Transferrin-binding Protein Complex Is the Receptor for Transferrin Uptake in *Trypanosoma brucei*

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Abstract. In *Trypanosoma brucei*, the products of two genes, *ESAG 6* and *ESAG 7,* located upstream of the variant surface glycoprotein gene in a polycistronic expression site form a glycosylphosphatidylinositol-anchored transferrin-binding protein (TFBP) complex. It is shown by gel filtration and membrane-binding experiments that the TFBP complex is heterodimeric and binds one molecule of transferrin with high affinity (2,300 binding sites per cell; K_D = 2.1 nM for the dominant expression site from *T. brucei* strain 427 and K_D = 131 nM for ES1.3A of the EATRO 1125 stock). The ternary transferrin–TFBP complexes with iron-loaded or iron-free ligand are stable between pH 5 and 8. Cellular transferrin uptake can be inhibited by 90% with Fab fragments from anti-TFBP antibodies. After uptake, the TFBP complex and its ligand are routed to lysosomes where transferrin is proteolytically degraded. While the degradation products are released from the cells, iron remains cell associated and the TFBP complex is probably recycled to the membrane of the flagellar pocket, the only site for exo- and endocytosis in this organism. It is concluded that the TFBP complex serves as the receptor for the uptake of transferrin in *T. brucei* by a mechanism distinct from that in mammalian cells.

TRANSFERRIN (TF)[1] is the major serum glycoprotein that transports iron to most tissues in mammals. Diferric-TF (holo-TF) binds to a specific cell-surface receptor, the ligand–receptor complex is endocytosed and delivered to endosomes (for reviews see Crichton and Charloteaux-Wauters, 1987; Huebers and Finch, 1987). The low pH of endosomes triggers the release of iron from holo-TF but the resulting iron-free TF (apo-TF) remains bound to the receptor and is recycled back to the cell surface where, at neutral pH , apo-TF dissociates from the receptor. The human TF receptor is a transmembrane glycoprotein composed of two identical disulphide-linked subunits of 90 kD; each monomer can bind one molecule of TF. The intracellular domain of the receptor subunits contains specific signals for uptake in clathrin-coated vesicles (Trowbridge et al., 1993).

African trypanosomes, the causative agents of sleeping sickness in humans and Nagana in cattle, are unicellular, flagellated protozoa that live extracellularly in the blood and tissues. The parasites require TF for growth (Schell et al., 1991*a*) and uptake has been proposed to occur by receptor-mediated endocytosis, because, in culture, clearance of radiolabeled TF is 200 times faster than the rate of pinocytosis (Coppens et al., 1987). After uptake by endocytosis in the flagellar pocket, an invagination of the cell membrane at the base of the flagellum (Balber, 1990; Webster and Russel, 1993; Overath et al., 1994), TF is delivered to tubular organelles and lysosomes between the flagellar pocket and the nucleus (Webster and Grab, 1988; Webster, 1989). Finally, Grab et al. (1992) showed by immunofluorescence experiments that TF is accumulated in lysosomes provided the incubations are performed in the presence of proteinase inhibitors. Therefore, these authors proposed that TF may be proteolytically degraded rather than recycled.

In search of a receptor for TF uptake, a complex designated TFBP (transferrin-binding protein) has been isolated from bloodstream form trypanosomes by affinity chromatography in a yield of ~3,000 molecules/cell that is composed of two proteins, pESAG 6 and pESAG 7 (for proteins derived from the expression site [ES]-associated genes 6 and 7, Schell et al., 1991*b*, 1993; Steverding et al., 1994). The respective genes, *ESAG 6* and *ESAG 7*, are located upstream of the variant surface glycoprotein gene in a polycistronic ES (Cully et al., 1985; Kooter et al., 1987; Alexandre et al., 1988; Pays et al., 1989). pESAG 6 is a heterogenously glycosylated protein of 50–60 kD modified by a glycosylphosphatidylinositol (GPI) anchor at the

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1. *Abbreviations used in this paper:* apo-TF, iron-free transferrin; ES, expression site; ESAG, expression site–associated gene; GPI, glycosylphosphatidylinositol; holo-TF, diferric transferrin; TF, transferrin; TFBP, transferrin binding protein.

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COOH terminus, while pESAG 7 is a 42-kD glycoprotein carrying an unmodified COOH terminus. The amino acid sequences of the two pESAGs are homologous over their NH_2-terminal half but diverge at the COOH termini (Pays et al., 1989). TF binding requires association of pESAG 6 and pESAG 7 as shown by coexpression in insect cells (Chaudhri et al., 1994), in procyclic forms of *Trypanosoma brucei* (Ligtenberg et al., 1994), and in *Xenopus* oocytes (Salmon et al., 1994). When isolated from trypanosomes grown in rodents, the complex is in part free and in part associated with TF. Most of it is membrane bound, but part is found in the soluble fraction of cell lysates (Steverding et al., 1994). Both TF and the TFBP complex can be demonstrated by immunoelectron microscopy in the lumen of the flagellar pocket, as well as on the flagellar pocket membrane and in intracellular vesicles (Steverding et al., 1994; Ligtenberg et al., 1994; Salmon et al., 1994).

The demonstration of an unusual TFBP complex in trypanosomes raised a number of questions regarding its biochemical properties and its function; some of these are considered in this article. In the first part, we show that the TFBP complex is a heterodimer that binds one molecule of TF with high affinity. The experiments reported in the second part lead to the proposal that the TFBP complex serves as the receptor for the uptake of TF. Our data will be compared with the results of Salmon et al. (1994) who arrived at a similar conclusion.

Materials and Methods

Reagents

Holo-TF_{bovine}, apo-TF_{human}, apo-TF_{rat}, BSA, leupeptin, chymostatin, pepstatin A, antipain, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), cycloheximide, chloramphenicol, puromycin, *p*-chloromercuribenzenesulfonic acid (PCMBS) and dibutyl phthalate were purchased from Sigma, Deisenhofen, FRG, PMSF from Serva, Heidelberg, FRG, paraffin oil (d = 0.85 g/ml) from Merck, Darmstadt, FRG, 5-(and 6) carboxy fluorescein succinimid ester and sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce Chemical Co.) from Bender and Hobein, Rottenburg, FRG, and sodium boro[^{3}H]hydride (5–20 Ci/mmol) and $^{55}FeCl_3$ (1–50 mCi/mg) from Amersham, Braunschweig, FRG.

Trypanosomes

T. brucei strain 427, variant clone MITat 1.4 (117a; Cross, 1975), and variant AnTat 1.3A from the EATRO 1125 stock (Van Meirvenne et al., 1975; Pays et al., 1989) were grown in mice and purified from blood by DEAE–cellulose chromatography (Lanham and Godfrey, 1970).

