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Expression of a glycosylphosphatidylinositol-anchored *Trypanosoma brucei* transferrin-binding protein complex in insect cells

(expression site-associated gene/baculovirus)

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ABSTRACT **The expression site-associated gene ESAG 6 was previously implicated in transferrin binding in the protozoan parasite *Trypanosoma brucei*. ESAG 6 and the closely related ESAG 7 of *T. brucei* strain AnTat1.3 have now been expressed in insect cells using the baculovirus expression system. Expression of ESAG 6 alone in insect cells gives rise to a glycosylated protein of ≈52 kDa, which is cell surface-associated through a glycosylphosphatidylinositol anchor at its C terminus. The ESAG 7 product of about 42 kDa is also glycosylated, but lacks the glycosylphosphatidylinositol modification, and is located intracellularly. No transferrin-binding activity is observed when either ESAG is expressed independently. However, their coexpression results in a cell surface complex of ESAG 6 and 7 products that specifically binds transferrin. This shows that both ESAG 6 and 7 products are necessary and sufficient for binding to transferrin.**

Trypanosoma brucei is widespread in Africa and causes a wasting disease—Nagana—in cattle and sleeping sickness in humans. In the mammalian host the parasite exists extracellularly in the blood and tissues, where it evades the immune response by sequentially expressing a repertoire of antigenically distinct surface proteins, encoded by the variant surface glycoprotein (VSG) genes. Each transcriptionally active VSG gene is located in a so-called expression site, which contains at least eight other cotranscribed expression site-associated genes (ESAGs) (1). Although highly immunogenic, the VSGs are unsuitable for immunization against the parasitic infection. Potential targets for protective immunization may be surface proteins that deliver essential nutrients to the parasite and that are invariant.

Living extracellularly, the parasite has access to the host's serum components. It has been demonstrated that *T. brucei* requires the major serum iron-transport protein, transferrin (TF), for growth (2). In *T. brucei*, a specific TF-binding protein (TFBP) that could function as a receptor in transferrin uptake was sought using TF-Sepharose as an affinity matrix (3). N-terminal sequencing of a purified TFBP isolated in extremely small amounts from *T. brucei* strain MITat 1.4 (clone 117a) revealed it to be identical to the products of two highly similar ESAGs, ESAGs 6 and 7 (3, 4). The putative products of these genes share about 85% sequence identity. Both contain a potential signal peptide, suggesting they are secreted or located on the surface. The ESAG 6 but not ESAG 7 product has a potential recognition site for attachment of a glycosylphosphatidylinositol (GPI) anchor at the C-terminal end. ESAG 6 was originally reported as encoding the TFBP (3). Subsequent work, however, has suggested that the data were misinterpreted (M. Ligtenberg and P. Borst, personal communication; this work; refs. 5 and 6) and that ESAG 6 and 7 products are involved in TF binding (this work; refs. 6 and 7).

In this paper we report the expression of both proteins individually and together in insect cells and unequivocally demonstrate that in this heterologous system, coexpression of ESAG 6 and 7 is required for TF binding and that this binding takes place at the cell surface.

MATERIALS AND METHODS

Isolation of Recombinant ESAG 6 and ESAG 7 Baculovirus. *Spodoptera frugiperda* (SF9) cells (Invitrogen) were maintained at 27°C either as monolayers in T75 flasks or in suspension in spinner flasks and were routinely cultured in TNMFH medium (Sigma) supplemented with 10% fetal bovine serum. Amphotericin (2.5 μg/ml) and gentamicin (50 μg/ml) were added for sterility in plaque assays and transfections. Viral techniques were carried out following standard procedures (8). The recombinant baculovirus transfer vectors (see Fig. 1) were cotransfected with BacPAK6 baculovirus DNA (Invitrogen) into SF9 cells using Lipofectin (Bethesda Research Laboratories) as recommended by the suppliers. Plaques were subjected to a further round of plating, after which DNA hybridizations confirmed their recombinant nature.

