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Purification of the membrane-form variant surface glycoprotein of *Trypanosoma brucei*

DIETMAR SCHELL and PETER OVERATH*
Max-Planck-Institut für Biologie, Corrensstrasse 38, D-7400 Tübingen (F.R.G.)

ABSTRACT

The membrane-form variant surface glycoprotein (mfVSG) is anchored in the plasma membrane of African trypanosomes by a diacylglycerol residue. On cell rupture the anchor is rapidly cleaved by an endogenous phospholipase C. A purification procedure is described which results in native mfVSG devoid of lipase activity. A total membrane fraction is prepared in the presence of the SH-inhibitor *p*-chloromercuribenzenesulphonic acid (pCMBS). Membrane proteins are solubilized in the presence of pCMBS and the detergent Zwittergent 3-12, conditions which inhibit the activity of the phospholipase. mfVSG is then purified by successive chromatography on rabbit anti-VSG affinity and cation-exchange columns (25% yield). The isolated protein is electrophoretically pure and partitions into the detergent phase on Triton X-114 phase separation, proving that it retains the diacylglycerol anchor.

INTRODUCTION

African trypanosomes, exemplified by *Trypanosoma brucei*, are unicellular flagellates which cause human sleeping sickness and Nagana in cattle. The surface of one cell is covered by 10^7 copies of a single protein, the membrane-form variant surface glycoprotein (mfVSG) which is anchored in the membrane by a glycosylphosphatidylinositol residue (GPI [1,2]). On cell rupture, an endogeous GPI-specific phospholipase C rapidly converts mfVSG to its soluble form (sVSG) and diacylglycerol [3–6]. In a recent investigation [7], sVSG was used as a model for analysing the denaturation–renaturation properties of a glycoprotein. In order to extend such biophysical studies to the membrane-form VSG, a purification procedure yielding native protein devoid of phospholipase C was required. Previous protocols for purifying mfVSG have used denaturing conditions [8–12], whereas the preparation described here was obtained under non-denaturing conditions and was free from both sVSG and phospholipase C.

EXPERIMENTAL

Materials

N^{α}-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK) (cat. no. 17013), *p*-chloromercuribenzenesulphonic acid (pCMBS) (cat. No. 16900), N-(2-hydroxymethyl)-piperazine-N′-(2-ethanesulphonic acid) (HEPES) (cat. No. 25245), 2-(N-morpho-

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lino)ethanesulphonic acid (MES) (cat. No. 29834), Triton X-100 (cat. no. 37238) and Triton X-114 (cat. No. 37243) were purchased from Serva (Heidelberg, F.R.G.), octyl glucoside (cat. No. O-8001) from Sigma (Deisenhofen, F.R.G.) and Zwittergent 3-12 (cat. No. 693015) from Calbiochem (Frankfurt, F.R.G.).

Cells

Trypanosoma brucei (variant clone MITat 1.2 [1]) was raised in rats and purified by DEAE-cellulose chromatography [13].

Antibodies

sVSG purified by DEAE-cellulose chromatography and isoelectric focusing [14] was used for the immunization of a New Zealand white rabbit [15]. The immunoglobulin G (IgG) was isolated from serum by ammonium sulphate precipitation and DEAE-cellulose chromatography. The purified antibody was coupled to activated CH-Sepharose 4B (Pharmacia–LKB, Freiburg, F.R.G.) as described by the manufacturer.

Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [16] using 4.5% acrylamide in the stacking gel and 10% in the separation gel. Proteins were rendered visible by staining with Coomassie Brilliant Blue R-250.

Detergent extract of membrane proteins

Cells ($4 \cdot 10^{10}$) were lysed in 40 ml of ice-cold 5 m*M* HEPES–0.1 m*M* TLCK–10 m*M* pCMBS (pH 7.0) using a Branson sonifier at 60 W for 2 min. The lysate was centrifuged at 4°C and 105000 *g* for 1 h. The pellet was taken up in 40 ml of 5 m*M* HEPES–25 μM TLCK–2.5 m*M* pCMBS (pH 7.0), centrifuged and extracted with 40 ml of 5 m*M* HEPES–0.1 m*M* TLCK–5 m*M* pCMBS–20 mg/ml Zwittergent 3-12 (pH 7.0) by sonication. Centrifugation yielded a clear detergent extract.

Affinity chromatography

The detergent extract was adjusted to 150 m*M* NaCl using a 1.5 *M* stock solution. A 10-ml volume of rabbit anti-VSG-Sepharose 4B with 10 mg of antibody per ml of gel was packed into an HR 10/10 column. The purification was performed in a fast protein liquid chromatographic (FPLC) system (Pharmacia–LKB). The column was equilibrated with phosphate-buffered saline (PBS)–0.2% Triton X-100 at a flow-rate of 0.5 ml/min. A 15-ml volume of the detergent extract was loaded from a 50-ml Superloop at a rate of 0.3 ml/min. The column was washed with 100 ml of PBS–0.2% Triton X-100 at 0.5 ml/min and the VSG was eluted in 2-ml fractions with 200 m*M* glycine–0.2% Triton X-100 (pH 2.5) at 0.5 ml/min in tubes containing 100 μl of 1 *M* Tris–Cl (pH 8.0). The column was used again after re-equilibration in PBS–Triton X-100 or stored in the presence of 0.1% sodium azide.

