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Receptors for the host low density lipoproteins on the hemoflagellate *Trypanosoma brucei*: Purification and involvement in the growth of the parasite

(receptor-mediated endocytosis/protozoan/plasma membrane antigen/immunization)

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ABSTRACT **The slender bloodstream form of *Trypanosoma brucei* shows receptor-mediated endocytosis of low density lipoprotein (LDL) particles of its hosts. We have purified the LDL receptor of this species nearly to homogeneity (about 1000-fold purification) and obtained monospecific polyclonal antibodies against it. As analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis, the purified receptor consists of a single subunit, with an apparent molecular mass of 86 kDa. Its isoelectric point is 5.9. On the average, each cell exposes 52,000 copies of low-affinity receptors (K_d of 250 nM) and 1800 copies of high-affinity receptors (K_d of 5.7 nM). According to indirect *en bloc* immunolabeling of fixed parasites, the receptor appears to be localized to the flagellar pocket membrane and the flagellar membrane and to be completely absent from the rest of the pericellular membrane. LDL is required for optimal growth of the trypanosome *in vitro*: cell growth can be inhibited either by removal of LDL from the culture medium or by antibodies against the purified LDL receptors. In both cases, growth is restored by the addition of excess LDL.**

African trypanosomes belonging to the *Trypanosoma brucei* complex are the causative agents of sleeping sickness in humans and of nagana in livestock. *T. brucei* is an extracellular parasite that circulates in the bloodstream and body fluids of the mammalian host, where it divides every 7 hr. Although cholesterol is the major sterol in the membranes of the bloodstream form, there is no evidence that this form is capable of synthesizing its own cholesterol *de novo* (1, 2). Consequently, *T. brucei* is assumed to incorporate this lipid intact from the host (2, 3). However, in the mammalian bloodstream, cholesterol is not freely available but is buried, partly as esters, within circulating low density lipoprotein (LDL) particles (4). We previously showed that the bloodstream form of *T. brucei* specifically takes up LDL from its hosts through its flagellar pocket (5). This uptake was characterized by an accelerated clearance (3 orders of magnitude higher than that of fluid-phase endocytosis), an almost complete dependence on calcium, saturation of binding, specific competition by the homologous protein, and sensitivity of uptake by intact cells to trypsin. The occurrence of receptor-mediated endocytosis was further supported by the presence of coated pits on the flagellar pocket membrane. These observations suggested to us that trypanosomes must have their own receptor for LDL. In this paper we report the isolation and partial characterization of this LDL receptor and offer evidence that antibodies directed against the receptor inhibit growth of trypanosomes in culture.

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MATERIALS AND METHODS

Isolation and Cultivation of Trypanosomes. Bloodstream forms of *T. brucei* stock 427 were grown in 300-g Wistar rats (6). Blood was withdrawn from highly infected rats (10^9 trypanosomes per ml of blood) by cardiac puncture under ether anesthesia. Trypanosomes were separated from blood cells by passage through a DEAE-cellulose column (7). They were washed three times in 65 mM phosphate buffer solution (pH 8) containing 55 mM glucose and were recovered each time by centrifugation at 4000 rpm for 10 min at 7°C in an SS-34 rotor of a Sorvall RC-5 centrifuge (Sorvall Instruments, Wilmington, DE).

Growth of trypanosomes was monitored *in vitro* by counting parasites after cultivation at 37°C on a feeder layer of fibroblast-like cells of *Microtus montanus* embryos (8), in 4 ml of culture medium supplemented with 1 ml of serum.

Lipoproteins. Rabbit LDL was isolated from freshly isolated plasma supplemented with KBr to increase plasma density and was recovered between densities of 1.019 and 1.063 g/cm^3 (9). Lipoprotein-free serum was obtained by centrifugation after the density was increased to 1.215 g/cm^3 (9). Lipoproteins were radiolabeled with ^{125}I by means of iodine monochloride (10) and dialyzed against 0.15 M NaCl containing 0.01% EDTA (pH 7.4). Specific radioactivity of ^{125}I-labeled LDL (^{125}I-LDL) was 200 dpm/ng of protein.

