclei, cell fragments, and intact flagella, while the supernatant contains small vesicles, large membrane fragments, cytosol proteins, and a few free flagella. Treatment of this supernate with DNAase I degrades residual pieces of DNA which might tend to produce clumping of membrane vesicles. Centrifugation of the DNAase-treated supernate at $30000 \times g$ produces a crude particulate fraction containing membrane pieces, rough and smooth microsomes, small granules, and cytoskeletal elements. Velocity sedimentation of the crude particulate fraction yields a band at the sample/sucrose interface which contains chiefly smooth membrane vesicles, as well as a pellet consisting of rough microsomes and cytoskeletal material. The interface fraction when centrifuged to equilibrium is resolved into four bands. Bands 1 (1.12/1.14 interface) and 2 (1.14/1.16 interface) contain the majority of the plasma membrane-derived activity of the preparation and the least amount of contaminating material from other subcellular fractions. Bands 3 (1.16/1.18 interface) and 4 (1.18/1.20 interface) are morphologically heterogeneous and contain variable amounts of protein. The yield of membrane from band 1 is approx. 500–1000 μg of protein.

Morphological appearance of gradient-purified membrane fractions

Electron microscopic analysis of band 1 (Fig. 1a) reveals a population of small (80–100 nm diameter) and large (170–250 nm diameter) vesicles and larger membrane pieces (up to 1 μm in length),