Cation-exchange chromatography

The pooled mfVSG-containing fractions were dialysed against buffer A [20 m*M* MES–0.2% Triton X-100 (pH 6.0)]. The sample was applied to a MonoS HR 5/5

cation-exchange column (50 mm × 5 mm I.D., bed volume 1 ml) equilibrated with 10 ml of buffer A. The whole cation-exchange purification was run at 1 ml/min. The column was washed with 10 ml of buffer A and then 10 ml of buffer B [20 m*M* MES–1% octylglucoside (pH 6.0)]. The bound proteins were eluted with 20 ml of a linear salt gradient of 0–400 m*M* NaCl using buffer B and buffer C [20 m*M* MES–1% octylglucoside–1 *M* NaCl (pH 6.0)]. At the end of the gradient the column was washed with buffer C and re-equilibrated with buffer A. mfVSG-containing fractions were pooled after assessing the purity of mfVSG by SDS-PAGE.

Protein determination

Protein was measured by the method of Peterson [17] using bovine serum albumin as a protein standard.

Triton X-114 phase separation

Phase-separation experiments [18] were carried out using 50 μl of precondensed Triton X-114 (2% in PBS) and 5 μl of the protein sample. A detergent concentration of 0.2% Triton X-100 in the protein solution did not interfere with the phase separation.

RESULTS AND DISCUSSION

The objective of the purification procedure was the isolation of native, phospholipase C-free mfVSG which could be used for subsequent physico-chemical studies. As mfVSG and the phospholipase show a similar behaviour on ion-exchange columns, an affinity column of immobilized anti-VSG antibody was chosen as a first

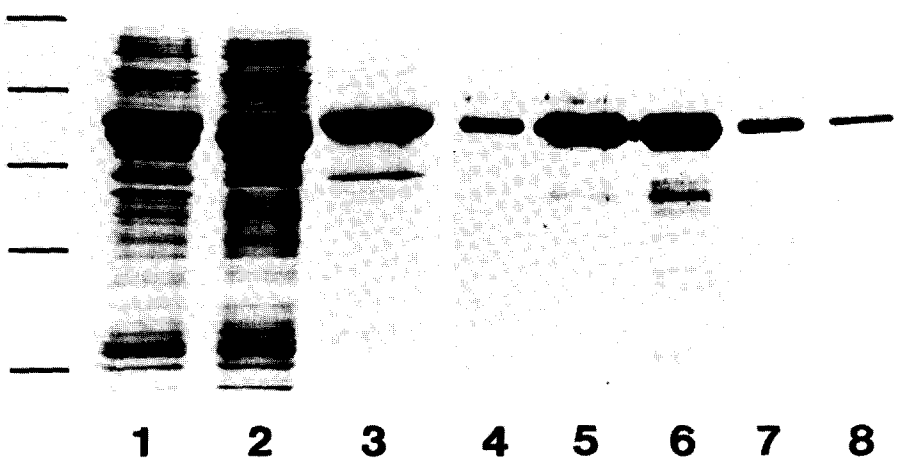

Fig. 1. SDS-PAGE of mfVSG. Lanes: 1 = homogenate of whole cells; 2 = supernatant of the homogenate after centrifugation (soluble proteins); 3 = detergent extract of membrane proteins after centrifugation; 4–8 = protein pattern produced by successive fractions eluted from the rabbit anti-VSG affinity column with glycine (pH 2.5). Molecular mass standards from top to bottom 116 000, 66 000, 45 000, 29 000 and 14 300 dalton.

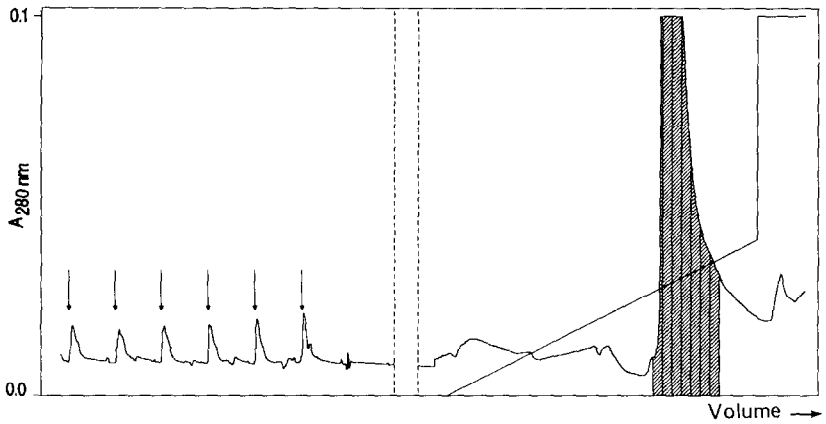

Fig. 2. Cation-exchange chromatography on a Mono S column: sequential sample application (6 times 500 µl), washing with buffer A. The detergent exchange to buffer B is not shown (space between vertical dotted lines); elution of mfVSG (shaded fractions) with a linear salt gradient. The graph is corrected to a common baseline for buffer A (before the dotted lines) and buffer B (after the dotted lines).