Production of Recombinant Proteins in SF9 Cells and Preparation of Cell Extracts. SF9 cells (1×10^6 per ml) were infected with recombinant virus at a multiplicity of 5–10 plaque-forming units per cell for 1 hr and harvested 48–72 hr postinfection. Subsequent steps were carried out at 4°C. Cells were washed in phosphate-buffered saline (PBS; 150 mM NaCl/2.8 mM KCl/1.5 mM KH_2PO_4/6.5 mM Na_2HPO_4, pH 7.4) and then resuspended in NTE buffer (150 mM NaCl/10 mM Tris/1 mM EDTA, pH 7.5) containing 100 μg of phenylmethylsulfonyl fluoride per ml, 1 μM pepstatin, 1 μM leupeptin, and 200 μM N^{α}-(*p*-tosyl)lysine chloromethyl ketone. Cells (1×10^7 per ml) were lysed by sonication and then centrifuged at 100,000 × *g* for 1 hr, yielding a supernatant and a pellet. Membrane proteins were solubilized from the pellet with 1.5% Triton X-100 in NTE buffer plus protease inhibitors by Dounce homogenization and sonication. After 2–3 hr on ice, a membrane extract was obtained following ultracentrifugation as above. For inhibition of the endogenous phospholipase C (PLC), the lysate was prepared in 10 mM *p*-chloromercuriphenylsulfonic acid, and Triton X-100 was replaced by 20 mg of Zwittergent 3-12 per ml (3).

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Abbreviations: TF, transferrin; TFBP, TF-binding protein; NPV, nuclear polyhedrosis virus; GPI, glycosylphosphatidylinositol; PLC, phospholipase C; GPI-PLC, GPI-specific PLC; ESAG, expression site-associated gene; PNGase F, N-glycosidase F; VSG, variant surface glycoprotein.

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When TF-binding activity was to be assayed, cells were infected as above and then washed extensively with TNMFH medium to remove the TF present in the fetal bovine serum. Cells were then resuspended in serum-free medium SF-900 II (GIBCO) and cultured for 48 to ≈72 hr, before being processed as above.

Antibodies and Immunochemical Techniques. All antibodies used were raised in rabbits, against the following: (*i*) synthetic polypeptides corresponding to the first 27 N-terminal amino acids of ESAG 6 and 7 products (anti-NTP); (*ii*) C-terminal amino acids G_{248}–A_{365} of ESAG 6, produced as a β-galactosidase fusion protein in *Escherichia coli* (anti-U2) (a kind gift from M. Ligtenberg and P. Borst, The Netherlands Cancer Institute, Amsterdam); (*iii*) a synthetic peptide corresponding to the C-terminal 10 amino acid residues of the ESAG 7 product (anti-CTP) (these are all fully described in ref. 6); (*iv*) the hydrophilic form of the GPI-anchored *Leishmania* protein, gp63, as a source of antibodies against the GPI anchor (anti-CRD) (9); (*v*) bovine TF (anti-bovine TF). For immunofluorescence assays, SF9 cells were infected for 1 hr with 10 plaque-forming units per cell of viral stock in T25 flasks; then 4×10^5 cells were placed onto 11-mm sterile, poly(L-lysine)-coated glass coverslips in 24-well tissue culture plates and incubated at 27°C for 48 hr. Blocking and antibody incubation steps were each performed for 30 min in 2% fish gelatin in HBS (25 mM Hepes/150 mM NaCl, pH 7.4), at 4°C. Cell surface fluorescence was detected as follows. After blocking, cells were incubated with rabbit antibody (6), followed by Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch). Cells were finally fixed in 3% formaldehyde at 4°C for 30 min, and coverslips were inverted onto microscope slides on a solution of 25 mg of 1,4-diazabicyclo(2.2.2)octane per ml in 50% glycerol in HBS and sealed with nail polish. For examination of internal fluorescence, cells were fixed, permeabilized in 0.02% digitonin for 30 min at room temperature, and then blocked and processed as above.

