Subcellular Localization of a Variable Surface Glycoprotein Phosphatidylinositol-specific Phospholipase-C in African Trypanosomes

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Abstract. African trypanosomes contain a membrane-bound enzyme capable of removing dimyristyglycerol from the membrane-attached form of the variable surface glycoprotein (mfVSG; Ferguson, M. A. J., K. Halder, and G. A. M. Cross, 1985, J. Biol Chem., 260:4963-4968). Although mfVSG phospholipase-C has been implicated in the removal of the VSG from the trypanosome surface (Cardoso de Almeida, M. L., and M. J. Turner, 1983, Nature (Lond.), 302:349-352; Ferguson, M. A. J., K. Halder, and G. A. M. Cross, 1985, J. Biol Chem., 260:4963-4968), its precise function and subcellular location have not been determined. We have developed a procedure for the separation of the cell fractions and organelles of Trypanosoma brucei brucei (and other trypanosome species) by differential sucrose and isopycnic Percoll centrifugation. These fractions were tested for mfVSG phospholipase activity using Trypanosoma brucei mfVSG labeled with 3H-myristic acid as substrate. The highest enzyme-specific activity was associated with the flagella and evidence is presented to suggest that it is localized in the flagellar pocket. Some activity was also associated with the Golgi complex. These results suggest that the mfVSG phospholipase is localized primarily in the membrane of the flagella pocket and possibly other membrane organelles derived from and associated with this structure, and may be part of the VSG-membrane recycling system in African trypanosomes.

The activity of mfVSG phospholipase amongst various trypanosome species was determined. We show that, in contrast to the bloodstream forms of Trypanosoma brucei, cultured procyclic Trypanosoma brucei and bloodstream Trypanosoma vivax had little or no mfVSG phospholipase activity. The activity found in bloodstream forms of Trypanosoma congolense was intermediate between Trypanosoma vivax and Trypanosoma brucei.

African trypanosomes, which cause sleeping sickness in man or nagana in livestock, have a 12-15-nm-thick coat of a variable surface glycoprotein (VSG) covering the entire surface of the organism (53). For Trypanosoma brucei and T. congolense, the generation of a new population of trypanosomes with a different surface coat has been shown to be the result of a clone-specific change in the expression of a particular VSG gene (14, 40). It appears clear that the remarkable ability of the trypanosome to alter its surface glycoprotein (14) may preclude the development of a conventional vaccine against trypanosomiasis. Since the integrity of the surface coat is known to be essential for parasite survival in the host, the purpose of this study was to investigate VSG synthesis, processing, and replacement on the cell surface. A clearer understanding of the cell biology of the VSG may suggest alternative approaches for the possible control of trypanosomiasis.

The VSGs from T. brucei are proteins that contain complex carbohydrate moieties of mannose, galactose, glucosamine, and myristilated phosphatidylinositol (17, 18, 39) conjugated to the protein through the alpha-carboxyl group of the carboxy-terminal amino acid via an ethanolamine residue (13, 28, 39). The biological importance to VSG of this carbohydrate side chain is evident since it is present on all variable antigens of T. brucei and T. congolense organisms so far studied and is probably responsible for cross-reactivity observed between soluble forms of VSGs (also known as the cross-reacting determinant [CRD]) (14). The carbohydrate side chain may be involved in anchoring the VSG to the plasma membrane (15, 17, 18, 39), and maintaining the integrity of VSG-VSG interactions within the coat (24).

1. Abbreviations used in this paper: CRD, cross-reacting determinant; GPDH, glycerol-3-phosphate dehydrogenase; LG fraction, large granule fraction; mfVSG, membrane-attached form of the variable surface glycoprotein; SG fraction, small granule fraction; sVSG, soluble form of the variable surface glycoprotein; VSG, variable surface glycoprotein.
It has been proposed that VSG is anchored to the plasma membrane by a hydrophobic domain within the CRD (16-18, 39). Although the precise structure of the CRD is not completely known, the presence of a covalently linked glycosyl-sn-1,2 dimyristoyl phosphatidylinositol in the VSG of trypanosomes has been revealed (18). The possibility that the VSG is anchored to the membrane via the myristyl residues is supported by the evidence that the membrane-attached form of the T. brucei VSG (mVSG) contains glycerol and myristic acid whereas the soluble form of VSG (sVSG) lacks these residues (15, 17, 39).

The conversion of the mVSG to sVSG may be mediated by the action of an endogenous phospholipase-C-like enzyme (also designated Enzyme-X; 9, 10, 15, 17, 18, 30, 39). It has been postulated that mVSG is selectively released in vivo from the plasma membrane of trypanosomes as a result of the removal of the diacylglycerol domain from the phosphatidylinositol (17, 18, 39), and that the VSG-releasing enzyme is possibly located in the plasma membrane (10, 15, 17, 18, 39). However, the intracellular localization of the enzyme responsible for the conversion of mVSG to sVSG is still uncertain. The present report is an attempt to address this problem.

**Materials and Methods**

**Biochemicals**

All reagents were of analytical grade and were obtained from the following sources. Uridine diphospho-D-[6-3H]galactose (640 GBq/mmol), [9,10(n)-3H]-myristic acid (2.04 TBq/mmol), H3-casein (1.1 MBq/mg protein), l-[3H]methionine (30 TBq/mmol), and Hyperfilm MP were obtained from Radiochemical Centre Limited (Amersham, England). Aqueosol and EN'HANCE were obtained from New England Nuclear (Boston, MA). [N-(2-acetamido)]-2-Amino ethanesulfonic acid (ACES), 2-(N-morpholino)-ethanesulfonic acid (MES), Hesep; and [N,N-bis-(2-hydroxyethyl)]glycine (BICINE) were from Research Organics Inc. (Cleveland, OH). Aristar grade sucrose was from BDH Chemicals (Poole, England). RPMI-1640 and Isco's culture media were from Flow Laboratories, Inc. (Irvine, Scotland). Zwittergent TM 3-14 was from Calbiochem-Behring Corp. (La Jolla, CA). Poly/sep 47 IEF buffer was from Polysciences, Inc. (Warrington, PA). Sephacyr S-200 and Percoll were from Pharmacia Fine Chemicals (Uppsala, Sweden). DE-53 was from Whatman Ltd., (Maldstone, Scotland). Zwittergent TM 3-14 was from Calbiochem-Behring Corp. (La Jolla, CA). Poly/sep 47 IEF buffer was from Polysciences, Inc. (Warrington, PA).