Preparation of Affinity Resins

Holo-TF_{bovine} was coupled to CNBr-activated Sepharose 4B (Pharmacia, Freiburg, FRG) as described by the manufacturer. The beads were washed by five cycles of glycine/NaCl buffer (50 mM glycine, 500 mM NaCl, pH 2.7) and PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2), which results in the release of iron from the immobilized TF (change in color from salmon pink to white). Part of the apo-TF_{bovine}–Sepharose was reloaded with iron by treatment with 100 mM NH_4HCO_3, 1 mg/ml ferric ammonium citrate (Van Driel et al., 1984). Anti-TF_{rat}–protein A–Sepharose was prepared as previously described (Steverding et al., 1994).

Radioactive Labeling of TF

Holo-TF_{bovine} was labeled with ^{3}H by reductive methylation using sodium boro[^{3}H]hydride as described by Means and Feeney (1968). Labeled protein was separated from low mol wt products by chromatography on Sephadex G-25 (PD-10 column; Pharmacia), equilibrated with PBS. The specific activity of the product was 110–530 cpm/ng.

Labeling of apo-TF_{human} with ^{55}Fe was performed using a modification of the method of Karin and Mintz (1981). First, iron was complexed by combining a solution of $^{55}FeCl_3$ (0.35 mCi; in 38.5 µl 0.1 M HCl) with 69 µl of 20 mM citric acid. After 5 min, the solution was diluted with PBSC (PBS plus 1 mM $NaHCO_3$) and the pH was adjusted to 7.5 with 0.4 M Na_2CO_3. After adjusting the volume with PBSC to 2 ml, 5 mg apo-TF_{human} in 500 µl PBSC were added and the mixture was incubated for 3 h. Excess ^{55}Fe-citrate was removed by gel filtration. The specific activity of ^{55}Fe-TF_{human} was 48,300 cpm/µg.

Labeling of Proteins with NHS-Fluorescein or NHS-LC-Biotin

IgG (purified by protein A–Sepharose chromatography from rabbit anti-TFBP or preimmune serum; cf., Steverding et al., 1994), holo-TF_{bovine} and apo-TF_{bovine} (prepared by treatment of holo-TF_{bovine} with 0.3 M Na-acetate, 10 mM EDTA, pH 5.5; cf., Coppens et al., 1987) were labeled at a molar ratio of 1:22 with NHS-fluorescein or NHS-LC-biotin, as described by the manufacturer. Unreacted reagents were removed using Centricon-30 (Amicon, Witten, FRG).

Purification of TFBP Complex

Trypanosomes (10^9/ml) were lysed on ice in lysis buffer (50 mM Na-Hepes, 2.5 mM EDTA, 2 mM EGTA, 200 µM TLCK, 400 µM PMSF, 10 µM leupeptin, 2 µM E64, 1 µM pepstatin A, pH 7.0) in the presence of 0.1% Triton X-100, sonicated three times for 2 min and then incubated for 10 min at 37°C with occasional shaking. Under these conditions, GPI-anchored proteins are cleaved by endogenous phospholipase C. The lysate was supplemented with Triton X-100 and 10× PBS to final concentrations of 2% and 1×, respectively, sonicated again three times for 2 min and centrifuged at 4°C for 1 h at 114,000 *g*. TFBP complex was precipitated from the supernatant with apo- or holo-TF_{bovine}–Sepharose or anti-TF_{rat}–protein A–Sepharose (9–37 µl beads/ml supernatant; cf., Steverding et al., 1994), by end-over-end rotation at 4°C for at least 8–11 h. After washing the beads five times with PBS/0.2% Triton X-100, bound proteins were eluted by shaking for 5–10 min at room temperature either with glycine buffer (50 mM glycine, 150 mM NaCl, 0.2% Triton X-100, pH 2.7) or sequentially with glycine buffer of pH 5.0–3.0. The acidic eluates were immediately neutralized with 1 M Tris-HCl, pH 8.0, and analyzed by SDS-PAGE and immunoblotting (Steverding et al., 1994).

Recombinant TFBP complex was produced in SF9 insect cells by coinfection with recombinant *ESAG 6* (ES1.3A) and *ESAG 7* (ES1.3A) baculovirus or by infection with a double expressor and harvested 65–72 h after infection (Chaudhri et al., 1994). Cells (1.7 × 10^9) were lysed in 42 ml PBS/2% Triton X-100 plus proteinase inhibitors by sonication, incubated overnight at 4°C, and then centrifuged at 100,000 *g*. The supernatant was passed over a 1.25-ml holo-TF_{bovine}–Sepharose column equilibrated in PBS. Bound proteins were eluted with 50 mM glycine, 150 mM NaCl, pH 3.5, and immediately neutralized by collecting 1-ml fractions into tubes containing 0.1 ml 1 M Tris-Cl, pH 8.0.

Gel Filtration Chromatography

Gel filtration of 100-µl samples was performed on a Superose 6 HR 10/30-column (Pharmacia) equilibrated with PBS, 0.02% NaN_3, pH 7.4, and a flow rate of 0.5 ml/min in an FPLC system. The column was calibrated with blue dextran 2000 (void vol), ferritin (440 kD), β-amylase (200 kD), IgG (150 kD), bovine-TF (77 kD), β-lactoglobin (37 kD), myoglobin (19 kD), and cytidine 5′-triphosphate (total volume).

Binding Assays

Binding studies were performed with membranes from trypanosomes or SF9 cells infected with recombinant virus. The membranes were prepared by lysis of cells in lysis buffer on ice in the presence of 10 mM PCMBS to inhibit the endogenous phospholipase C. After centrifugation for 10 min at 14,000 *g*, the membrane pellet was washed twice with ice-cold 50 mM glycine, 150 mM NaCl, pH 3.5, and resuspended in PBS, 100 µM PCMBS by Dounce homogenization. Membranes (1–5 × 10^8 and 0.2–1 × 10^7 cell equivalents/ml for trypanosomes and SF9 cells, respectively) were incubated with varying amounts of holo-^{3}H-TF_{bovine} in the presence of 1 mg/ml

fish gelatin in 1 ml PBS, 100 μM PCMBS by end-over-end rotation at room temperature. After 1 h, the membranes were separated by centrifugation for 5 min at 14,000 g and washed once with 1 ml ice-cold PBS, 100 μM PCMBS. The membrane pellets were then dissolved in 0.2 ml 2% SDS by boiling and the tubes were rinsed once with 0.1 ml 2% SDS. Nonspecific binding was determined in the presence of a 1,000-fold excess unlabeled holo-TF_{bovine}. In the case of SF9 cells, nonspecific binding was determined by use of membranes from uninfected SF9 cells. The bound radioactivity was determined by liquid scintillation counting in 4 ml Aquasafe 300 Plus (Zinsser, Frankfurt/Main, FRG).