Purification of TFBP Complex. Holoform bovine TF (30 mg) was coupled to 1 g of CNBr-activated Sepharose (Pharmacia LKB), according to the manufacturer's instructions. For analytical preparations, 40 μl of beads was added to the extracts obtained from 1×10^7 cells (equivalent to 500 μg of protein) and shaken for 2 hr at 4°C. As a control, extracts were incubated with bovine serum albumin coupled to Sepharose beads. After incubation, the beads were washed five times with 0.5% Triton X-100 in PBS, resuspended in 100 μl of 50 mM glycine/150 mM NaCl/0.2% Triton X-100, pH 3.5, and shaken vigorously for 1 hr at room temperature. The beads were centrifuged, the supernatant containing the eluted proteins was collected, and the pH was adjusted to 7.5 using Tris buffer. Samples were then analyzed by SDS/PAGE and immunoblotting (6). For preparative purification of the proteins, cells infected with a double expressor recombinant virus (unpublished results) were harvested 65–72 hr postinfection. Cells (4×10^7 per ml) were lysed in PBS/2% Triton X-100 plus protease inhibitors by sonication, incubated overnight at 4°C, and then centrifuged at 100,000 × *g*. The supernatant was passed over a 1.25-ml bovine TF-Sepharose column, preequilibrated with PBS. Bound proteins were eluted in 50 mM glycine/150 mM NaCl, pH 3.5.

RESULTS

Expression of ESAG 6 and 7 in SF9 Cells. Recombinant baculovirus vectors were constructed as depicted in Fig. 1. Recombinant viruses obtained following cotransfection of the vectors with baculovirus DNA into SF9 cells were designated ES6-NPV and ES7-NPV, respectively. The kinetics of ES6-NPV and ES7-NPV expression in SF9 cells were analyzed by immunoblotting (Fig. 2*A*). Infection of SF9 cells with ES6-NPV resulted in a time-dependent expression of several immunoreactive bands ranging from about 52 kDa to 55 kDa, as predicted for a heterogeneously glycosylated ESAG 6 product. ES7-NPV infection induced a prominent 42-kDa band and a weaker 45-kDa band, corresponding to expression of ESAG 7. In cells infected with either ES6-NPV or ES7-NPV, an additional lower molecular mass band, expressed with the same kinetics, was recognized by the antiserum. This was observed in all independently isolated viral clones examined. No immunoreactive bands could be detected in lysates of uninfected cells or cells infected with wild-type virus.