**Organisms**

*Trypanosoma brucei* clones MITat 1.2, 1.52, ILNat 1.1, *T. congolense* clone ILNat 2.1, and *T. vivax* ILDat 1.2 (subclone 1392) were grown from cryopreserved stablates in lethally irradiated rats (600-900 rad). The organisms were isolated from the infected blood using isopycnic Percoll gradients (21). Infected blood was mixed 1:2 with 90% Percoll containing 1% glucose, 0.73 % NaCl (buffered to pH 7.4), and centrifuged at 15,000 rpm for 15 min in a JA20 rotor. The trypanosomes were collected, the pH was adjusted to 8.0 with 1 M Tris-base, and the trypanosomes immediately passed through a DE-53 column equilibrated in phosphate-buffered saline glucose (PSG), pH 8.0 (33) supplemented with nucleosides (0.1 mM adenosine, 0.05 mM thymoxanthine, 0.05 mM thymidine) as well as Balz's additions; namely, 0.2 mM 2-mercaptoethanol and 2 mM pyruvate (2). After elution the pH was adjusted back to pH 7.4 with Heps. Platelets and other blood cells are effectively removed by these isolation conditions.

However, the ATP contents of the isolated parasites are several-fold higher than those isolated in PSG by previous workers (19, 35).

**Subcellular Fractionation**

For each experiment ~10⁴ trypanosomes were washed in SHK (250 mM sucrose, 50 mM Hepes, 25 mM KCl, pH 7.4, at 5°C) buffer containing known inhibitors of the major trypanosomal proteinases (36; 50-100 µg/ml of leupeptin, E-64, antipain, and chymostatin). The parasites were disrupted by passage through a French Pressure Cell under a chamber pressure of 2,500 psi.

Fractions containing lysosomes, glycosomes, and flagella were prepared at 0-5°C as follows: EDTA was added to the homogenate (to a final concentration of 1 mM; SHKE, pH 7.4), and unbroken cells, nuclei, and cell debris were sedimented at 700 g for 10 min in a JA-20 rotor (Beckman Instruments Inc., Palo Alto, CA). The resulting pellet was designated the crude nuclear pellet. The resulting supernatant was centrifuged at 2,800 g for 10 min to produce a large granule (LG) fraction; this was followed by a small granule (SG) fraction produced at 15,000 g for 10 min. Centrifugation of the (psect-SG) supernatant at 123,000 g for 90 min in a 50.2 Ti rotor (Beckman Instruments Inc.) gave the crude microsomal pellet fraction and the soluble supernatant.

The LG and SG fractions were further fractionated by isopycnic centrifugation in Percoll. The LG and SG fractions were made 57.6% (vol/vol) with respect to Percoll in SHKE (pH 7.4) and layered under a discontinuous gradient consisting of 43.2, 28.8, and 20.3% Percoll in the same buffer. Centrifugation was for 30-35 min at 25,000 rpm in either an SW-41 or an SW-27 rotor. Fractions banding between the 28.8/43.3 % and 43.3/57.6 % Percoll interfaces were made 57.6 % with regard to Percoll and centrifuged again in either the gradient described above or in continuous Percoll gradients in SHKE. Fractions on top of and within the 20.3 % Percoll layer (crude flagella) were adjusted to 28.8 % Percoll and layered under a discontinuous gradient containing 20.3, 17.3, and 14.4 % Percoll in SHKE buffer, pH 7.4, and centrifuged again as above to obtain the lighter membrane fractions. Depending on yields, the fractions were washed with SHKE buffer and stored at −80°C. For some preparations, the entire fractionation was done in the presence of 5 mM magnesium instead of the EDTA.

To obtain endoplasmic reticulum and Golgi fractions, 5 mM MgSO₄ was added to the homogenate (in SHKM, pH 7.4) and processed using established procedures as described by Grab et al. (23) except that the separation...
of smooth endoplasmic reticulum from rough endoplasmic reticulum components was done in a vertical VTi50 rotor (50,000 rpm for 1 or 2 h) instead of an SW-27 swinging bucket rotor. The latter requires longer centrifugation times. These fractions were washed with SHKE buffer before further analysis or storage by freezing at -80°C. These fractionation procedures, developed for T. brucei bloodstream forms, may be used for T. congolense and T. vivax bloodstream forms as well as for cultured procyclic T. brucei.

**Morphology**

For electron microscopy, suspended fractions were fixed in a fixative containing picric acid, formaldehyde, and glutaraldehyde (29) in 100 mM sodium phosphate buffer, pH 7.2. After fixation at room temperature for 1 h, the fractions were centrifuged in an Eppendorf microfuge for 1-15 min to obtain a visible pellet. The samples were washed with 100 mM sodium cacodylate buffer, treated with 1% CaO in sodium cacodylate buffer en bloc, stained with uranyl acetate, dehydrated, and embedded in Epon-Araldite. Thin sections were cut across the entire thickness of the pellet, stained with uranyl acetate and lead citrate, and examined in the electron microscope.