Transferrin Uptake Experiments

Trypanosomes (2 × 10^7 cells/ml) were incubated with 50 μg/ml holo-^3H-TF_{bovine} or ^{55}Fe-TF_{human} in medium (Baltz medium supplemented with 1% BSA; Baltz et al., 1985) in the presence and absence of proteinase inhibitors (50 μg/ml each of leupeptin, antipain, chymostatin, and E64) at 37°C and 5% CO_2 in air. In some experiments, protein synthesis inhibitors (25 μg/ml each of chloramphenicol and puromycin and 50 μg/ml cycloheximide; cf., Stieger et al., 1984) were present. For pulse–chase experiments, trypanosomes (4 × 10^7 cells/ml) were incubated with 2.5 μg/ml holo-^3H-TF_{bovine} in medium for 30 min, then harvested by centrifugation through oil (95% dibutyl phthalate, 5% paraffin oil) and incubated in fresh medium in the presence of nonradioactive holo-TF_{bovine} after resuspension to the original density. Competition studies were performed by preincubating trypanosomes (2 × 10^7 cells/ml) in the presence of proteinase inhibitors with 130 μg/ml anti-TFBP Fab fragments or preimmune Fab fragments in medium for 15 min at 37°C and TF uptake was started by the addition of holo-^3H-TF_{bovine} at a final concentration of 0.4 μg/ml. At various times, 1-ml samples were taken and cells were harvested by centrifugation through 100 μl oil. The aqueous phase was discarded and the tubes were washed once (pulse–chase experiments and competition studies) or twice (TF uptake experiments) by carefully overlaying the oil with 1 ml PBS. Finally, the oil was dissolved in 1 ml ethanol. After removal of the ethanolic solution, the trypanosome pellets were dissolved in 0.2 ml 2% SDS by boiling and the tubes were rinsed once with 0.1 ml 2% SDS. The supernatants of the pulse–chase experiments were treated with TCA (15% final concentration) for 30 min on ice. After centrifugation, the protein precipitates were dissolved with 1 M NaOH/2% SDS at 100°C and then processed for liquid scintillation counting.

Fluorescence and Immunoelectron Microscopy

For fluorescence microscopy, trypanosomes (2 × 10^7 cells/ml) were incubated with 50 μg/ml flourescein-labeled holo- or apo-TF_{bovine} or with 100 μg/ml fluorescein-labeled anti-TFBP IgG in medium in the presence or absence of proteinase inhibitors as described above. After 2 h, cells were fixed on ice with 2% formaldehyde/0.05% glutaraldehyde, applied to poly-L-lysine–coated microscope slides, and treated with 0.0001% 4,6-diamidino-2-phenylindole in PBS. The slides were mounted in Mowiol 4-88 (Hoechst Frankfurt/Main) (Rodriguez and Deinhardt, 1960) and inspected in a fluorescence microscope (Axioplan; Carl Zeiss, Inc., Thornwood, NY) using a ×100 Plan-Neofluar objective.

For immunoelectron microscopy, cells were incubated with 50 μg/ml biotin-labeled holo-TF_{bovine} in the presence or absence of proteinase inhibitors as described above, fixed with 2% formaldehyde/0.05% glutaraldehyde in PBS, and embedded in Lowicryl HM20 (Roth, Karlsruhe) (Steverding et al., 1994). Double-labeling experiments were performed by first incubating freely floating sections with rabbit anti-biotin antibodies (Enzo Diagnostics, New York) or mouse anti-*Leishmania mexicana* cysteine proteinase antibodies (Ilg et al., 1994) and protein A–6 nm gold. After transferring the sections downside down to pioloform- and carbon-coated grids, the opposite side was labeled with rabbit anti-recombinant pESAG 6 antibodies (Ligtenberg et al., 1994) and protein A–13 nm gold. Discrete labeling on the two sides of the sections was confirmed by shadowing with platinum/carbon (Steverding et al., 1994).

Results

Molecular Mass of the TFBP and the TF-TFBP Complex

Previous experiments suggested that the TFBP complex contains pESAG 6 and pESAG 7 in approximately equimolar amounts (Chaudhri et al., 1994; Steverding et al., 1994). To determine the molecular mass of the complex, we used gel filtration of the recombinant product from insect cells. The complex eluted at a relative molecular mass of 135 kD (Fig. 1 *a*). Considering that highly glycosylated proteins tend to show an anomalously large elution volume (Andrews, 1965), this value was relatively close to the sum of the molecular masses of pESAG 6 and pESAG 7 as determined by SDS–gel electrophoresis (95 kD, Chaudhri et al., 1994). In fact, *N*-glycosidase F treatment under native conditions (15,000 U PNGase F in 60 μl PBS, 23 h, 37°C) resulted in a partially deglycosylated complex (average molecular mass 85 kD as determined by SDS–gel electrophoresis), which eluted at a mass of 96 kD. This estimate is nearly the same as the molecular mass expected for a completely deglycosylated dimer (80 kD, cf., Chaudhri et al., 1994). Therefore, we would like to suggest that the complex is a heterodimer.

Gel filtration chromatography of the recombinant TFBP complex preincubated with high concentrations of holo-TF_{bovine} (2 mg/ml) resulted in the elution of two peaks (Fig. 1 *b*). The first peak at a molecular mass of 231 kD contained TF, pESAG 6, and pESAG 7, suggesting a TFBP complex/TF stoichiometry of 1:1 (135 kD + 77 kD). A quantitative evaluation of this peak and the second peak corresponding to free TF confirmed this stoichiometry, because 100 μg TFBP complex was associated with 78 μg TF (expected 81 μg). In addition, chromatography of the ternary complex formed with holo-^3H-TF_{bovine} (0.76 mg/ml) showed that 34.9 μg (0.45 nmol) ligand was associated with 46.7 μg (0.49 nmol) TFBP complex. The ternary complex obtained by incubation of TFBP complex from *T. brucei* clone MITat 1.4 with TF likewise eluted at a molecular mass of 230 kD (Fig. 1 *c*).

Binding Characteristics of Holo-TF and Apo-TF to the TFBP Complex

Binding of holo-^3H-TF_{bovine} to the TFBP complex derived from ES1.3A (Pays et al., 1989) was studied in membranes from trypanosomes or from baculovirus-infected insect cells. Scatchard analysis indicated a single class of binding sites with K_D values of 108 ± 22 nM or 169 ± 20 nM, respectively. A much lower value, K_D = 3.6 ± 1.5 nM, was determined for clone MITat 1.4 expressing the polypeptides from the so-called dominant ES (Zomerdijk et al., 1991). Representative binding curves and Scatchard plots for the two trypanosome clones are shown in Fig. 2, *a* and *b*.

The number of TF-binding sites present in the membrane preparations of both *T. brucei* clones was ~2,300/cell (AnTat 1.3A, 2,300 ± 800/cell; MITat 1.4, 2,200 ± 700/cell). These estimates were lower than the number of TFBP complex molecules that can be isolated from a detergent extract of a cell lysate (AnTat1.3A, 3,300 ± 600/cell; MITat 1.4, 3,700 ± 400/cell). The discrepancy could arise from a partial inaccessibility of TFBP complex in closed inside-out membrane vesicles. Also, only 75% of the total amount of TFBP complex is present in the membrane fraction of a cell lysate (Steverding et al., 1994). As judged by thin sectioning electron microscopy, the membrane fraction from trypanosomes represented a heterogenous mixture of vesicles and sheetlike structures which were absent in the soluble fraction.