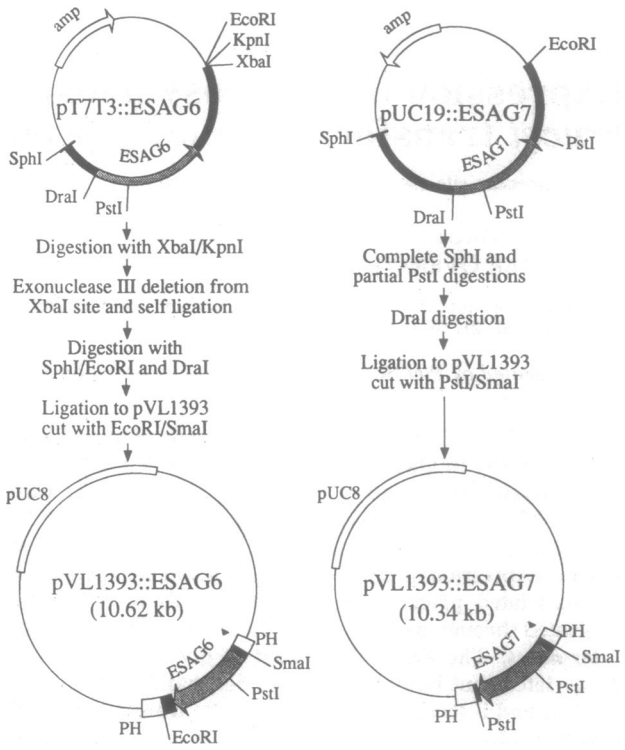

FIG. 1. Construction of recombinant transfer vectors pVL1393::ESAG6 and pVL1393::ESAG7. Genomic clones of ESAG 6 and ESAG 7 from strain AnTat 1.3 were obtained from E. Pays (Univesite Libre de Bruxelles, Brussels). The open reading frames of ESAG 6 and 7 are 1206 and 1022 nt, respectively. The ESAG 6 insert in pT7T3::ESAG 6 included 1.5 kb of 3′ sequence. There were no suitable restriction sites 3′ of the stop codon. The region downstream of ESAG 6 was shortened by exonuclease III digestion. A fragment was isolated using *Eco*RI and *Sph* I sites in the polylinker and then digested with *Dra* I, to yield ESAG 6 with 130 nt of 3′ untranslated sequence and 12 nt upstream of the ATG codon. This was ligated into pVL1393 (Invitrogen) digested with *Sma* I and *Eco*RI. Plasmid pUC19::ESAG7 was digested with *Sph* I and partially digested with *Pst* I. This fragment was then further digested with *Dra* I, to yield ESAG 7 with 12 nt upstream of the start codon and 30 nt downstream of the gene. This was ligated into pVL1393 cut with *Sma* I and *Pst* I. Recombinant clones were analyzed by detailed restriction mapping. PH, polyhedron flanking sequences. The black regions refer to upstream and downstream regions of the ESAG 6 and 7 inserts.

To investigate the distribution of the ESAG 6 and 7 products, soluble proteins, detergent-soluble membrane proteins, and insoluble proteins from both sets of infected cells were analyzed by immunoblotting (Fig. 2*B*). ESAG 6 product was consistently found associated mainly with the membrane fraction but could also be detected in the cell soluble fraction. ESAG 7 product was distributed between the soluble and membrane fractions. Treatment of pelleted membranes with detergent released considerable amounts of ESAG 6 product and the majority of ESAG 7 product. None of the lower

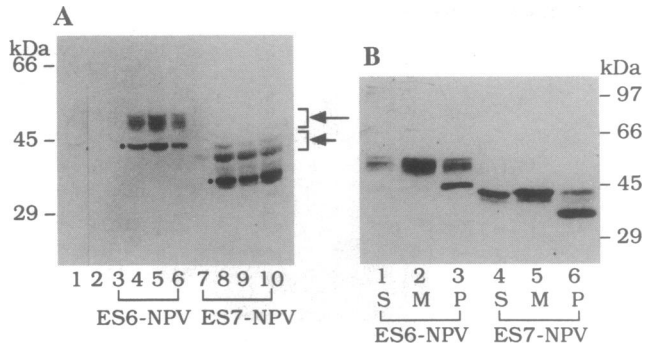

FIG. 2. Immunoblot analysis of ESAG 6 and 7 expression (*A*) and distribution (*B*) in SF9 cells, probed with anti-NTP antiserum. Each lane corresponds to cell lysates of 2×10^5 cells or cell fractions (*B*) normalized to 2×10^5 cells. (*A*) Lane 1, uninfected SF9 cells; lane 2, SF9 cells infected with wild-type virus [*Autographa californica* nuclear polyhedrosis virus NPV)]; lanes 3–6 and 7–10, lysates of cells infected with recombinant virus, harvested 24, 48, 72, and 96 hr postinfection, respectively. The long arrow points to ESAG 6 product; the short arrow points to ESAG 7 product. The dots refer to the additional lower molecular mass bands described in the text. (*B*) ES6-NPV- and ES7-NPV-infected cells were lysed 48 hr postinfection in the absence of detergents and ultracentrifuged; soluble proteins in the supernatant (S) are shown in lanes 1 and 4. Pelleted membranes were solubilized in 1.5% Triton X-100 and ultracentrifuged again. Lanes 2 and 5, detergent-soluble membrane proteins (M); lanes 3 and 6, pellet of detergent insoluble proteins (P).

molecular mass bands could be solubilized under these conditions, suggesting that they were in insoluble aggregates.