**Biochemical Analysis**

Acid phosphatase was assayed using as substrate either beta-glycerophosphate (52) or 4-methylumbelliferylphosphate (51). Acid protease was assayed using 13C-methylated casein or benzyloxycarbonyl-Phe-Arg-7-amido-4-methylcoumarin as substrate (37). Glycerol-3-phosphate dehydrogenase was assayed according to the method of Rovis and Baekkeskov (50). The transfer of galactose from UDP-galactose to endogenous protein substrates (galactosyltransferase) and adenylyl cyclase were assayed as described by Grab et al. (23). Malate dehydrogenase was measured according to the method of Opperdoes et al. (47). Protein determination was performed according to the method of Neville (43) except that a slab gel (2 mm thick by 200 mm long) containing a linear 10-20% polyacrylamide gradient was used.

**Preparation of 3H-Myristic-labeled MITat 1.2 mVSG**

1H-Myristic acid (185 MBq) was dried in a rotary evaporator, washed several times with absolute ethanol/benzene 1:1 (vol/vol) to remove the toluene, and once with absolute ethanol. The labeled fatty acid (in ethanol) was dried for 1 h at 65°C, and exposed to Hyperfilm MP film at -80°C for reproducible. No proteolysis occurred during incubation with the various inhibitors (data not shown). The isolated lysosome-like organelles (Fig. 2 A) were spherical with a rim of high density substance and a clear center. These membrane-bounded structures

![Image](https://example.com/image.png)

**Figure 2.** (A) Lysosome fraction from the 28.8/43.2% Percoll interface. (B) Glycosome fraction from the 43.2/57.6% Percoll interface. Bar, 2 μm.

**mVSG Phospholipase Assay**

The assay mixture for VSG phospholipase contained, in a volume of 30 μl, 2.26 μg labeled mVSG (8,500 cpm/μg VSG protein), and 7.5 ng membrane protein (in 15 μl SHKE) or Sephacryl S-200 enzyme protein (diluted in 15 μl SHKE), 50 mM Hepes, pH 7.4, 0.025% polyethylene glycol (PEG; M, 6,000, wt/vol), 0.3% wt/vol Triton X-100, protease inhibitors (20 μg/ml each of E-64, leupeptin, chymostatin, and antipain). Under the conditions stated, the protein substrate-to-enzyme ratio was 300:1, while the ratios of substrate phosphatidylinositol to total lipid, glycolipid, total phospholipid, and membrane phosphatidylinositol were at least 11:1, 107:1, 161:1, and 160:1, respectively, based on the data of Baekkeskov et al. (1). After a 15-30 min incubation at 37°C the reaction was terminated by boiling in 100 μl of 0.3% (wt/vol) Zwittergent TM 3-14 after which 300 μl of 0.3 M NaCl/90% methanol (vol/vol) was added followed by 800-900 μl of n-hexane. The sample was vortexed until emulsification occurred and then centrifuged in an Eppendorf microfuge to separate the phases. The release of labeled myristic acid into the hexane phase was monitored by liquid scintillation spectrometry. The above assay was done in duplicate or triplicate and was found to be reproducible. No proteolysis occurred during incubation with the various fractions when [35S]methionine-labeled mVSG was the substrate.

**TLC of the hexane-extractable product was done on heat-activated (100°C for 1 h) 0.25-mm-thick silica gel 60 TLC plates in a solvent system containing pentane, diethyl ether, glacial acetic acid (80:20:1; vol/vol/vol) (41).** The plates were dried for 30 min at 60°C, sprayed with ENHANCE, dried for 1 h at 65°C, and exposed to Hyperfilm MP film at ~80°C (Amersham).

**Sephacryl S-200 Column Chromatography of mVSG Phospholipase**

Flagellar membrane fractions which banded on top of 14.4% Percoll (in SHKE) were solubilized in 2% (3-(3-cholamidopropyl)dimethyl-ammonio)propane-1-sulfonate (CHAPS) (wt/vol), containing the protease inhibitors E-64 and leupeptin (20 μg/ml each) and centrifuged at 100,000 g for 1 h to remove undissolved cytoskeletal components. After dialysis against 0.1% CHAPS in 10 mM Hepes, 0.05% PEG, pH 7.0 (CHP buffer), the sample was applied to a 1.6 × 95-cm Sephacryl S-200 column equilibrated in the same buffer. The active fractions were pooled. IEF was done on a Poly-Sep 47 column (6). Product analysis was conducted by TLC (41) as described above.

**Results**

**Subcellular Fractionation: Morphological and Biochemical Analysis**

Discontinuous Percoll gradients produced highly enriched preparations of lysosomes, glycosomes, and flagella. An important feature of the isolation procedure appears to be the hypertonicity of the 250 mM SHKE buffer (~410 mosmols) more as isotonic buffers resulted in fractions with poor purity (data not shown). The isolated lysosome-like organelles (Fig. 2 A) were spherical with a rim of high density substance and a clear center. These membrane-bounded structures
tions. In contrast, morphologically recognizable glycosomes
were identifiable in this and all other Percoll-derived frac-
tions. As can be seen in Table I, the fraction containing the
part of the fixed pellet obtained from the fraction derived
from the 20.3/28.8% Percoll interface (fraction I; Fig. 4 B).
Little lysosomal protease/peptidase activity was associated
with the glycosome fraction (Table I). The proteolytic activ-
ity against benzylxycarbonyl-Phe-Arg-7-amido-4-methyl-
comarain, with the exception of the Golgi fraction, showed
a slight lag phase (2–4 min) before full (linear) activity was
observed. In the case of the Golgi fraction, however, the
activity was unstable and rapidly diminished after the addition
of the enzyme to the reaction mixture. When the proteolytic
activity of the lysosomes was measured as described by
Lonsdale-Eccles and Mpimbaza (36), it was observed to
have an apparent molecular mass of 30,000 D, but upon the
addition of a trace of serum (0.1%) additional bands were ob-
served at ~100,000 D (38).