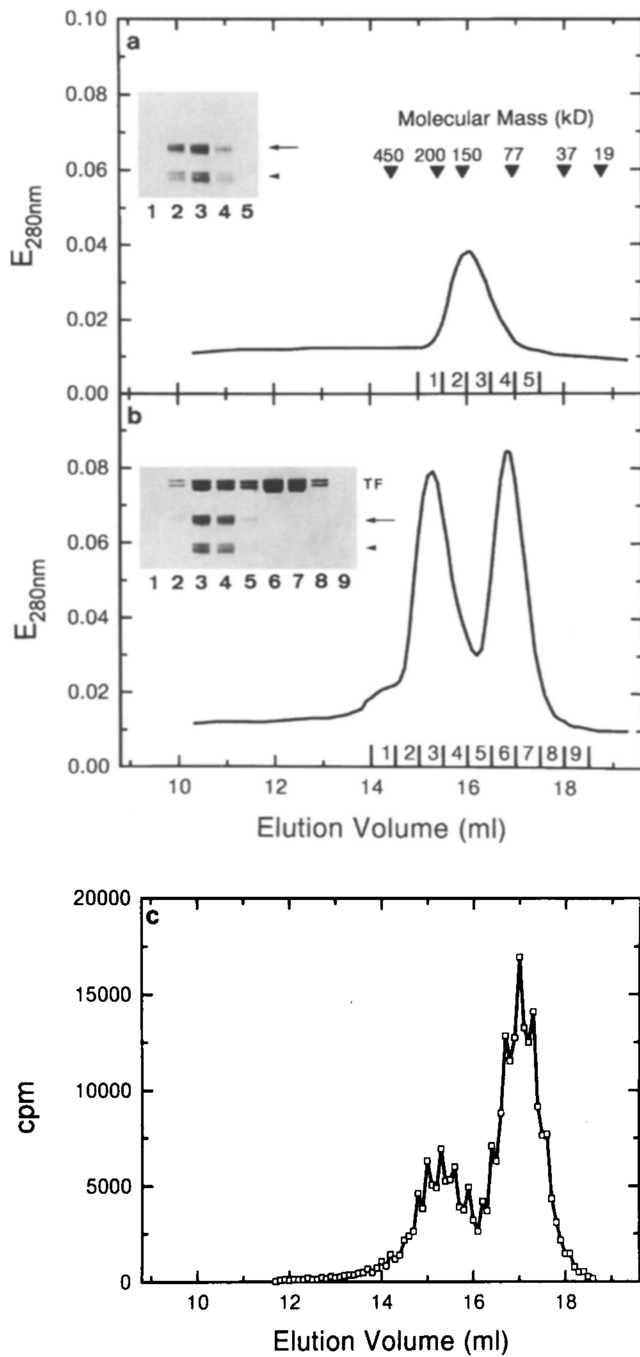

Figure 1. Superose 6 chromatography of TFBP and TF–TFBP complex. (*a*) Elution profile of 100 μg recombinant TFBP complex isolated from insect cells. 500-μl fractions were collected and analyzed by SDS-PAGE and Coomassie blue staining (*inset*) demonstrating the coelution of pESAG 6 (*arrow*) and pESAG 7 (*arrowhead*). (*b*) Elution profile of the recombinant TF–TFBP complex formed by incubation of 100 μg TFBP complex with 200 μg holo-TF_{bovine} in 100 μl for 1 h at room temperature. The first peak (fractions 2–4) contained TF, pESAG 6, and pESAG 7, the second peak (fractions 5–8) corresponded to free TF. (*c*) Elution profile of the ternary complex isolated from *T. brucei* variant clone MITat 1.4 with 21 μg/ml holo-^{3}H-TF_{bovine}. The left peak corresponds to the ternary complex, the right peak to free transferrin.

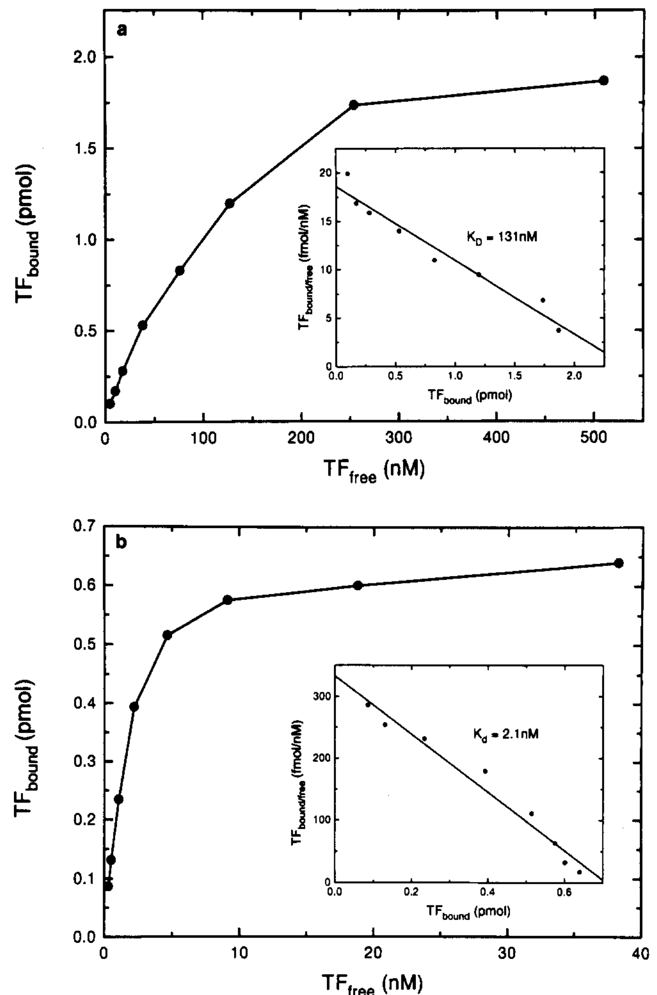

Figure 2. Binding of holo-^{3}H-TF_{bovine} to membranes of *T. brucei* variant clones AnTat 1.3A (ES 1.3A) (*a*) and MITat 1.4 (DES) (*b*). Insets show the Scatchard analysis of the binding data.

In view of the mechanism of iron uptake in mammalian cells, it appeared of interest to study the pH dependence of ligand–TFBP complex interaction. TFBP complex purified from clone AnTat 1.3A was first bound to immobilized holo- or apo-TF at pH 7.2 and then eluted at different pH values. Fig. 3 shows that both ternary complexes were stable down to pH 5; dissociation occurred between pH 4.5 and 3.5. It remains open whether under these latter conditions dissociation of the TFBP complex from holo-TF occurred after release of iron. The same binding and dissociation behavior was observed for the TFBP complex from clone MITat 1.4. In addition, we confirmed the observation of Schell et al. (1991*b*) that, in contrast to the mammalian system (Fernandez-Pol and Klos, 1980; Rudolph and Regoeczi, 1987; Turkewitz et al., 1988), the apo-TF–TFBP complex is not dissociated in the presence of the chaotropic SCN^--ion (0.5 M in 100 mM Tris/0.2% Triton X-100, pH 8).