Localization of ESAG 6 and 7 Products in Infected Cells. The cellular location of the ESAG 6 and 7 products was investigated by immunofluorescence microscopy using antiserum specific for either ESAG 6 product (anti-U2) or ESAG 7 product (anti-CTP) (Fig. 3; for specificities of antibodies, see Fig. 4*B*). This demonstrated that in live cells infected with the individual viruses, ESAG 6 product was located at the cell surface. However, ESAG 7 product could only be detected after the cells were permeabilized, indicating that it was located intracellularly. Cells coinfected with ES6-NPV and ES7-NPV still showed ESAG 6 product on the surface, but now ESAG 7 product could also be detected at the surface of live cells. This suggested that the two proteins form a complex.

ESAG 6 and 7 Products Form a TFBP Complex. To determine whether ESAG 6 or ESAG 7 products, or both, are required for binding to TF, singly or coinfected cells grown in serum containing bovine TF were incubated with an anti-bovine TF antibody. This clearly showed that only when both proteins were expressed in the same cell did binding to TF take place at the cell surface (Fig. 3). No fluorescence was detected with these antibodies in uninfected cells (data not shown). To confirm the specific nature of the interaction of the ESAG 6 and 7 products with TF, lysates of cells infected with either ES6-NPV or ES7-NPV, or both together, were incubated with bovine TF coupled to Sepharose. Proteins eluted under acidic conditions were then analyzed on immunoblots probed with antiserum recognizing both ESAG 6 and 7 products, anti-NTP (Fig. 4*A*). No proteins were found in the eluates from singly infected cells (lanes 4 and 5), nor in the eluate from an incubation of a lysate from doubly infected cells with bovine serum albumin-Sepharose (lane 6). Both ESAG 6 and 7 products were present in the TF-Sepharose eluate from the doubly infected cells (lane 7). The identity of these two proteins was confirmed by immunoblotting with anti-U2 and anti-CTP antisera (Fig. 4*B*, lanes 2 and 3, respectively). Silver staining of the eluates showed that no additional proteins were present (Fig. 4*B*, lane 1). These data unequivocally demonstrated that in insect cells both ESAG 6 and 7 products are necessary and sufficient for binding to TF and form a TFBP complex. Further analysis of the extract from doubly infected cells after incubation with the TF-Sepharose revealed that a significant amount of ESAG 6 and 7 products remained in the lysate, and this could not be removed by further incubations with additional TF-

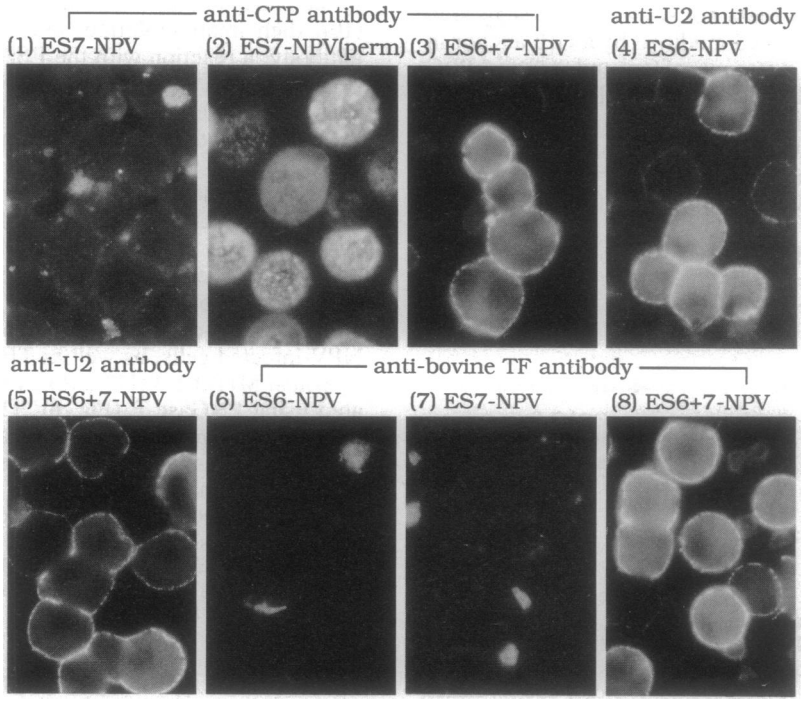

FIG. 3. Localization of the ESAG 6 and 7 products in SF9 cells by immunofluorescence microscopy. Antibody incubations were performed on live cells, except in panel 2, where cells were permeabilized. Panels 1–5: ESAG 6 and 7 products were identified using anti-U2 and anti-CTP antibodies, respectively; panels 6–8, live cells grown in serum containing TF were infected with either (or both) recombinant virus, and the presence of TF bound to the surface was detected with anti-bovine TF antibody.