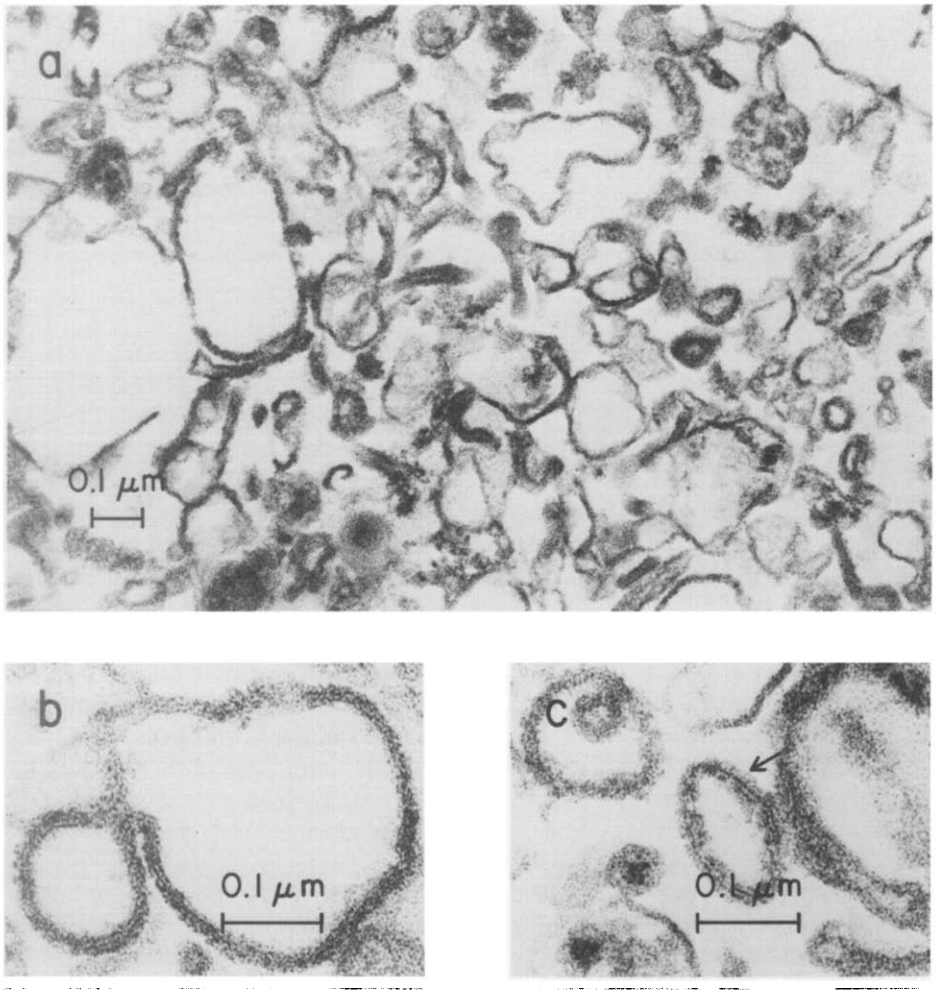

Fig. 1. Transmission electron micrographs of membrane fraction 1. Membrane fractions were prepared and analyzed by electron microscopy as described. (a) Smooth membrane vesicles and large fragments as well as osmiophilic granules are visible. Some vesicles and fragments appear to be covered with electron-dense material (×63500). (b) Membrane fragment with electron-dense coating (×131500). (c) Smooth membrane vesicles with electron-dense coating and lipid bilayer structure visible (arrow (×131500). Bars=0.1 μm.

some of which are covered with an electron-dense material resembling the surface coat seen on intact trypanosomes (Fig. 1b). The lipid bilayer structure of the membrane can be seen clearly beneath this material at high magnification (Fig. 1c, arrow) and the vesicles are of the smooth membrane type expected in a fraction of such low density. The second major morphological element in this fraction is a population of osmiophilic vesicles, which may correspond to the lipid granules reported by Steiger [30] in his study of the ultrastructure of intact trypanosomes. There is no visible microtubular array subtending any of the membrane fragments, nor are there intact flagella, paraxial rods, nuclear debris, or ribosome-like particles in this fraction. There is some lightly staining amorphous background material present which may represent surface coat glycoprotein which has become detached from the membrane vesicles. Bands 2–4 also contain membrane vesicles of the type described above (data not shown); however, ribosome-like particles, paraxial rod fragments, mi-

TABLE I

RECOVERY OF CELLULAR COMPONENTS IN ISOLATED MEMBRANE FRACTIONS

Fraction	% of original				