Binding Experiments. Trypanosomes were cultivated for 48 hr in lipoprotein-free medium, harvested, resuspended at a density of 10^8 cells per ml, and incubated at 4°C in 137 mM NaCl/5.4 mM KCl/0.34 mM Na_2HPO_4/0.44 mM KH_2PO_4/3.6 mM $CaCl_2$/0.8 mM $MgSO_4$/1% (wt/vol) bovine serum albumin/1% (wt/vol) glucose together with the labeled lipoproteins. The pH of this mixture was adjusted to 7.4. After incubation, the cells were washed and cell-associated radioactivity was determined as described (5).

Purification of the LDL Receptor. Membranes from 43 g (wet weight) of trypanosomes were prepared and processed as described by Schneider *et al.* (11), except that the trypanosomes had to be homogenized by sonication because of their organized subpellicular microtubular system. At each step of purification, aliquots were taken for assay of ^{125}I-LDL binding activity and protein content. High-affinity ^{125}I-LDL binding was determined by the standard phosphatidylcholine/acetone precipitation assay at an ^{125}I-LDL concentration of 10 μg of protein per ml (11).

Membranes were solubilized with 1% (vol/vol) Triton X-100 and a first purification was performed by DEAE-cellulose chromatography at pH 6. Further purification was obtained by affinity chromatography with LDL coupled to Sepharose 4B. The receptor bound to the column was eluted

Abbreviations: LDL, low density lipoprotein; ^{125}I-LDL, ^{125}I-labeled LDL.

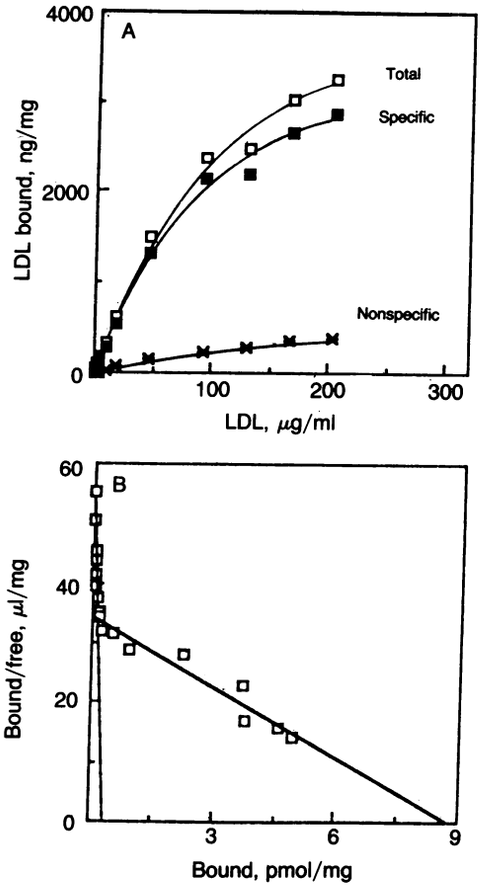

FIG. 1. Concentration-dependent binding of ^{125}I-LDL to intact trypanosomes. (*A*) Trypanosomes (10^8 cells per ml) were incubated for 6 hr at 4°C in incubation medium containing various concentrations of rabbit ^{125}I-LDL. After the cells were washed, cell-associated radioactivity was measured and expressed as ng of ^{125}I-LDL per mg of cell protein (1 mg of protein represents 10^8 cells). Total binding (□) was corrected for nonspecific binding (✱), which was measured in the presence of 4 mM EDTA, to yield specific binding (■). Incubation was extended up to 12 hr with identical results (data not shown). (*B*) Scatchard plot analysis of specific binding.

with suramin, a polysulfonated polycyclic hydrocarbon that interferes with the LDL–receptor interaction (11).