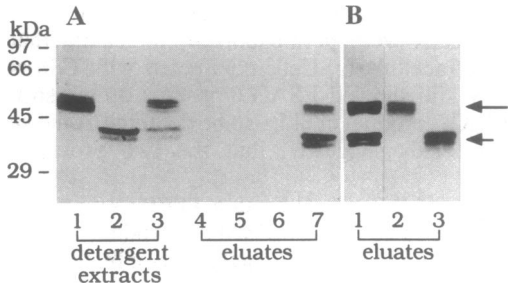

FIG. 4. TF binding to a complex of ESAG 6 and 7 products. (*A*) The immunoblot was probed with anti-NTP antiserum. Samples in all lanes correspond to 1×10^6 cells. Lanes 1–3, detergent extracts before incubation with TF-Sepharose: ES6-NPV- (lane 1), ES7-NPV- (lane 2), and ES6+7-NPV- (lane 3) infected cells. Lanes 4, 5, and 7, eluates from TF-Sepharose; lane 6, eluate from bovine serum albumin-Sepharose (control); lanes 4 and 5 are eluates from single infections of ES6-NPV and ES7-NPV, respectively; lanes 6 and 7, eluates from ES6+7-NPV-infected cells. (*B*) TFBPs eluted from TF-Sepharose analyzed on SDS/PAGE by silver staining (lane 1); immunoblotting with anti-U2 antiserum (lane 2) and anti-CTP antiserum (lane 3). The long arrow points to ESAG 6 product; the short arrow points to ESAG 7 product.

conjugated beads (data not shown). This implied that only a proportion of the proteins formed a complex able to recognize TF.

Purification of the proteins on a preparative scale from either doubly infected cells or cells infected with a double expressor recombinant virus (data not shown) yielded in both cases a maximum of 7.5 μg of functional TFBP complex per mg of total cell protein.

Detection of a GPI Anchor on the ESAG 6 Product and Evidence for an Endogenous Phosphatidylinositol-PLC (PI-PLC) Activity in SF9 Insect Cells. In trypanosomes, ESAG 6 product has a GPI anchor (6). Immunoblotting experiments with TFBP complex derived from insect cells and anti-CRD antibodies, which preferentially recognize the GPI anchor after cleavage by PI-PLC, revealed that the ESAG 6 product reacted with this antibody (data not shown). This suggested (*i*) ESAG 6 product was indeed modified by a GPI anchor in insect cells and (*ii*) the presence of an endogenous PLC activity in the insect cells. To test this, the complex was purified from soluble and membrane extracts of doubly infected cells grown in the absence of TF, using a procedure adapted from work with *T. brucei* where the endogenous enzyme was inhibited using *p*-chloromercuriphenylsulfonic

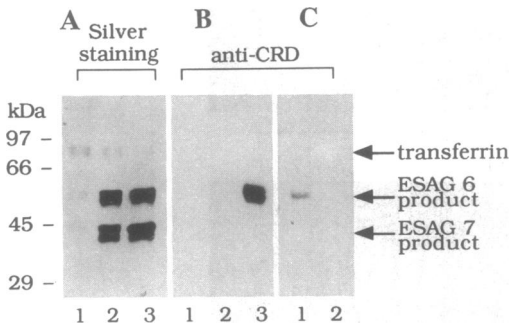

FIG. 5. ESAG 6 product expressed in insect cells is modified by a GPI anchor. TFBP complex was purified from soluble and detergent extracts of infected cells under GPI-PLC inhibitory conditions (lanes 1 and 2, respectively, of *A* and *B*) and from whole cell detergent extracts under noninhibitory conditions (lanes 3 of *A* and *B*). Lanes 1 and 2 in *C* show the detergent-soluble protein prepared under GPI-PLC inhibitory conditions. In lane 1 the complex was incubated with *T. brucei* GPI-PLC as described in ref. 10; the complex in lane 2 had no enzyme added. Purified proteins were silver stained (*A*) and analyzed by immunoblotting with antisera as indicated (*B* and *C*).