	Protein	DNA	RNA	^{125}I-labeled surface proteins	^{3}H-labeled glycoproteins
Sonicate	100.0	100.0	100.0	100	100.0
Crude particulate	20.0	7.0	25.0	41.0	33.3
82000× *g* interface	4.0	–	–	9.7	8.7
Gradient band 1	0.3–1.0	0.1	1.0	1.7	1.0
Gradient band 2	0.2–0.5	0.4	1.0	2.2	2.0
Gradient band 3	0.5	2.1	–	2.4	–
Gradient band 4	0.01	2.9	–	–	–

crotubule fragments and electron dense, granular background material all become more prominent components with increasing fraction density across the gradient.

Plasma membrane recovery and enrichment

The data in Table I demonstrate that gradient band 1 exhibits reduced levels of DNA and RNA relative to other cell fractions while containing up to 1% of total cell protein and retaining up to 1.7% of iodinatable surface proteins and glycoproteins, most of which consists of variable surface coat glycoprotein. The specific activities of adenyl cyclase and ouabain-sensitive, $(Na^+ + K^+)$-ATPase are increased 5-fold in gradient band 1 (Table II) relative to the sonicate, while 5′-nucleotidase activity was undetectable and 3′-nucleotidase activity [31] although present, was found primarily in soluble fractions. When cell surface proteins are iodinated, or when glycoproteins are labeled by metabolic incorporation of [^{3}H]mannose, there is an increase in the relative specific activity of each label in membrane fraction 1 relative to the cell sonicate. Since the variable surface coat glycoprotein is the principal component of this cell surface label, we have demonstrated that this protein in the isolated membrane fractions represents material originally associated with the plasma membrane, and is not present as a result of release and non-specific re-absorption during the isola-