In addition to lysosomes and glycosomes, the LG subfrac-
tion was found to contain an extra-membrane band that con-
sistently migrated slightly below the 20.3/28.8% interface,
and which biochemical evidence suggested may contain rem-
ants of mitochondrial membranes (data not shown). Occa-
sionally, we found a very dense membrane fraction which
resided deep within the 57.6% Percoll layer. This layer may
contain morphologically intact mitochondria in low yield.
However, it appears that most of the mitochondria were dis-
rupted in the French Press since malate dehydrogenase, a
known mitochondrial marker (47, 51), was found in the solu-
ble fraction (Table I).

Flagella were found in both the SG and LG fractions.
However, the flagella were readily separated from lysosomes
and glycosomes by centrifugation in Percoll. In the presence
of EDTA, the crude flagellar fractions containing microtu-
bule-associated membranes, trypanosome skeletons, and
other vesiculated membranes were found either within and/
or on top of the 20.3% Percoll layer (Fig. 3 A). These could
be separated from each other by a second centrifugation
through a lighter Percoll gradient containing EDTA. The
17.3/14.4% Percoll interface (fraction IV) contained flagella
with fewer trypanosome skeletons and more membrane than
those seen in Fig. 3 A (see Fig. 3 B). The presence of tubular
structures, with diameters of more than twice the diameter
of microtubules, were found with the flagellar obtained from
the fraction banding within the 17.3% layer (fraction III) just
above the 20.3/17.3 % interface (fraction II; Fig. 3, C and D).
They are too large to be pellicular microtubules and are of
the wrong shape to be the flattened cisternae of the Golgi
apparatus. It is possible that they could be the collecting tubules
described by Langreth and Balber (34) but seem not to be
interconnected. They layered on the top of the fixed pellet.
Also present were occasional glycosome-like structures (cen-
ter, Fig. 3 D). The flagella fraction which was derived from
the 20.3/17.3% Percoll interface (fraction II) contained axo-
nome profiles and much membrane (Fig. 4 A). However, the
membranes did not seem closely associated with the flagella.
Some pellicular microtubules were also present. Flagellar
profiles with paraxial rods were abundant and membranes
that were not directly attached to the flagella were present in
part of the fixed pellet obtained from the fraction derived from
the 20.3/28.8% Percoll interface (fraction I; Fig. 4 B). A different part of the same pellet also contained a few flagel-
lar profiles and was rich in membranes (Fig. 4 C) which

(density = 1.082 g/ml) were usually enriched in the SG frac-
tion and consistently banded between the 28.8/43.2% Percoll
interface. The sizes were similar to those of metrizamide-pu-
rified rat liver lysosomes (57). Biochemical characteriza-
tion of these trypanosomal organelles is given by Lonsdale-Eccles
et al. (37). Some Percoll particles (~25 nm in diameter)
were identifiable in this and all other Percoll-derived frac-
tions. In contrast, morphologically recognizable glycosomes
(Fig. 2 B) were divided between the LG and SG fractions
and could be obtained from the 43.2/57.6% Percoll interface.
These spherical microbody-like organelles (density = 1.091
g/ml) contained densely staining crystalloid centers and were
similar to those obtained by Opperdoes et al. (48). Little
stratification was observed within the lysosome and glyco-
some fractions.

Biochemical analyses confirm the morphological observa-
tions. As can be seen in Table 1, the fraction containing the
highest specific activity of glycerol-3-phosphate dehydroge-
nase (GPDH), an enzyme marker for glycosomes (47, 48,
50), was found in the glycosomal fraction identified as such
by electron microscopy (Fig. 2 B). The relative specific ac-
tivity of GPDH as compared to the total homogenate was
similar to the purified glycosomes obtained by Opperdoes et
al. (48): 7.3 vs. 8.2, respectively (see Table I). However,
some glycosomal enzyme activity was also observed in the
lysosome fraction. Since GPDH activity was low in the high-
speed soluble supernatant fraction, this suggested that the
Table I. Biochemical Analysis of MITat 1.2
Membrane Fractions

| Fraction          | AC  | AP* | GT  | AP4 | GDPH | MDH |
|-------------------|-----|-----|-----|-----|------|-----|
| Membrane          |     |     |     |     |      |     |
| Homogenate        | 1.0 | 1.0 | 1.0 | 1.0 | 1.0  |     |
| Crude nuclear pellet | 1.4 | 1.0 | 0.6 | 0.2 | 0.3  |     |
| LG fraction       | 2.3 | 2.1 | 1.0 | 0.7 | 0.3  |     |
| SG fraction       | 3.4 | 2.0 | 1.0 | 1.3 | 0.1  |     |
| Crude microsomal pellet | 1.4 | 2.5 | 1.4 | 1.6 | 1.5  | 0.1 |
| High-speed soluble supernatant | 0.3 | 0.1 | 0.5 | 0.1 | 1.8  |     |
| Rough endoplasmic reticulum |     | 0.4 | 2.2 | 1.3 | 1.0  | 0.0 |
| Smooth endoplasmic reticulum | 1.0 | 0.3 | 1.3 | 0.8 | 0.0  |     |
| Golgi fraction    | 2.2 | 14.7| 5.7 | 0.5 | 0.3  | 0.1 |
| Lysosome          | 1.1 | 5.3 | 11.9| 3.7 | 0.1  |     |
| Glycosome         | 0.4 | 1.4 | 11.9| 7.3 | 0.0  |     |
| Flagella I        | 1.9 | 1.4 | 11.8| 0.7 | 0.0  |     |
| Flagella II       | 2.6 | 2.1 | 1.0 | 0.7 | 0.2  | 0.1 |
| Flagella III      | 2.1 | 2.2 | 0.5 | 0.4 | 0.3  |     |
| Flagella IV       | 2.8 | 2.5 | 0.9 | 0.3 | 0.8  | 0.0 |
| Flagella V        | 5.1 | 3.9 | 0.9 | 0.1 | 0.0  | 0.0 |