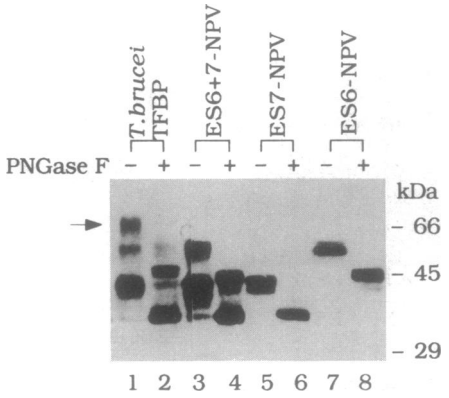

FIG. 6. Immunoblot analysis of deglycosylated ESAG 6 and 7 products, compared with the *T. brucei* deglycosylated proteins. The blot was probed with anti-NTP antiserum. +, Samples were incubated with 5 milliunits of PNGase F for 18 hr at 37°C; −, samples were controls with no enzyme. Lanes 1–4, proteins purified by elution from TF-Sepharose. Lanes 5–8, detergent-solubilized proteins of cells infected with recombinant virus. The arrow refers to the additional highly glycosylated form of ESAG 6 product in *T. brucei*. Detergent extracts of ES6-NPV and ES7-NPV and purified proteins from insect cells correspond to 1×10^6 cells; the TFBP complex from *T. brucei* is equivalent to 3×10^8 cells.

acid and Zwittergent 3-12 (3) (Fig. 5). The complex was also purified from whole cell detergent extracts in the conventional manner. Purified proteins were identified by silver staining (Fig. 5*A*), which showed that hardly any complex was obtained from the soluble fractions of cell lysates. Immunoblotting with the anti-CRD antiserum showed a strong immunoreactive band of about 52 kDa, corresponding to ESAG 6 product, only in the sample prepared under noninhibitory conditions; no cleavage of ESAG 6 product's GPI anchor could be detected under the extraction conditions known to inhibit the *T. brucei* endogenous GPI-PLC. However, when the complex prepared under conditions inhibitory to the phospholipase was incubated with *T. brucei* GPI-PLC (10), then immunoblotted, and probed with the anti-CRD antibody, a reaction with the ESAG 6 product was detected (Fig. 5*C*). This confirmed that the ESAG 6 product in insect cells is modified by a GPI anchor and that under normal extraction conditions, the protein is cleaved by an endogenous PLC.

N-Glycosidase F (PNGase F) Deglycosylation of ESAG 6 and ESAG 7 Products. As can be seen from Figs. 2, 4, and 5, both ESAG 6 and 7 products appeared as multiple bands, which may signify heterogeneous glycosylation. To analyze the glycosylation of the recombinant proteins, purified protein and detergent-extracted proteins of ES6-NPV- and ES7-NPV-infected cells, as well as TFBP complex isolated from *T. brucei* MITat 1.4 (117a) (6) as a control, were deglycosylated using PNGase F (Fig. 6). The deglycosylated sizes of ESAG 6 and 7 products are 45 kDa and 36 kDa, respectively, in both trypanosomes and insect cells. This confirms the sizes predicted from the amino acid sequence for the mature nonglycosylated proteins. The additional higher molecular mass band present in the *T. brucei*-isolated proteins appears to be a differentially glycosylated form of ESAG 6 product (6), which does not appear in ESAG 6 product isolated from insect cells.

DISCUSSION

In this study we have successfully expressed *T. brucei* ESAG 6 and ESAG 7 in insect cells using the baculovirus system. The gene products are posttranslationally modified and form a complex at the cell surface, which binds specifically to TF.