TABLE II

ENRICHMENT FOR PLASMA MEMBRANE MARKERS IN SUBCELLULAR FRACTIONS OF *T. BRUCEI* YTat1 BLOODSTREAM TRYPOMASTIGOTES

Fraction	Specific activity		Relative specific activity	
	Adenyl cyclase [a]	Ouabain-sensitive $(Na^+ + K^+)$-ATPase [b]	^{125}I-labeled surface proteins [c]	^{3}H-labeled glycoproteins [d]
Sonicate	7.24	2.58	1.9	35.1
Crude particulate	14.70	–	3.3	69.6
82000× *g* interface	–	–	6.1	–
Gradient band 1	35.75	12.9	27.5	140.0
Gradient band 2	13.67	7.7	18.8	120.0
Gradient band 3	15.23	–	11.4	–
Gradient band 4	7.23	–	–	–

[a] Specific activity = pmol cyclic AMP/mg per min.
[b] Specific activity = nmol P_i/mg per min.
[c] Relative specific activity = ^{125}I cpm × 10^{-5}/mg protein.
[d] Relative specific activity = ^{3}H cpm × 10^{-3}/mg protein.

TABLE III

NONSPECIFIC ABSORPTION OF IODINATED VARIABLE SURFACE GLYCOPROTEIN TO SUBCELLULAR FRACTIONS DURING MEMBRANE ISOLATION

Fraction	^{125}I cpm ($\times 10^{-6}$)	% Total cpm
Sonicate	17.50	100.0
12000× *g* supernate	20.00	114.0
12000× *g* pellet	0.10	0.9
30000× *g* supernate	15.10	86.3
30000× *g* pellet	1.40	8.2
82000× *g* supernate	0.58	3.3
82000× *g* interface	0.13	0.74
82000× *g* pellet	0.51	2.92
Equilibrium gradient band 1	0.006	0.03
Equilibrium gradient band 2	0.002	0.01
Equilibrium gradient band 3	<0.001	<0.01

tion. Purified variable surface coat glycoprotein isolated from *T. brucei* YTat1 by lectin affinity chromatography was iodinated and mixed with a suspension of unlabeled YTat1 cells. The cells were then sonicated, and subcellular fractions monitored for acid-insoluble ^{125}I counts (Table III). Gradient band 1 contained 0.03% of the original counts which represents less than 2% of the surface label usually recovered in this fraction during an isolation (see Table I).

II. Polypeptide composition of membrane fractions

Polypeptide profiles

When subcellular fractions of *T. brucei* YTat1 are analyzed by electrophoresis the principal component of all fractions is the variable surface coat glycoprotein, $M_r = 58000$ (Fig. 2a and 2b, band 'k'). Other major components include a doublet at $M_r = 55000$ and 53000 thought to represent α and β tubulin monomers ('l') which are constituents of the trypanosome flagellum and subpellicular microtubule array. Similarly, actin-like bands, $M_r =$ 41000 and 43000 ('m') and a triplet of $M_r =$ 15000–17000 ('o') are prominent. Most of these cytoskeletal elements initially seen in the sonicate and crude particulate fractions are progressively removed from purified membrane by differential centrifugation and are ultimately found in the microsomal pellet (lane 82 K_p). Tubulin and variable surface coat glycoprotein bands are difficult to resolve due to the large amounts present and to similarities in molecular weight. However, in lane 1 of Fig. 2a there appears to be a depletion of cytoskeletal proteins relative to variable surface coat glycoprotein. Cytoskeletal proteins increase in prominence in the denser membrane fractions shown in lanes 2 and 3 and in the microsomal pellet, lane 82 K_p.

The profile in Fig. 2b represents a similar experiment with a different preparation of YTat1 cells. The gel is overloaded to stress the minor polypeptide bands in the molecular weight range of 42000–200000. Most of these bands are seen in all gradient purified membrane fractions, but the amounts may vary. Prominent polypeptides in the plasma membrane fraction (lane 1) have estimated $M_r = 200000$, 170000, 125000–135000, 92000–100000, 84000–86000 and 76000. The 76 kDa polypeptide band is seen not only in particulate fractions, but also in the high speed supernatant (lane 30 K_s). This polypeptide is thought to be identical with the soluble 76 kDa polypeptide which co-purifies with variable surface coat glycoprotein on lentil lectin affinity chromatography [16]. Similarly, some of the bands in the cluster at $M_r = 92000$–100000 (lane 1, bands d, e, f) which are seen in gradient purified membranes also appear in the high speed supernatant and may be extrinsic membrane polypeptides.