* Specific activities relative to the homogenate control. AC, adenyly cyclase; AP, acid phosphatase; GT, galactosyltransferase; AP4, acid proteinase; GPDH, glycerol-3-phosphate dehydrogenase; MDH, malate dehydrogenase.
† Using 4-methylumbelliferylphosphate as substrate. Identical results were obtained with beta-glycerophosphate.
‡ Determined in preparations made in the absence of protease inhibitors using benzylxycarbonyl-Phe-Arg-7-amido-4-methylcomarain (37); results were essentially identical to those obtained with radiolabeled casein.
§ Flagella fraction I was from the 28.8/20.3% Percoll interface; II was from the 20.3/17.3 % interface; III was within the 17.3% Percoll layer; IV was from the 17.7/14.4% interface; and V was from top of the 14.4% Percoll layer.

glycosomal contamination of the lysosome fraction is due to
a nonsoluble glycosomal element. Some isolations were per-
formed in the absence of protease inhibitors to establish the
contribution of the lysosomal fractions to the other fractions.

* Specific activities relative to the homogenate control. AC, adenyly cyclase; AP, acid phosphatase; GT, galactosyltransferase; AP4, acid proteinase; GPDH, glycerol-3-phosphate dehydrogenase; MDH, malate dehydrogenase.
Figure 3. (A) Crude flagella fraction from the first Percoll gradient (entire 20.2% layer). Contains flagella with associated membranes as well as without. This fraction also contains other flagella-free membranes as well as trypanosome skeletons. (B) Fraction from 14.4/17.3% Percoll interface. Similar to Fig. 3 A but with fewer trypanosome skeletons and more membranes. Many flagellar profiles have membranes associated with them. (C) This fraction banded within the 17.3% Percoll layer above the 17.3/20.3% interface. It contains many flagellar profiles and, as shown in a higher magnification micrograph (D), tubular structures having more than twice the diameter of microtubules. Bars: (A–C) 4 μm; (D) 2 μm.

Figure 4. (A) Flagella fraction derived from the 17.3/20.2% Percoll interface. Contains axoneme profiles and membranes not associated with the flagella. Some pellicular microtubules are also present. (B) Flagella profiles with paraxial rods abundant are found in the fraction banding at the 20.2/28.8% Percoll interface. (C) Different part of the pellet seen in B. This fraction is rich in membranes many of which appear to be coated vesicles. A few flagellar profiles are present. (D) Higher magnification of C. Bars: (A–C) 4 μm; (D) 1 μm.
were recognizable under high magnification as coated vesicles (Fig. 4 D). Large vesiculated membrane profiles were found to band on top of the 14.4% Percoll layer (fraction V). These light Percoll membranes with diameters ranging from 500 to 1,000 nm (Fig. 5 A) were similar in size to the membrane surrounding the flagella (400-1,200 nm) when cut in cross section, as seen in Fig. 2, A and B. This fraction contained little or no GPDH, acid proteinase, galactosyltransferase, or malate dehydrogenase, but was rich in adenyl cyclase and acid phosphatase, which are enzymes reported to be in the flagella pocket (34, 51, 56).

mfVSG Phospholipase Distributions

Using 3H-myristic acid–labeled MITat 1.2 mfVSG as the substrate, we have examined the distribution of mfVSG phospholipase in both intact trypanosomes and in the morphologically defined subcellular fractions. The amount of the enzyme activity was identical in slender or stumpy T. brucei bloodstream forms, while this activity was less in T. congolense bloodstream forms and present in low amounts in both T. vivax bloodstream and T. brucei procyclic culture forms (Table II). This suggests that, unlike T. brucei and to a lesser degree T. congolense, T. vivax either lacks the enzyme or that its enzyme cannot use T. brucei mfVSG as substrate. The latter interpretation may be the more plausible because it appears that the enzyme is immunologically cryptic in T. vivax compared with that in T. brucei and T. congolense (Fish, W. R., D. L. Grab, and G. W. N. Mpimbaza, unpublished observations).

The endogenous membrane-bound mfVSG phospholipase activity in T. brucei homogenates was independent of magnesium, manganese, or calcium cations, and did not need sulfhydryl reducing agents for activity. However, ~15-20% activation occurred with either 10–25 mM dithiothreitol (DTT) or with 5 mM EDTA; i.e., no further activation occurred when the membranes were incubated with DTT and EDTA together. This does not seem to be due to the method of trypanosome preparation or lysis as membranes we prepared as described by Hereld et al. (27) again showed only the minimal 15–20% activation by these agents.

Using a citric acid/MES/ACES/Hepes/BICINE (pH 3–9) mixed buffer, the enzyme showed optimal activity at pH 7.5 and little activity below pH 6.0. This differs from T. brucei phospholipase A1, which, unlike VSG phospholipase, is mostly soluble with a pH optimum around 6.0. A particle-bound enzyme with a pH optimum of 5.2 is also present in low amounts (47). The reaction was dependent on both mfVSG concentration and time of incubation (data not shown) and was inhibited by phosphatidylinositol, by the detergent Zwittergent 3-12 and 3-14, and by unlabeled mfVSG (but not by VSG). Although the mechanism of the Zwittergent inhibition is not obvious, it is clear that the detergent can effect VSG-VSG interactions and is an effective inhibitor of mfVSG phospholipase (22, 24–26). This Zwittergent effect also oc-