The formation of a functional TF-binding complex in insect cells demonstrates the value of the baculovirus system for the expression of trypanosome genes and indicates that the processing of the gene products is similar to that in trypanosomes. The amount of recombinant protein recovered from the TF-affinity column (maximal expression of 0.75% of total cell protein) was about 100 times more than that isolated from trypanosomes. Furthermore, not all coexpressed ESAG products could be recovered after incubation with TF-Sepharose. The discrepancy between the amounts of the individual proteins produced and the quantity of functional complex detected is commonly observed in the baculovirus system (11, 12). This may be due to several factors: not all cells are doubly infected, infected cells may have difficulty in assembling an excess of recombinant proteins, or the kinetics of optimal translation of the two proteins may not overlap to any great extent.

The additional lower molecular mass band found in both ES6-NPV- and ES7-NPV-infected cell lysates (Fig. 2) may result from incorrect translation or posttranslational processing or cleavage by a host cell protease. Additional forms of recombinant proteins are occasionally observed in the baculovirus system (13, 14).

ESAG 6 product is modified by a GPI moiety at its C terminus and this anchors both ESAG 6 and ESAG 7 products at the cell surface. ESAG 7 product itself has no transmembrane domains and, expressed alone, is found intracellularly in insect cells, where it may be retained in the endoplasmic reticulum (15). The two proteins may fold independently and their nonhomologous C-terminal regions may be required for dimerization; the N-terminal domains of ESAG 6 and 7 products may then form a TF-binding site. Alternatively, the nonhomologous regions may be involved in binding site formation. Both of these models can explain the requirement for the formation of heterodimers. Under normal extraction procedures ESAG 6 product was isolated with its anchor already cleaved, apparently by an endogenous PLC. The fact that this enzyme is inhibited under the same conditions as those for the trypanosome enzyme indicates that it may require a thiol group for activity. Judging by its immunoreactivity with specific antisera, only a small proportion of the GPI anchor of ESAG 6 product extracted under inhibitory conditions could be cleaved by *T. brucei* GPI-PLC, even after prolonged incubation times (Fig. 5*C*). The specificity of the *T. brucei* enzyme may be more restricted than the insect cell enzyme with regard to variation in the structure of GPI anchors (reviewed in ref. 16). The observation that a PLC activity exists in insect cells is not surprising, given that many eukaryotic cells express this enzyme (17, 18). Expression of a GPI-anchored membrane protein in this system has already been demonstrated (19).

Both ESAG 6 and 7 products were heterogeneously glycosylated in insect cells. Baculovirus-infected insect cells are capable of a high degree of oligosaccharide processing, the larger N-linked complex-type oligosaccharide formation occurring late in postinfective times (20, 21). The additional highly glycosylated form of the ESAG 6 product found in trypanosomes is not, however, synthesized by the insect cells. This may be due to cell culture conditions, which can influence glycosylation (reviewed in ref. 22). It is not known if this difference in the glycosylation profile of the ESAG 6 product from *T. brucei* and insect cells has any bearing on the affinity of the complex for TF.

The human TF receptor, which shows no sequence homology to the TF-binding protein of *T. brucei*, has also been expressed in insect cells and was shown to function in TF binding (23). Our data, and that from ref. 23, show that SF9 cells appear not to express any endogenous TF receptors. This should facilitate future studies of TF uptake by the TFBP complex in insect cells, as a model for uptake in trypanosomes. Due to the GPI anchor of the complex, this is unlikely to be via uptake in clathrin-coated vesicles, as is the case for the mammalian TF receptor (24). A more plausible mechanism for TF uptake could be by bulk membrane flow, as has been proposed for a GPI-anchored derivative of the CD4 receptor (25). Future studies must focus on understanding this uptake mechanism and further characterizing the heterodimeric receptor complex. Antibodies raised against complex purified from insect cells should also allow an investigation into whether these can be used to hinder TF uptake in trypanosomes, thereby inhibiting parasite growth.

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