Many of the polypeptide bands observed in plasma membrane-enriched fraction 1 are also seen in the other gradient-purified membrane fractions, and it is likely that surface membrane fragments are present in all of these gradient fractions. However, the gel profiles, the enrichment data in Table II and the electron microscopic evidence, suggest that fraction 1 has a higher content of purified plasma membrane material than other subcellular fractions. Thus, our studies have concentrated primarily on polypeptides of fraction 1.

Membrane glycopeptides

In order to identify glycoprotein components of the trypanosome membrane, and to exclude the possibility that bound host serum glycoproteins contribute to the polypeptide profile, cells of *T. brucei* YTat1 were metabolically labeled by incubation in [^{3}H]mannose, and subcellular frac-

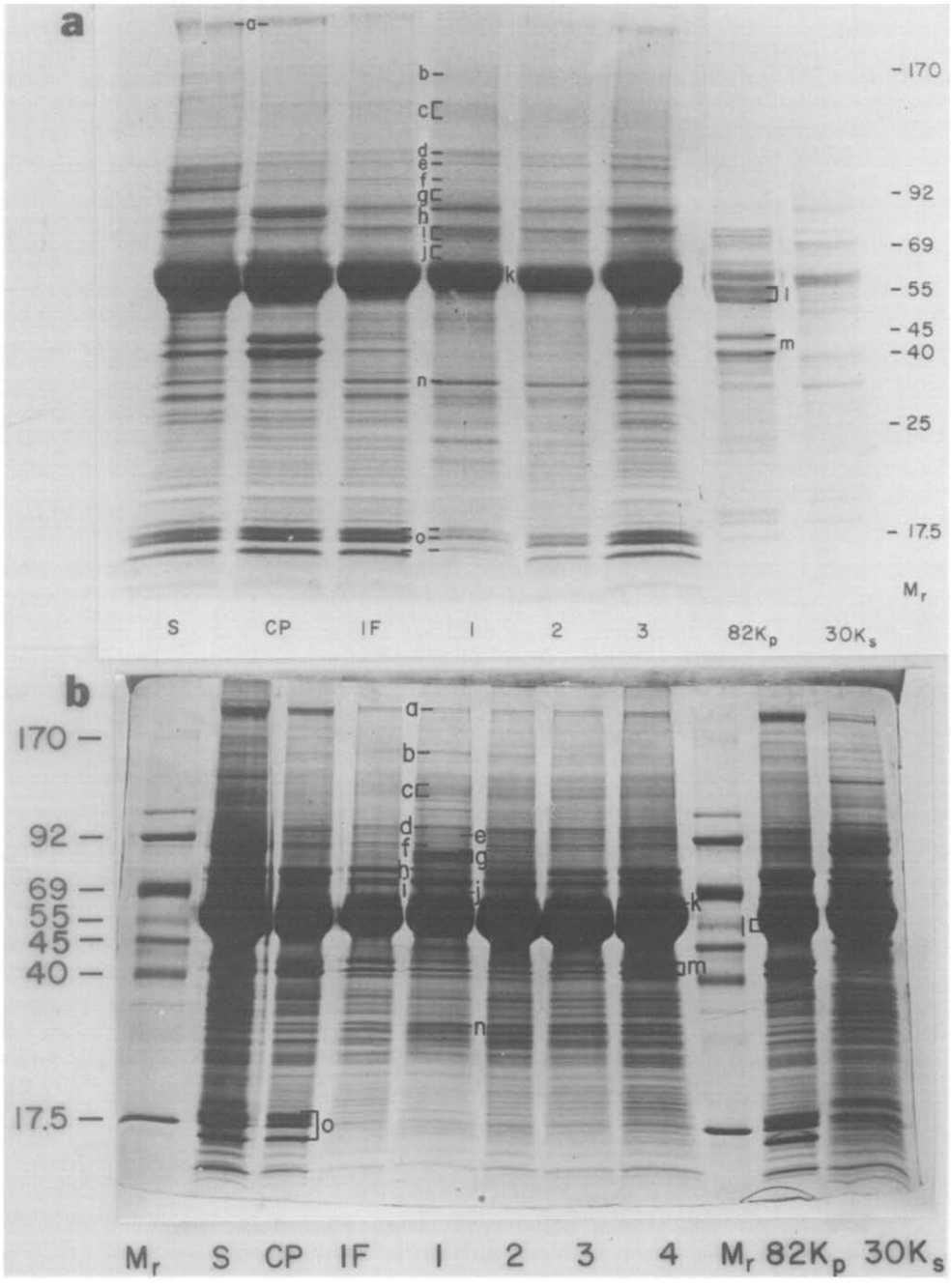

Fig. 2. Coomassie blue polypeptide profiles of *T. brucei* YTat1. Long slender bloodstream trypomastigotes were isolated, and subcellular fractions prepared as described. Aliquots were withdrawn from the preparation at the designated stages, processed, and analyzed by electrophoresis in sodium dodecyl sulfate 6–20% polyacrylamide gradient slab gels. Molecular weight standards $\times 10^{-3}$, M_r; sonicate, S; crude particulate, CP; 82000 $\times$ *g* velocity gradient interface, IF; equilibrium gradient-purified membrane fractions, 1, 2, 3 and 4; 82000 $\times$ *g* velocity gradient pellet, 82 K_p; 30000 $\times$ *g* supernate, 30 K_s. Estimated molecular weights $\times 10^{-3}$ of polypeptide bands are: a, 190–200; b, 170; c, 125–135; d, 100; e, 96; f, 92; g, 84, 86; h, 76; i, 72; j, 61–65; k, 58; l, 51, 53; m, 41, 43; n, 34; o, 17, 16 and 15. (a) Lanes S–3 contain 100 μg protein; lanes 82 K_p and 30 K_s contain approx. 50–75 μg protein. (b) Samples from a different preparation of YTat1. Each lane contains approx. 150 μg of protein.