Electrophoresis. One-dimensional electrophoresis (12) was carried out in a polyacrylamide (7.5–15% linear gradient) slab gel (0.7 × 140 × 180 mm) containing 4% NaDodSO$_4$. Each sample (1–50 μl) was diluted in 40 mM Tris·HCl, pH 6.8/4 mM Na$_2$EDTA/4% (wt/vol) NaDodSO$_4$/20% (wt/vol) dithiothreitol/40% (vol/vol) glycerol/0.4% bromophenol blue. After heating at 100°C for 4 min, the samples were applied to the gel and electrophoresed at 100 V for 3 hr at 10°C. The gel was stained with 0.05% Coomassie blue.

Antibodies. Rabbits were immunized with 70 μg of the most purified receptor preparation, obtained from the affinity chromatography column, in Freund's complete adjuvant (Difco).

The antiserum was characterized by immunoblotting. Proteins of receptor preparations were transferred for 20 hr from the NaDodSO$_4$/polyacrylamide slab gels to nitrocellulose paper according to the method of Burnette (13) as modified by Beisiegel *et al.* (14). The nitrocellulose paper was saturated with 4% bovine serum albumin and then incubated for 4 hr at room temperature with the antiserum (dilution, 1:100) in 10 mM Tris·HCl, pH 8/2% bovine serum albumin and washed. Bound antibodies were detected by autoradiography after incubation with ^{125}I-labeled donkey anti-rabbit IgG antibodies.

Specific anti-LDL-receptor antibodies were affinity-purified on the 86-kDa protein adsorbed onto nitrocellulose. A nitrocellulose transfer was prepared and incubated with the antiserum as above, and then the band corresponding to 86 kDa was excised and transferred to 0.2 M glycine/HCl buffer (pH 2.8) containing 0.2% gelatin (15) at room temperature. After 2 min, the eluate was neutralized with unbuffered 1 M Tris.

Ultrastructural Immunolocalization. Gold particles 15 nm in diameter were obtained by reduction of 0.3 mM tetrachloroauric solution with 1.2 mM sodium citrate (16) and covered with protein A (17). To localize the LDL receptors on the surface of the trypanosomes, a suspension of parasites (10^8 cells per ml) was fixed for 15 min at room temperature in 4% formaldehyde/1% glutaraldehyde/0.1 M sodium cacodylate buffer, pH 7.4. Cells were incubated with antiserum (dilution, 1:5), washed, labeled with protein A–gold conjugates (5 × 10^{11} particles per ml), and processed for electron microscopy (5).

Other Assays. The protein content of lipoproteins and receptor preparations was determined by the method of Lowry *et al.* (18), with bovine serum albumin as a standard.

RESULTS

We first determined the affinity of the putative receptor for LDL on the bloodstream form of *T. brucei*, as well as the number of receptors per cell. Binding studies were performed with intact cells after 6 hr of incubation at 4°C, to allow adsorption of the LDL particles to their receptors to reach equilibrium. Fig. 1*A* shows the binding of LDL as a function of its concentration, and Fig. 1*B* shows the same data analyzed according to Scatchard (19). This analysis suggests that, on the average, each trypanosome has 52,000 low-affinity binding sites with an equilibrium dissociation constant (K_d) of 250 nM, and about 1800 high-affinity binding sites with a K_d of 5.7 nM.

We next purified the LDL receptor from *T. brucei* by using the rapid two-step procedure developed for the LDL receptor

Table 1. Purification of LDL receptor from bloodstream form of *T. brucei*

Step	Protein, mg			LDL-binding activity			Purification factor
	Applied	Recovered	(%)	ng	(%)	ng/mg	
Sonication	—	3386*	(100)	7737	(100)	2.29	1
High-speed sedimentation (100,000 × *g* for 60 min)	3311	848	(25)	5919	(76.5)	6.98	3
Solubilization	825	375	(11)	3060	(39.6)	8.16	3.6
DEAE-cellulose chromatography (pooled peak fractions)	368	13.3	(0.39)	1249	(16)	93.9	41
LDL-affinity chromatography (pooled peak fractions)	5.163	0.534	(0.016)	1043	(13.5)	1953	855†

*Starting from 43 g of trypanosomes.
†In the most purified fraction, purification was 1100-fold.