2. It should be noted that high trypanosomal membrane concentrations can cause an adventitious release of 3H-radioactivity from mfVSG into n-hexane. This release is independent of pH throughout the 3.0–9.0 range and the activity is found in the chloroform/methanol extract of the membranes. The reaction occurs after proteolysis of the substrate by papain and protease K and after boiling the extract in the presence of detergents. It can also be mimicked by relatively high concentrations of phospholipids: the best effectors being phosphatidylinositol, phosphatidylycholine, and sphingomyelin. We suspect that the effect may be due in part to a detergent-like effect on the labeled mfVSG leading to the release of some noncovalently bound polar lipid since the product(s) released does not migrate off the origin on TLC in a neutral solvent system (Fig. 6). However, we cannot completely rule out the possibility of some nonenzymatic covalent isotope exchange reactions nor that at higher membrane concentrations the effects of other phospholipases (e.g., D) may be of some consequence. However, the latter possibility seems unlikely in view of the broad pH range over which the effect is observed.
Figure 6. TLC of the reaction products released into n-hexane. Membranes, purified enzyme, or phosphatidylinositol were incubated in a volume of 500 μl until completion of the enzymatic reaction. The n-hexane phase was dried down under a flow of (CaCl₂-dried) nitrogen gas and the residue was washed several times in chloroform/methanol (2:1, vol/vol). TLC was done as described in Materials and Methods. (Lane 1) Homogenate; (lane 2) light Percoll-derived membrane fraction (14.4%); (lane 3) partially purified VSG phospholipase; (lane 4) mfVSG incubated with 4 μg phosphatidylinositol. The arrows refer to the migration of (a) free myristic acid; (b) an unknown lipid (possibly 1,3 diacylglycerol); (c) 1,2-diacylglycerol; (o) origin.

curs in the presence of other detergents such as NP-40 or Triton X-100 (Grab, D. J., unpublished observations).

Since African trypanosomes are known to contain both phospholipase A₁ and A₂ (32, 42, 46), we used TLC to examine the products of mfVSG hydrolysis to determine the nature of the products released and the specificity of our assay system. The major hydrolysis product was found to be 1,2-diacylglycerol, with minor amounts of free myristic acid and some unknown lipid, possibly 1,3-diacylglycerol (Fig. 6). This confirms the phospholipase-C nature of the reaction assayed.

A comparison of Table I with Table III shows that the highest relative specific activity for T. brucei mfVSG phospholipase was associated with membranes presumably derived from the flagellar pocket. In Table III, column A shows data averaged from MITat 1.2, MITat 1.52, and ILTat 1.1; and column B shows data for MITat 1.2 which had a specific activity of the homogenate of 38 fmol myristate released per min/ng membrane protein. Although the yield was only 0.5%, little evidence for an endogenous activator or inhibitor was found under the assay conditions used (Table III). The activity from these flagellar fractions could be solubilized with detergent. Relatively high activity for the mfVSG phospholipase was also found in the Golgi fraction and in those flagella fractions where the membrane is associated with the flagella axoneme (fractions III and IV). Because any individual experiment must of necessity deal with a single clone and consist of parasites in different stages in development, it was necessary to determine whether the distributions shown in column B of Table III were representative of different clones and developmental stages. As can be seen from column A, which is a composite of three T. brucei clones, the same general pattern is internally consistent although detailed variations must exist because the standard errors are fairly large for some fractions. Similar distributions of activity were also obtained for both T. brucei ILTat 1.1 stumpy forms and T. congolense ILNat 2.1 (data not shown).

To confirm the localization of this VSG phospholipase within the flagellar pocket we attempted to manipulate the

**Table III. Distribution of VSG Phospholipase in Different Subcellular Fractions from Trypanosoma brucei**

| Fraction                      | Yield (%) | Relative specific activity* |
|-------------------------------|-----------|-----------------------------|
| Homogenate                    | (100)     | 1.0                         | 1.0 |
| Crude nuclear pellet          | 39.7      | 1.6 ± 0.6                   | 1.5 |
| LG fraction                   | 8.7       | 1.9 ± 0.5                   | 2.2 |
| SG fraction                   | 9.6       | 2.1 ± 0.5                   | 2.1 |
| Crude microsomal pellet       | 18.5      | 1.6 ± 0.6                   | 1.4 |
| High-speed soluble supernatant| 11.0      | 0.2 ± 0.1                   | 0.2 |
| Rough endoplasmic reticulum   |           | 1.3 ± 0.3                   | 1.3 |
| Smooth endoplasmic reticulum  |           | 1.6 ± 0.4                   | 1.3 |
| Golgi fraction                |           | 2.6 ± 0.6                   | 3.5 |
| 1st Percoll gradient†         |           |                             | 3.1 (5 B) |
| Top 20.3% (in magnesium)      |           |                             | 3.1 (5 B) |
| Flagella (entire 20.3%)       | 14.2      | 2.4 ± 0.2                   | ND (3 A) |
| Flagella (within 28.8%)       |           | 2.4 ± 0.2                   | 2.5 |
| Lysosomes                     | 0.7       | 1.6 ± 0.4                   | 1.3 (2 A) |
| Glycosomes                    | 0.3       | 0.8 ± 0.2                   | 1.1 (2 B) |
| Flagella subfractions         |           |                             | 1.1 |
| I                             |           | 1.9 ± 0.6                   | 1.8 (4, B-D) |
| II                            |           | 1.9 ± 0.3                   | 1.4 (4 A) |
| III                           |           | 2.5 ± 0.2                   | 2.4 (3, C and D) |
| IV                            |           | 2.5 ± 0.6                   | 2.6 (3 B) |
| V                             | 0.5       | 3.9 ± 0.8                   | 3.7 (5 A) |

* Specific activity relative to the homogenate. (Column A) Fractionation of MITat 1.2, MITat 1.52, and ILTat 1.1 in EDTA (data from 3–5 determinations ± standard error). (Column B) Data for enzyme assay with EDTA prepared membranes for MITat 1.2 from which most electron micrographs in the text were taken (average of duplicate determinations). (Column C) Fractionation MITat 1.2 in magnesium (average of duplicate determinations).