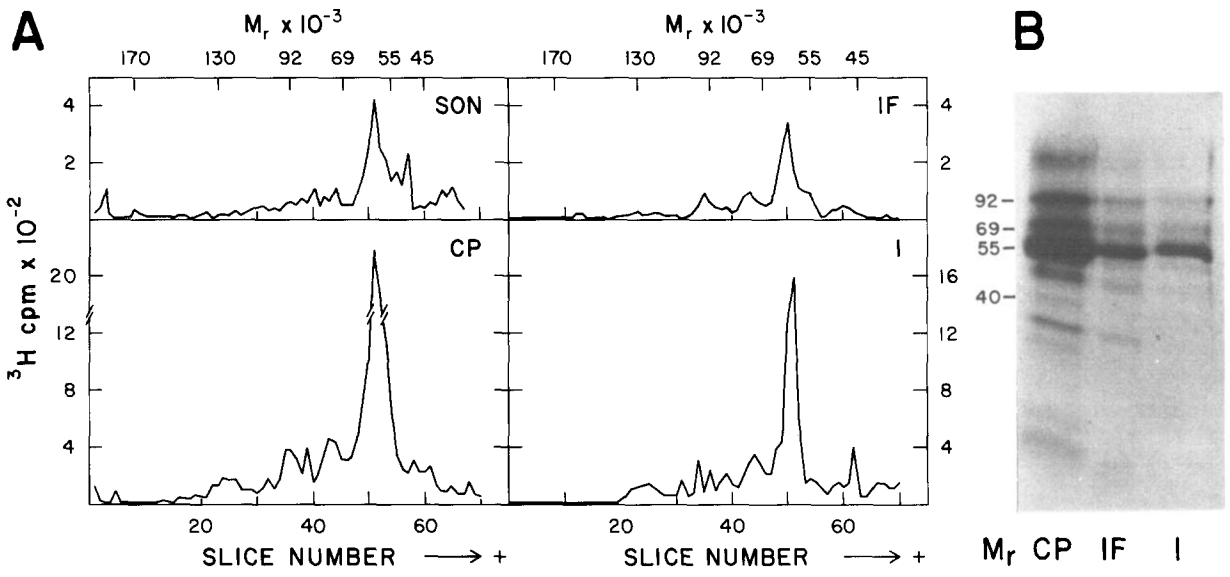

Fig. 3. Electrophoretic profiles of glycopeptides of *T. brucei* YTat1 long slender bloodstream trypomastigotes. Trypanosomes were incubated at $5 \cdot 10^7$/ml for 90 min at 37°C in Dulbecco's modified Eagle medium +0.1% bovine serum albumin with 15 μCi [^{3}H]mannose/ml to metabolically label cell glycoproteins. Labeled cells were then washed by centrifugation and subcellular fractions isolated as described in Methods. (A) Aliquots of sonicate (SON), crude particulate (CP), 82000 × *g* velocity gradient interface (IF), and membrane fraction 1 (1) were analyzed by electrophoresis as described in Fig. 2. Gels were then sliced into 1 mm fractions and ^{3}H cpm/slice determined by scintillation spectrometry as described in Methods. Counts are normalized on the basis of mg protein per gel. $M_r \times 10^{-3}$, molecular weight standards. (B) Aliquots of crude pellet, interface and fraction 1 analyzed by electrophoresis and fluorography. M_r, molecular weight standards $\times 10^{-3}$.

tions were isolated and analyzed by electrophoresis. The sliced gel profiles of whole cells, crude particulate fraction, velocity gradient interface material and membrane fraction 1 are shown (Fig. 3A) along with an autoradiographic profile of crude pellet, interface, and fraction 1 samples (Fig. 3B). The most prominent glycopeptide in all fractions is the variable surface coat glycoprotein band at $M_r = 58000$. However, in both the sliced gel profiles and the autoradiogram, small but distinct areas of incorporated radioactivity with estimated molecular weights of 130000, 92000–100000, 86000, 76000, 42000 and 30000 are seen in the particulate fractions. Some of these polypeptides correspond to discrete bands or clusters seen in the stained gel profile although we have not confirmed identity with these bands.

Polypeptides and glycopeptides of the cell surface of YTat1

When intact trypanosomes are subjected to lactoperoxidase-catalyzed iodination, the variable surface coat glycoprotein is the major cell surface component observed, but several other iodinated polypeptide bands can also be identified in gel profiles. These co-purify with isolated membrane fractions, and several are also found in both the 30000 × *g* supernate and in the microsomal pellet. They are shown in the autoradiogram in Fig. 4, which has been overexposed with respect to the variable surface coat glycoprotein band in order to emphasize minor bands. In whole cells (lane a) additional bands are seen at $M_r = 42000$, 65000–76000, 86000–100000 and 130000. In the sonicate (lane b) an additional band is observed at $M_r =$ 45000, and may represent a partial degradation product of variable surface coat glycoprotein since it segregates into the soluble fraction (lane g). Subcellular fractions demonstrate distinct distribution patterns for the labeled polypeptides. The 76, 86, and 92 kDa bands are seen in the soluble as well as particulate fractions while material at