Uptake and Degradation of Transferrin

Incubation of trypanosomes with ^{3}H-TF_{bovine} led to an initial rise in cell-associated radioactivity corresponding to a

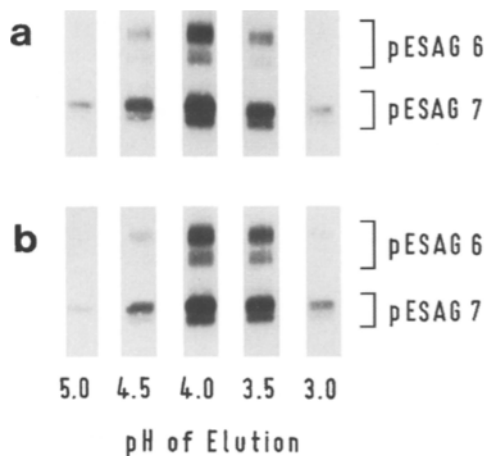

Figure 3. Immunoblot analysis of TFBP complex from clone AnTat 1.3A eluted from apo- or holo-TF_{bovine}–Sepharose at various pH values. TFBP complex was precipitated with apo- or holo-TF_{bovine}–Sepharose at pH 7.2 and bound proteins were eluted sequentially with glycine buffers (50 mM glycine, 150 mM NaCl, 0.2% Triton X-100) between pH 5 and 3. Aliquots of the eluates from apo-TF_{bovine}–Sepharose (*a*) or holo-TF_{bovine}–Sepharose (*b*) were subjected to SDS-PAGE and immunoblotting with anti-TFBP antibodies.

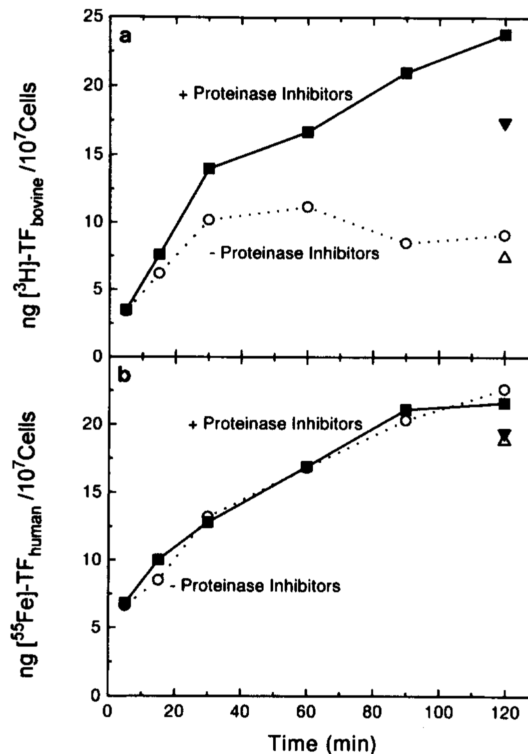

Figure 4. Uptake of holo-^{3}H-TF_{bovine} (*a*) or ^{55}Fe-TF_{human} (*b*) in the absence (*open symbols*) or presence (*closed symbols*) of proteinase inhibitors by *T. brucei* MITat 1.4. Triangles represent uptake experiments in the presence of additional protein synthesis inhibitors. Trypanosomes were incubated with 50 μg/ml TF as described in Materials and Methods. Data are the mean of two independent experiments.

rate of 15 ng/10^7 cells/h; after 60 min, a steady state was reached at 9 ng/10^7 cells (Fig. 4 *a*). This corresponds to a concentration of 7,000 molecules/cell, a value about twice as high as the TFBP complex content/cell. In the presence of proteinase inhibitors, uptake continued for 2 h (Fig. 4 *a*). The latter kinetics were also observed for the uptake of ^{55}Fe-TF_{human}, whether inhibitors were present or not (Fig. 4 *b*). These experiments suggested that, after uptake, the TF polypeptide was proteolytically degraded and the breakdown products were then released from the cells; in contrast, iron was retained. Indeed, when ^{3}H-TF uptake was performed in the absence of proteinase inhibitors and the parasites were then transferred to fresh medium, the cell-associated radioactivity decreased exponentially and, concomitantly, there appeared TCA-soluble degradation products in the culture medium (Fig. 5 *a*). These products had a molecular mass <3 kD, because they were not retained by the membrane of a centrifugal microconcentrator with a 3-kD cutoff. The presence of proteinase inhibitors during the ^{3}H-TF pulse prevented the degradation of TF during the chase (Fig. 5 *b*).

Incubation of clone MITat 1.4 trypanosomes with fluorescein-labeled holo-TF in the presence of proteinase inhibitors resulted in an intensely fluorescent region between the kinetoplast and the nucleus in all cells. Essentially no labeling was observed when the experiment was performed in the presence of a large excess of unlabeled holo-TF. Weak labeling close to the nucleus occurred when the proteinase inhibitors were omitted (data not shown). Similar results were obtained with fluorescein-labeled apo-TF and with variant AnTat 1.3A. To view the labeled compartment at a higher magnification, cells were incubated with biotinylated TF and then processed for on-section immunoelectron microscopy. Probing ultrathin resin sections with rabbit anti-biotin antibodies and protein A gold revealed labeling of large vesicles close to the nucleus in cells treated with proteinase inhibitors (Fig. 6 *a*), whereas in cells not treated with proteinase inhibitors, labeling of this compartment was very weak (not shown). The lysosomal nature of this compartment was confirmed by double labeling with a cross-reacting mouse antiserum against lysosomal cysteine proteinases of *L. mexicana* (Ilg et al., 1994; results not shown, compare below).

Involvement of the TFBP Complex in TF Uptake

The results presented in the last section establish that, as proposed by Grab et al. (1992), internalized TF is delivered to lysosomes, where the polypeptide is degraded. The following experiments are consistent with the view that TF uptake is mediated by the TFBP complex. First, when live trypanosomes were incubated with fluorescein-labeled anti-TFPB IgG in the presence of proteinase inhibitors, fluorescent label was again accumulated in a region close to the nucleus (Fig. 7, *a* and *b*), while in their absence the cells remained essentially unlabeled (not shown). Uptake of the antibody was inhibited in the presence of an excess of TF (Fig. 7, *c* and *d*) and no labeling was observed with fluorescein-labeled preimmune IgG (not shown). Therefore, specific IgG and TF compete for binding to the TFBP complex and the IgG–TFBP complex is delivered to lysosomes. Second, when ultrathin resin sections of cells treated with biotinylated TF and proteinase inhibitors were double labeled for pESAG 6 and biotin (Fig. 6 *a*) or a lysoso-