step for separating the glycoprotein from the enzyme. The cells were lysed in the presence of the detergent Zwittergent 3-12 and high concentrations of the sulphydryl reagent pCMBS in order to inhibit the phospholipase [4,12]. The detergent extract was

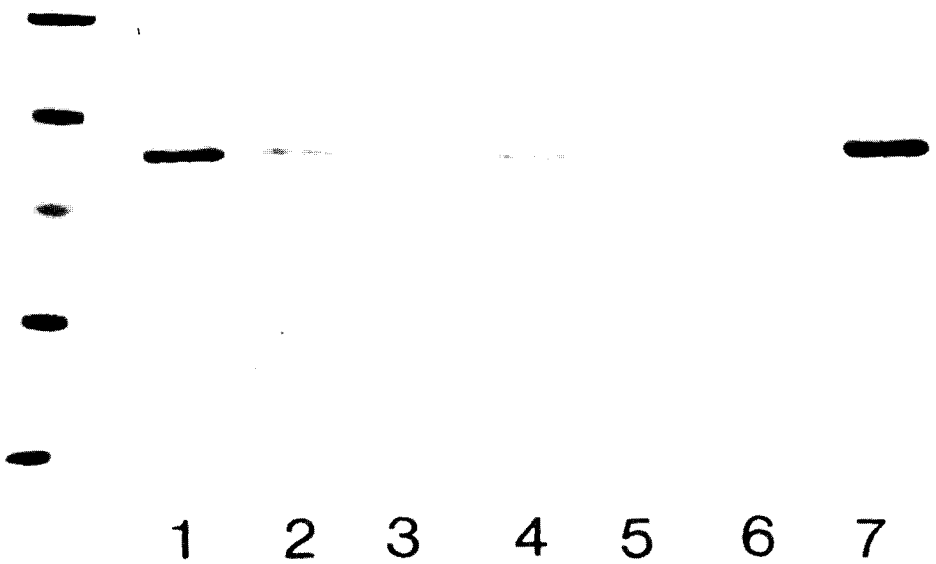

Fig. 3. Triton X-114 phase separation of mfVSG and sVSG. mfVSG eluted from the Mono S column was either directly applied to an SDS polyacrylamide gel (lane 1) or first subjected to a Triton X-114 phase separation (lane 2, detergent phase; lane 3, aqueous phase). Alternatively, mfVSG was first incubated for 1 h at 39°C and then subjected to a phase separation (lane 4, detergent phase; lane 5, aqueous phase). Lanes 6 and 7 refer to the detergent and aqueous phases, respectively, of a sample of sVSG. Molecular mass standards as in Fig. 1.

passed over the affinity column; mfVSG was eluted with glycine buffer (pH 2.5) and immediately neutralized. Fig. 1 shows the SDS-PAGE of the starting material and of the fractions eluted from the affinity column. The prominent band at an apparent M_r of 55000 is mfVSG. A multitude of minor impurities could be removed by chromatography on a cation-exchange column to which the protein binds at pH 6.0 and can be eluted at about 290 m*M* NaCl (Fig. 2). If the protein was eluted with a salt gradient using the detergent Triton X-100 instead of octylglucoside, the mfVSG eluted at 150 m*M* NaCl. On either column about half of the mfVSG could not be recovered. The final product (6 mg of protein, yield *ca.* 25%) was pure as judged by SDS-PAGE (Fig. 3, lane 1), even on highly overloaded gels (not shown).

In order to investigate whether the isolated product was mfVSG, *i.e.*, that it retained the diacylglycerol anchor, a phase separation using Triton X-114 [18] was performed. As shown in Fig. 3 (lanes 2 and 3), the protein was entirely recovered in the detergent phase as expected for an amphiphilic molecule. This pattern was not changed if the protein was first incubated for 1 h at 39°C (lanes 4 and 5). According to this criterion, mfVSG is free from phospholipase C. As a control, sVSG was entirely recovered in the aqueous phase (lanes 6 and 7). The fact that mfVSG was entirely recovered in the detergent phase is a strong argument for its native state because the denatured protein is recovered in the aqueous [19].

In conclusion, the procedure yields mfVSG devoid of contaminating GPI-specific phospholipase C in adequate yield.

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