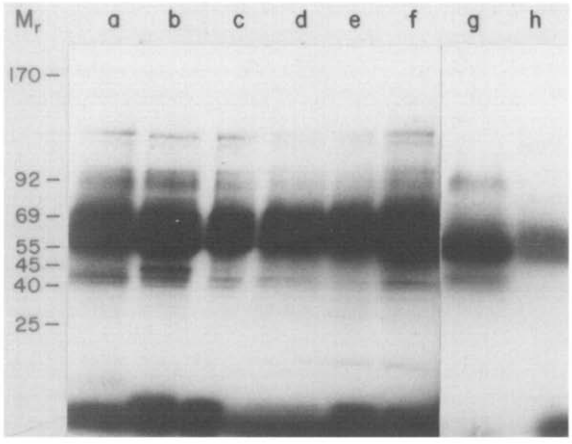

Fig. 4. Autoradiographic profile of surface polypeptides of *T. brucei* YTat1. Lactoperoxidase-catalyzed iodination of $1 \cdot 10^9$ long slender bloodstream trypomastigotes was performed as described in Methods. Labeled cells were mixed with $10 \cdot 10^9$ unlabeled carrier cells, and subcellular fractions prepared. Aliquots were withdrawn from the preparation at the designated stages and analyzed by electrophoresis and autoradiography as described in Methods. M_r, molecular weight standards $\times 10^{-3}$; a, intact trypanosomes before addition of carrier cells; b, sonicate; c, crude particulate; d, 82000 $\times g$ velocity gradient interface; e, membrane fraction 1; f, membrane fraction 2; g, 30000 $\times g$ supernate; h, 82000 $\times g$ velocity gradient pellet.

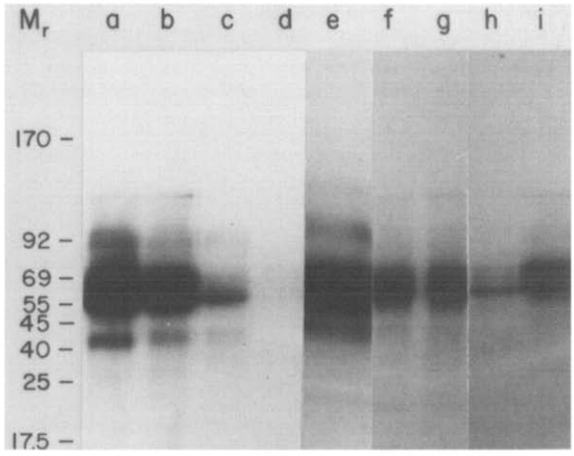

Fig. 5. Autoradiographic profile of iodinated surface glycopeptides of *T. brucei* YTat1. Trypanosomes were prepared and iodinated as described in the legend to Fig. 4. Sonicate (b) and partially purified membrane from the 82000 $\times g$ velocity gradient interface (f) were prepared as described in Fig. 1. Each sample was extracted with sodium deoxycholate (1% final concentration) then centrifuged for 1 h at 30000 $\times g$ to remove insoluble material. 500 μl aliquots of the detergent extracts of the sonicate (c) and the membrane fraction (g) were each mixed with an equal volume of packed Con A-Sepharose beads which had been equilibrated with 5 mM Tris-HCl buffer (pH 7.4) containing 0.5% sodium deoxycholate. Unbound protein (d,h) was removed by centrifugation and processed for electrophoretic analysis as described in Methods. The beads were washed with buffer alone, and with buffer containing 0.1% sodium dodecyl sulfate to remove nonspecifically bound protein. They were then resuspended in 500 μl of a 1 : 3 dilution of electrophoresis solubilization buffer and placed in a boiling water bath for 3 min to elute bound glycoproteins (e,i). Aliquots of each sample were analyzed by electrophoresis and autoradiography. a, iodinated whole cells; b–e, sonicate-derived samples; f–i, membrane-derived samples; $M_r \times 10^{-3}$, molecular weight standards.

65000–76000 apparently segregates to the microsomal pellet. The 42 and 130 kDa polypeptides are found only in particulate fractions.

In order to determine whether these iodinated polypeptides were also glycopeptides, a sonicate fraction (Fig. 5, lane b) and an interface membrane fraction (lane f) were prepared from iodinated cells (lane a). Deoxycholate solubilized polypeptides from these fractions were analyzed by affinity chromatography on Con A-Sepharose. The detergent extract of the sonicate (lane c) contains polypeptides with estimated $M_r = 42000$, 58000, 65000–76000, 86000–100000 and 130000. The 130 kDa polypeptide band cannot be seen in the detergent extract in lane c due to the small sample applied to the gel, but this material is sufficiently concentrated in the column eluate to detect its presence. All of these iodinated polypeptides appear to bind and elute from the lectin (lane e), and little or no iodinated material appears in the unbound fraction (lane d). When the interface membrane fraction is analyzed, a similar pattern is obtained, bu the 86–100 kDa material is reduced in this sample (lanes f–i) possibly because it is lost from the particulate fraction during membrane isolation.