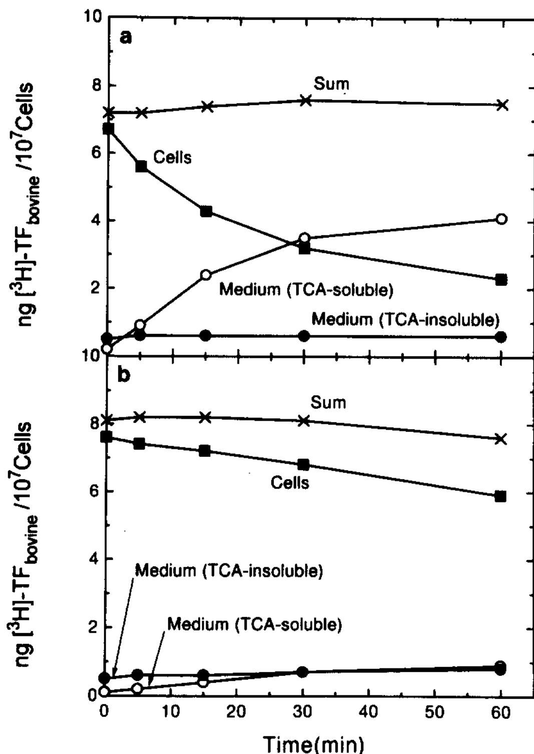

Figure 5. Release of degradation products of holo-^{3}H-TF$_{bovine}$ by clone MITat 1.4. Trypanosomes were preincubated with 2.5 μg/ml holo-^{3}H-TF$_{bovine}$ in the absence (*a*) or presence (*b*) of proteinase inhibitors, harvested, and incubated in fresh medium in the presence of nonradioactive TF$_{bovine}$ (2.5 μg/ml). ■, cell-associated TF; ○, TCA-soluble and ●, TCA-insoluble TF degradation products released into the medium; X, sum of radioactivity from cell associated TF and its degradation products. Data are the average of three independent experiments.

mal marker (Fig. 6 *b*), the gold particles colocalized in the same compartment. In cells not treated with proteinase inhibitors, the pESAG 6 labeling in the lysosomal compartment was very weak or missing (Fig. 6, *c* and *d*). In addition, the lysosomes accumulated electron-dense material in the lumen, when cells were incubated with proteinase inhibitors (compare Fig. 6, *a* and *d*). Whether this material contains membranes remains to be demonstrated. Third, the competition of anti-TFBP antibodies and TF for common binding sites could also be demonstrated for holo-^{3}H-TF$_{bovine}$ uptake. In variant AnTat 1.3A, anti-TFBP Fab fragments inhibited the accumulation of TF by 90%, while Fab fragments of the preimmune serum were without effect (Fig. 8). In contrast, at a concentration of 1 μg ^{3}H-TF/ml only a 30% inhibition of uptake was observed when Fab fragments were replaced by IgG (200 μg/ml).

In comparison to the short half-life of TF after uptake into trypanosomes (Fig. 5), TFBP complex appeared to be metabolically stable. First, the presence of protein synthesis inhibitors did not significantly influence the uptake of TF (Fig. 4, *triangles*). Since protein synthesis was inhibited by >98% under these conditions (Stieger et al., 1984), these results indicate that the TFBP complex is not degraded to a large extent during a 2-h incubation and remains available for TF uptake. Second, indirect evidence indicates that the rate of de novo synthesis of TFBP complex is low. When trypanosomes were incubated for 2 h in the presence of proteinase inhibitors, the amount of TFBP complex increased by the factor 1.4 as compared with untreated control cells (Fig. 9). Assuming that under these conditions no loss due to proteolysis occurred, the net increase should reflect the amount of newly synthesized TFBP complex.

Discussion

Subunit Structure, Affinity, and Number of Binding Sites

The TFBP complex of *T. brucei* is a heterodimer composed of one molecule of pESAG 6 containing a COOH-terminal GPI anchor and one molecule of pESAG 7 devoid of this modification. The two subunits are considered to associate side by side in the same orientation (Chaudhri et al., 1994), because a complex produced by recombinant techniques composed of a pESAG 7 containing the GPI anchor and an anchor-deficient pESAG 6 binds TF (Salmon et al., 1994). In the presence of high concentrations of TF, the heterodimer (apparent molecular mass 135 kD before and 96 kD after partial deglycosylation) derived from the insect cells binds one molecule of ligand. In gel filtration experiments the ternary complex from insect cells (Fig. 1 *b*) or trypanosomes (Fig. 1 *c*) elutes with an apparent molecular mass of 230 kD. While this estimate is in agreement with the results of Salmon et al. (1994) using the *Xenopus* oocyte system (240 kD), we disagree with their conclusion that this value suggests the binding of two molecules of TF per heterodimer. These authors did not determine the molecular mass of the functional TFBP complex first isolated by TF-affinity chromatography, but subjected material obtained from the extracellular medium of oocytes coinjected with *ESAG 7* and truncated *ESAG 6* mRNAs directly to gel filtration. The apparent molecular mass of 80 kD obtained for this product was similar to that obtained when pESAG 6 or 7 were expressed separately. In the insect cell system, a large fraction of coexpressed pESAG 6 and 7 does not assemble to a functional complex (Chaudhri et al., 1994). If this is also true for the *Xenopus* system, most of the 80-kD products analyzed by Salmon et al. (1994) may be monomeric and thus nonfunctional pESAG 6 or 7.

Binding experiments of radiolabeled TF to membranes of trypanosomes or insect cells indicate a single type of binding site (Fig. 2). The difference in the K_D values between the complex produced from *ESAGs* expressed from ES1.3A (108 nM for trypanosomes, 169 nM for insect cells) or the dominant ES (3.6 nM) is considered to be caused by variations in the amino acid sequence (Kooter et al., 1988; Pays et al., 1989; Hobbs and Boothroyd, 1990). These K_D values are of the same order of magnitude as those reported for the mammalian TF receptor (2 nM, Klausner et al., 1983; 110 nM, Octave et al., 1981). Therefore, the trypanosomal complex is expected to be saturated by the high concentrations of TF present in blood (25–50 μM). The number of binding sites per trypanosome (2,300 molecules) is in good agreement with the amount of TFBP complex that can be isolated from detergent cell ly-