† Flagella fraction I was from the 28.8/20.3% Percoll interface; II was from the 20.3/17.3% interface; III was within the 17.3% layer; IV was from the 17.3/14.4% interface; and V was from top of the 14.4% Percoll layer.

Note: % Yield = % total enzymatic activity. Terms in parenthesis refer to the electron micrograph figure in the text. ND, not determined.
densities of the various fractions and try to correlate these density changes with enzyme activity. Parallel changes in the enzyme activity and morphological structures were observed when the Percoll density centrifugation gradients were run in the presence of 5 mM magnesium instead of EDTA. The density of the flagellar fraction, morphologically similar to crude flagella (see Fig. 3 A), increased dramatically with the band migrating to within a few millimeters above the lysosome band within the 28.8% Percoll layer. The mfVSG phospholipase activity also shifted with this fraction (column C in Table III). Again this activity was solubilized by detergents. Although we do not understand the mechanism involved in the magnesium-induced transition, it is known that the concentration of magnesium in the isolation medium is critical when isolating flagella from other trypanosomatids (49). In addition, after centrifugation in magnesium, a membrane fraction was consistently observed on top of the 20.3% layer well away from the shifted flagella and other membranes, which had a high specific activity for mfVSG phospholipase (column C in Table III). Morphologically and biochemically this fraction resembles the EDTA-derived light Percoll flagella membranes (compare Fig. 5, A and B). This minor fraction banded on top of 14.4% Percoll. Collectively, the data suggest that the 14.4% Percoll layer (fraction V) and the 20.3% fraction obtained on Mg-Percoll gradients are identical.

Studies Using Partially Purified Lipase

Using the light density membranes derived from EDTA-containing Percoll gradients as starting material, we have partially purified the mfVSG phospholipase on Sephacryl S-200 columns in the presence of CHAPS (Fig. 7). The molecular weight of the enzyme, as assessed by gel filtration, was ~43,000 which corresponds closely with the estimated value of 37,000-39,800 on SDS-PAGE by other laboratories (8, 20, 27). Sometimes the lipase tended to form higher molecular weight aggregates if the initial solubilization was done in 1% instead of 2% CHAPS. Like the crude enzyme, the partially purified mfVSG phospholipase did not require any metal ions for activity. However, it was stimulated somewhat better (~30%) than the enzyme in crude homogenates when incubated in the presence of DTT. Although Bulow and Overath (8) found that the enzyme was inhibited by CHAPS, no inhibition was observed under the assay conditions used here (<0.025% detergent in the assay). However, the presence of CHAPS may have affected the apparent pl of the enzyme which was 2.79 on IEF columns in Poly-Sep 47 buffer (data not shown).

Discussion

In this report we have presented biochemical evidence that a mfVSG-specific phospholipase-C activity is present within, or enclosed by, membranes of the flagella and flagellar pocket, as well as in the Golgi fraction. The principal evidence for the flagellar pocket localization of the enzyme is based on the finding that the fraction with the most mfVSG-specific phospholipase activity is high in two flagella pocket markers. Acid phosphatase is an enzyme activity found in the flagellar pocket, lysosomes, and in the Golgi apparatus of trypanosomes (23, 34, 51; Table I). Since this fraction is low in galactosyltransferase, a Golgi marker (23, 34), and in acid proteinase, a lysosome marker (35, 38, 51), the membrane fraction (which banded on top of 14.4% Percoll; fraction V) contains little or no lysosomes or Golgi fraction and must, therefore, be rich in flagellar pocket membranes. Furthermore, the high adenylyl cyclase levels in these membranes, a postulated flagella pocket marker (56), support our conclusions. Other evidence for the flagellar pocket (and associated membranes derived from it) localization stem from the following arguments. Membranes isolated by Percoll centrifugation from fractions rich in flagella have the same dimensions as those membranes surrounding axonemes with attached paraxial rods. When the density of the flagella is changed with magnesium, part of the enzymatic activity is also seen to shift with the membrane-associated flagella components. In addition the association of the mfVSG-specific phospholipase with flagella pocket membranes is also supported by the preliminary finding (our unpublished results) that after incubation of membrane fractions (isolated from horseradish peroxidase-loaded trypanosomes) with diamino-benzidine and hydrogen peroxide, only the density of the flagella fractions increased in a manner similar to that de-

Figure 7. Sephacryl S-200 column chromatography of mfVSG phospholipase. MIat 1.2 membranes isolated on 14.4% Percoll were solubilized in 2% CHAPS with protease inhibitors as described in Materials and Methods. After centrifugation, the lysate in 2% CHAPS was applied onto a 1.6- x 95-cm Sephacryl S-200 column equilibrated in 0.1% CHAPS/Hepes/PEG buffer containing protease inhibitors. Protein was monitored at 226 nm and enzyme assayed as described. Protein standards (BSA, ovalbumin, chymotrypsinogen-A, ribonuclease-A; Pharmacia Fine Chemicals) were run in the absence of detergent.

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It has been reported that mfVSG phospholipase-C is a thiol-dependent enzyme. However, the role of thiols in mfVSG-lipase activation is not clear. Several laboratories report a 1.4-5.4-fold increase in enzyme activity with high concentrations of DTT (10-25 mM) (8, 20, 27). However, the variations reported by several groups in enzyme stimulation by thiol reagents and chelators may simply reflect the purity of the water or other reagents used in the isolation buffers.