Discussion

The variable surface coat glycoprotein is the major surface protein of *Trypanosoma brucei*, and constitutes up to 5% of the total cell protein [1]. The study of this molecule [32–34] and its metabolism in the parasite [23,35] has produced

insights into the mechanism of antigenic diversity in trypanosomes; however, complete understanding of the role of this protein in the regulation of host-trypanosome interaction necessitates the identification of the other proteins in the trypanosome plasma membrane with which it might interact. Some of these proteins may also be independently involved in regulating trypanosome development. If any of these minor membrane components are common to several variants, strains and/or developmental stages of the parasite they would present common sites for rational approaches to diagnosis, prophylaxis or treatment of the disease. We have selectively identified several potentially significant membrane polypeptides in bloodstream trypomastigotes of the YTat1 strain of *T. brucei* with respect to their membrane distribution, surface exposure and glycosylation.

Our plasma membrane-enriched fraction is similar both in equilibrium density and in recovery of protein to the membrane preparation of Rovis and Baekkeskov [13]. In contrast, the plasma membrane preparation isolated from *T. brucei* by Voorheis et al. [6] contains significant amounts of nucleic acid and cytoskeletal elements. Our preparation is virtually free of these non-membrane components and retains sufficient variable surface coat glycoprotein to permit its use as a membrane marker. Gradient fraction 1 is enriched in the plasma membrane marker enzymes adenyl cyclase and ouabain-sensitive ($Na^+ + K^+$)-ATPase at specific activities comparable to those previously reported for *T. brucei* [6,13]. We have also confirmed the apparent lack of 5′-nucleotidase activity in this species [13,36]. Although cell surface-associated 3′-nucleotidase activity has been reported in Leishmania [31], this enzyme is not enriched in membrane fractions of *T. brucei* YTat1, and cannot be used as a surface marker in this strain.

Gel profiles of subcellular fractions reveal certain unique distribution patterns among membrane polypeptides. The variable surface coat glycoprotein occurs in both soluble and particulate fractions, supporting the conclusion that it is an extrinsic membrane protein. The 76, 86 and 92–100 kDa externally disposed glycopeptides demonstrate distribution patterns similar to the major surface glycoprotein. This suggests that these relatively minor polypeptides may also be extrinsic, and may be additional components of the surface coat of the parasite.

Distribution patterns of the 42 and 130 kDa polypeptides suggest that these externally disposed glycopeptides may be intrinsic membrane components. Since they are not major bands in Coomassie blue-stained gel profiles, they may have a non-structural role in the membrane. The 170 kDa polypeptide seen in particulate fractions, although not an externally disposed glycopeptide, does appear to be an intrinsic membrane component.

These surface glycopeptides, whether extrinsic or intrinsic membrane components, are potentially useful probes for structural and functional studies of the trypanosome surface. The functional relationship between these minor membrane polypeptides and the major variable surface coat glycoprotein is unknown, but topographic interactions at the cell surface are presently under investigation (Strickler, J.E., Patton, C.L. and Mancini, P.E., unpublished data). Functional aspects of the involvement of minor surface glycoproteins in cellular regulatory processes are also being investigated. We have assayed protein kinase activity and identified several endogenous acceptor polypeptides in membrane fractions from *T. brucei* YTat1 and *T. brucei* 427. Phosphorylated polypeptides have $M_r = 170000$, 130000, 100000, 76000 and 53000. Variable surface coat glycoprotein does not appear to be phosphorylated (Mancini, P.E., and Patton, C.L., unpublished data). The relationship between these phosphorylated polypeptides and the cell surface glycopeptides described above has not been established, but it is tempting to speculate that some of them may be identical. Further structural and immunologic studies of these cell surface polypeptides should help elucidate their possible role in regulation of host/parasite interaction and antigenic variation.

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