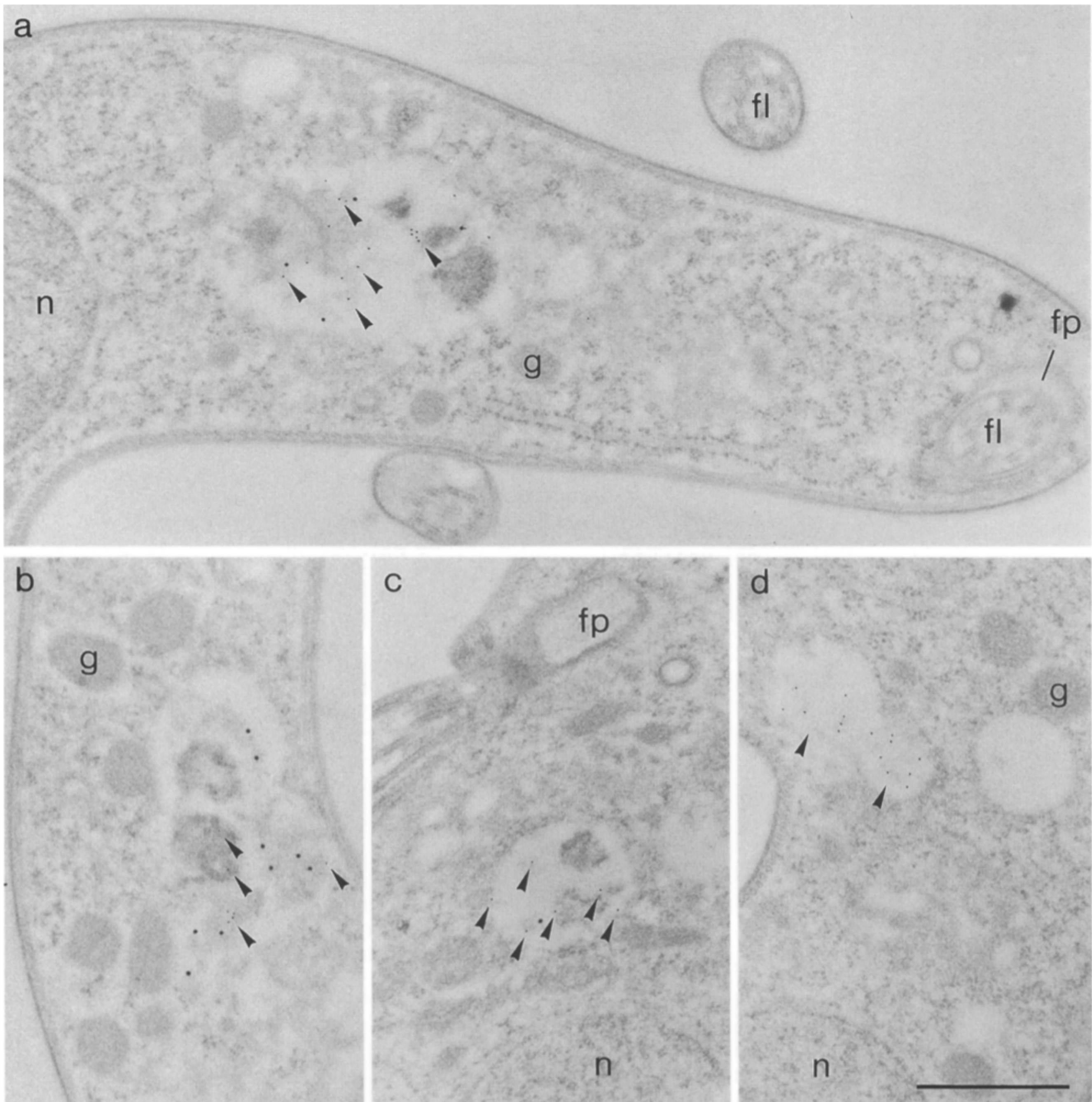

Figure 6. Ultrastructural localization of TFBP complex, TF, and lysosomal cysteine proteases by on-section immunolabeling of Lowicryl HM20–embedded clone MITat 1.4. Trypanosomes isolated from mouse blood were incubated with 50 μg/ml biotin-labeled holo-TF_{bovine} in the presence (*a* and *b*) or absence (*c* and *d*) of proteinase inhibitors and processed for immunoelectron microscopy. For double-labeling experiments, sections were probed on one side with rabbit anti-biotin antibodies/protein A–6 nm gold and on the other side with rabbit antibodies directed against recombinant pESAG 6 (anti-U2)/A–13 nm gold (*a*) or with mouse anti-cysteine proteinase antibodies/protein A–6 nm and anti-U2 antibodies/protein A–13 nm gold (*b–d*). Arrowheads point to 6-nm gold particles. *n*, nucleus; *fl*, flagellum; *fp*, flagellar pocket; *g*, glycosomes. Bar, 0.5 μm.

sates by binding to immobilized TF (Steverding et al., 1994).

Several groups have studied TF binding by incubation of radiolabeled TF to trypanosomes at low temperature. Incubation of cells for 45 min resulted in the binding of only ~900 molecules/cell (Ligtenberg et al., 1994). This binding was considered to be insignificant, because it was similar to that of insect-stage trypanosomes, which do not express the complex. In contrast, extended incubations (6 h) led to the association of high amounts of TF with the parasites (150,000 binding sites/cell, K_D = 1 μM, Coppens et al., 1987; 20,000 binding sites/cell, K_D = 830 nM, Salmon et al., 1994). The discrepancy between these latter estimates and our value suggests that under these conditions trypano-

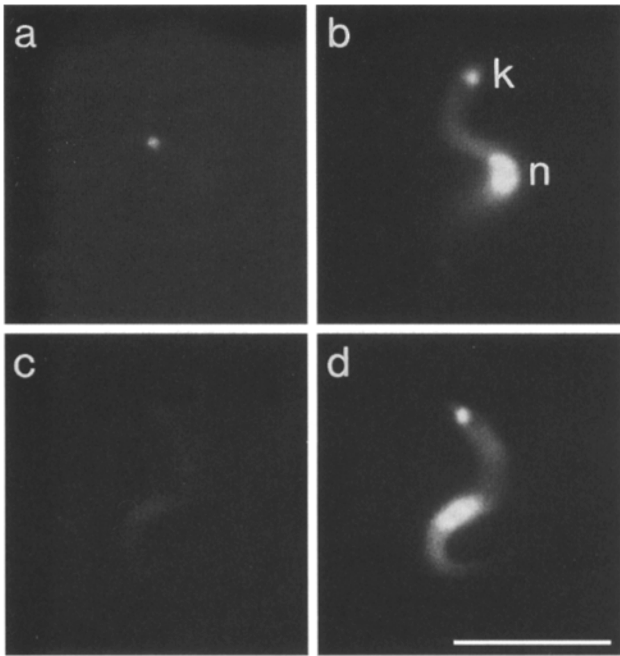

Figure 7. Immunolocalization of endocytosed fluorescein-labeled anti-TFBP IgG in clone MITat 1.4. Trypanosomes were incubated with proteinase inhibitors and 100 μg/ml fluorescein-labeled anti-TFBP IgG in absence (*a* and *b*) or presence (*c* and *d*) of 50 μg/ml holo-TF_{bovine} as competitor. (*a* and *c*) Fluorescence images; accumulated anti-TFBP IgG was observed between kinetoplast (*k*) and nucleus (*n*). (*b* and *c*) Corresponding DNA staining. Bar, 5 μm.