It is not clear what role the mfVSG phospholipase plays in trypanosomes. The enzyme is neither more active nor distributed differently between T. brucei slender or stumpy bloodstream forms of the parasites. Furthermore, the idea that the enzyme may be involved in the release of VSG during differentiation of bloodstream forms into uncoated procyclic forms is inconsistent with its disappearance before the release of the surface glycoprotein (7, 10). Thus, this release of VSG from the parasite may simply be due to dying parasites (5), or to some other complex interactions due to the use of inappropriate isolation/incubation conditions (35, 37). Consequently, if the VSG-specific phospholipase is involved in the release of VSG from the parasite surface as they are transformed to the supposedly VSG-free procyclic forms (14), then this release is not due to a simple increase in the total amount of enzyme. Our proposition is that the mfVSG phospholipase may be involved in surface coat recycling via an endocytotic mechanism. The involvement of phospholipase-C in VSG traffic within the parasite has also been postulated by Bülow and Overath (8). If the enzyme is in the same lipid bilayer as the VSG (8), activation of the lipase within the flagellar pocket or endosomes would release the VSG from the membrane and be taken up by the endocytotic system. In addition to removal of VSG from the membrane, the enzyme, with the help of an acid proteinase, could release the CRD for subsequent recycling into the VSG synthetic pathway (Webster, P., and D. J. Grab, manuscript submitted for publication). The release of diacylglycerol from VSG could also regulate protein kinase C, if present, within the parasite (see references 4, 39). However, the release of the enzyme either actively or as the result of dying parasites could have some other far-reaching effects on the host. We have shown that under certain conditions, living trypanosomes can release a phospholipase-C which is capable of using mfVSG as substrate (35). Decay-accelerating factor, a membrane-bound glycoprotein that inhibits amplification of the complement system (44), as well as acetylcholinesterase, have been shown to contain a phosphatidylinositol membrane-binding domain similar, if not identical, to the CRD (12, 39). Anemia is a hallmark of African trypanosomiasis. It is thus tempting to speculate that if trypanosomes released mfVSG phospholipase, either actively, or from dying organisms, it could remove the decay-accelerating factor from the surface of cell membranes, such as erythrocytes. Erythrocyte membranes altered as a result of decay-accelerating factor removal would then be susceptible to hemolysis and/or removal from the circulation by the reticuloendothelial system as occurs in patients with paroxysmal nocturnal hemoglobinuria (45).

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References

1. Baekkeskov, S., L. Rovis, and V. Verjee. 1979. The lipids of African trypanosomiasis. ILRAD Annual Report, 64-65.
2. Baltz, T., D. Baltz, C. Giroud, and J. Crockett. 1985. Cultivation in a semi-defined medium of animal infective forms of Trypanosoma brucei. EMBO (Eur. Mol. Biochem. J.) 4:1273-1277.
3. Bangs, J. D., D. Herald, J. L. Krakow, G. W. Hart, and P. T. Englund. 1985. Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein. Proc. Natl. Acad. Sci. USA. 82:3207-3211.
4. Bell, R. M. 1986. Protein kinase C activation by diacylglycerol second messengers. Cell. 45:631-632.
5. Black, S. J., R. S. Hewett, and C. N. Sendashonga. 1982. Trypanosoma brucei variable surface antigen is released by degenerating parasites but not by actively dividing parasites. Parasite Immunol. (Off. J.) 4:233-244.
6. Brown, W. C., and D. J. Grab. 1985. Biological and biochemical characterization of bovine interleukin 2. Studies with cloned bovine T cells. J. Immunol. 133:3184-3190.
7. Bülow, R., and P. Overath. 1985. Synthesis of a hydrolase for the membrane variant surface glycoprotein is repressed during transformation of Trypanosoma brucei. FEBS (Fed. Eur. Biochem. Soc.) Lett. 187:105-110.
8. Bülow, R., and P. Overath. 1986. Purification and characterization of the membrane-form variant surface glycoprotein hydrolase of Trypanosoma brucei. J. Biol. Chem. 261:11918-11923.
9. Cardoso de Almeida, M. L., and M. J. Turner. 1983. The membrane form of a trypanosome variant surface glycoprotein. Nature (Lond.) 302:349-352.
10. Cardoso de Almeida, M. L., L. M. Allan, and M. J. Turner. 1984. Purification and properties of the membrane form of a trypanosome variant surface glycoproteins (VSGs) from Trypanosoma brucei. J. Protozool. 31:53-60.
11. Courtney, P. J., J. Quintart, and P. Bauhdin. 1984. Shift of equilibrium density induced by 3,3′-diaminobenzidine cytochemistry: a new procedure for the analysis and purification of peroxidase-containing organelles. J. Cell Biol. 98:870-876.
12. Davitz, M. A., M. G. Low, and V. Nussenzweig. 1986. Release of decay-accelerating factor (DAF) from the cell membrane by phosphatidylinositol-specific phospholipase C (PIPLC). J. Exp. Med. 162:1150-1161.
13. Duvalier, G., A. Nouvelot, C. Richet, T. Baltz, and P. Degland. 1983. Presence of glycerol and fatty acids in the C-terminal end of a variant surface glycoprotein from Trypanosoma equiperdum. Biochem. Biophys. Res. Commun. 114:119-125.
14. Englund, P. T., S. L. Hågduk, and J. C. Marini. 1982. The molecular biology of trypanosomes. Annu. Rev. Biochem. 51:695-726.
15. Ferguson, M. A. J., and G. A. M. Cross. 1984. Myristylation of the membrane form of a Trypanosoma brucei variant surface glycoprotein. J. Biol. Chem. 259:3011-3015.
16. Ferguson, M. A. J., M. Duszenko, G. S. Lamont, P. Overath, and G. A. M. Cross. 1986. Biosynthesis of Trypanosoma brucei variant surface glycoproteins. N-glycosylation and addition of a phosphatidylinositol membrane anchor. J. Biol. Chem. 261:356-362.
17. Ferguson, M. A. J., K. Halder, and G. A. M. Cross. 1984. Trypanosoma