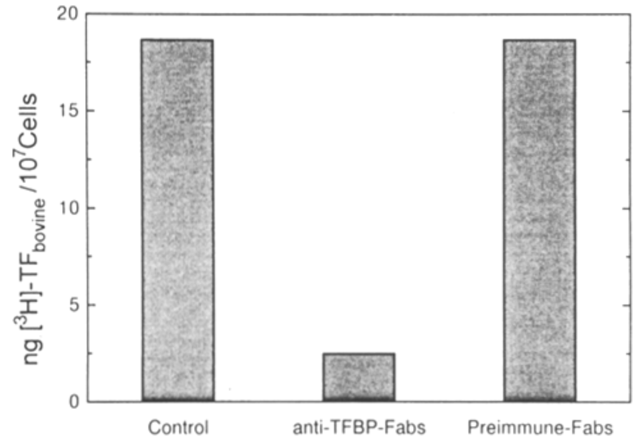

Figure 8. Inhibition of holo-^{3}H-TF_{bovine} uptake by anti-TFBP Fabs in clone AnTat 1.3A. Trypanosomes were incubated in the presence of proteinase inhibitors and 0.4 μg/ml holo-^{3}H-TF_{bovine} with 130 μg/ml anti-TFBP Fabs or preimmune Fabs, respectively. After 2 h at 37°C, trypanosomes were harvested and the cell-associated radioactivity was determined. Data are the average of two independent experiments. Control refers to incubation without Fab fragments.

somes associate with TF by a mechanism that does not involve the TFBP complex.

Transferrin Uptake by the TFBP Complex

Several observations suggest that the TFBP complex is the trypanosomal TF receptor. First, based on the results of our purification method, pESAG 6 and pESAG 7 are the only TF-binding proteins in trypanosomes (but see Grab et al., 1993) and, second, part of the complex in cells is associated with TF, because anti-TF antibodies coprecipitate a fraction of TFBP complex from detergent lysates (Steverding et al., 1994). Third, TF inhibits the uptake of anti-TFBP IgG (Fig. 7) or anti-TFBP Fab fragments inhibit TF uptake (Fig. 8). Salmon et al. (1994) have previously shown that anti-pESAG7 leads to a partial inhibition of TF uptake. The extent of inhibition depends on whether one uses IgG (not very efficient) or Fab fragments (up to 90% inhibition, cf. Fig. 8), on the ratio of anti-TFBP IgG to TF and, most likely, on the affinity of TF and the IgGs for the binding site of the particular pESAG 6/7 complex. An important implication of these results is that in view of the high TF concentration in the blood, it will be very difficult or impossible to inhibit TF uptake with antibodies against the complex in vivo and thus deprive the parasites of an essential growth factor. This question requires more systematic investigations preferentially with monoclonal antibodies. However, immunization experiments with the TFBP complex in mice have so far been totally unsuccessful (Steverding, D., unpublished results). Whether, in addition, a mechanism that distracts the immune response from the binding site is operative (Borst and Rudenko, 1994) remains to be demonstrated. Last, provided TF degradation is prevented by proteinase inhibitors, both TFBP complex and TF accumulate in lysosomes (Fig. 6). The concomitant lysosomal localization after proteinase inhibitor treatment observed in this study is characteristic for all trypanosome cells. Therefore, in the presence of proteinase inhibitors, the lysosomes appear to serve as a sink for ternary complexes.

We consider that after assembly in the endoplasmic reticulum, the pESAG 6/7 heterodimer is translocated via the Golgi complex to the flagellar pocket membrane and may then be in a diffusional equilibrium with the cell surface. However, binding of TF may only occur in the membrane lining the flagellar pocket, possibly because there

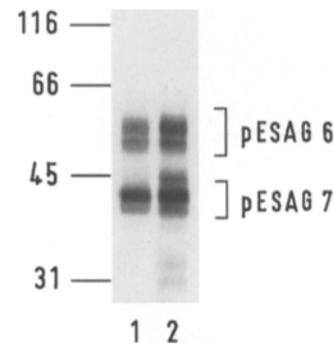

Figure 9. Stability of TFBP complex in clone AnTat 1.3A. Trypanosomes were incubated for 2 h with 15 μg/ml TF_{rat} in the absence (lane *1*) or presence (lane *2*) of proteinase inhibitors. After lysis, TFBP and TF–TFBP complex were precipitated with a mixture of holo-TF_{bovine}–Sepharose and anti-TF_{rat}–protein A–Sepharose. Bound proteins were eluted with glycine buffer (50 mM glycine, 150 mM NaCl, 0.2% Triton X-100, pH 2.7) and aliquots were analyzed by immunoblotting using anti-TFBP antibodies. Filter strips corresponding to the bands were excised, and the bound alkaline phosphatase was determined spectroscopically using *p*-nitrophenyl-phosphate as a substrate, thus providing a relative and semiquantitative estimate of TFBP complex in either fraction.

the variant surface glycoprotein coat is not so dense. The ternary complex is then internalized by endocytosis and delivered to lysosomes. There, TF is degraded, but iron is retained and the degradation products and, most likely, the metabolically relatively stable TFBP complex are recycled to the flagellar pocket. Recycling of the receptor appears likely considering that iron uptake in 120 min (18,000 molecules/cell cf., Fig. 4 *b*) is about six times higher than the amount of receptor. Since the ternary complexes with holo-TF and apo-TF are stable down to a pH of 5.0, the sequence of events leading to ligand dissociation and degradation remains undefined. A similar intracellular pathway has been demonstrated for the variant surface glycoprotein (for review see Duszenko and Seyfang, 1993) and a lysosomal glycoprotein (Brinkman and Balber, 1994). Whether endocytosis and recycling of the TFBP complex occurs by bulk membrane flow or whether other proteins are involved in this process remains to be determined.

The origin, physical state, and functional role of TF–TFBP complex in the lumen of the flagellar pocket (Ligtenberg et al., 1994; Salmon et al., 1994; Steverding et al., 1994) remains to be resolved. The amount of complex detected by immunoelectron microscopy can be highly variable both within a given cell population and between different populations, e.g., high in a fraction of cultured parasites (Steverding et al., 1994) and generally low in cells isolated from mouse blood and incubated with biotinylated TF (Fig. 6). In contrast, all trypanosomes accumulate TF and TFBP to a similar extent in lysosomes once protein degradation is inhibited.

The present paper extends the profound differences in primary structure, subunit organization, mode of membrane anchorage, and binding characteristics of holo- and apo-TF between the trypanosomal and the mammalian receptor to the ligand uptake mechanism. Whereas in mammalian cells, iron is dissociated from the transferrin–TF receptor complex in acidic endosomes and apo-TF remains bound to the receptor and is recycled to the cell surface where it can mediate further circles of iron uptake, TF is degraded after uptake in trypanosomes. This mechanism resembles the uptake of low density lipoproteins or of asialoglycoproteins by the low density lipoprotein receptor or the asialoglycoprotein receptor, respectively, in mammalian cells. TF uptake in trypanosomes may be characteristic for endocytic uptake systems developed early in evolution while uptake of TF in mammalian cells may be considered to be an advanced mechanism that allows multiple use of this iron carrier.

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