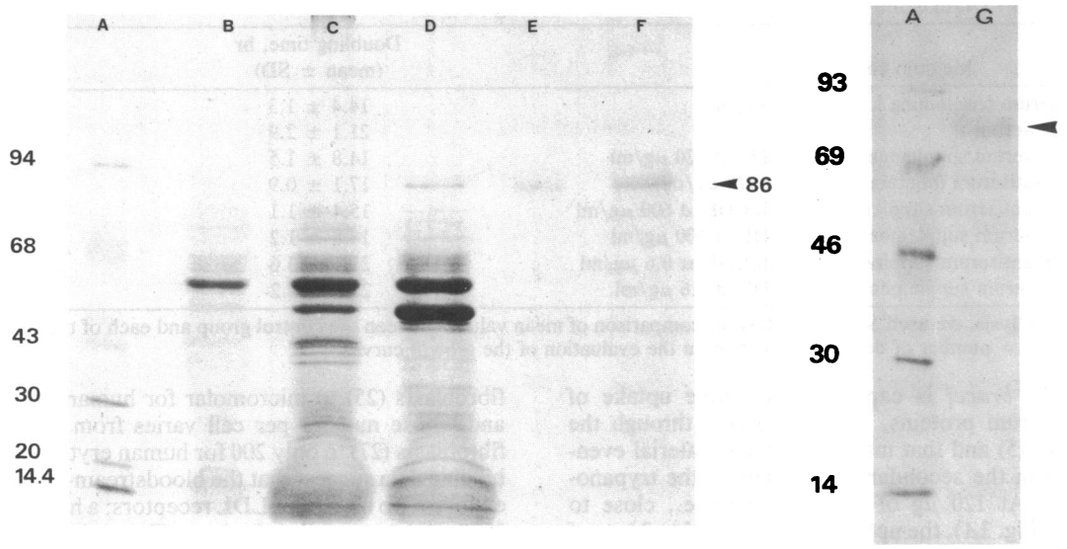

FIG. 2. (*Left*) NaDodSO$_4$/polyacrylamide gel electrophoresis at various stages of purification. Lane A, molecular size markers from Pharmacia (Uppsala, Sweden) (phosphorylase *b*, 94 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14.4 kDa); lane B, sonicated cells (50 μg); lane C, pelleted membranes (50 μg); lane D, DEAE-cellulose peak fraction (50 μg); lanes E and F, LDL-affinity peak fraction (2.5 μg and 5 μg). (*Right*) Autoradiograph of nitrocellulose blot replica. Lane A, ^{14}C-labeled methylated size markers from Amersham (Buckinghamshire, U.K.) (phosphorylase *b*, 93 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14 kDa); lane G, immunoblot of the DEAE-cellulose fraction.

of bovine adrenal cortex (11). The final preparation was purified 850-fold, expressed as LDL-binding activity, with an overall yield of about 14% (Table 1). This preparation was markedly enriched in a single polypeptide with an apparent molecular mass of 86 kDa (Fig. 2). This protein was acidic, with an isoelectric point of 5.9 as determined by isoelectric focusing (data not shown).

Antiserum directed against the purified receptor, as well as antibodies specific for the 86-kDa protein, strongly inhibited LDL binding (Fig. 3), confirming that the 86-kDa protein is the LDL receptor.

LDL was essential for optimal growth of *T. brucei in vitro* (Table 2), where the normal doubling time is about 14.4 hr. When the serum added to the growth medium was depleted of lipoproteins, this resulted in a significant increase of division time from 14.4 ± 1.3 hr to 21.1 ± 2.9 hr ($P < 0.005$). Normal growth was restored by the addition of LDL alone. In the presence of an LDL concentration in the range achieved by addition of normal serum (120 μg/ml), the antiserum directed against the LDL receptor also caused a significant increase in division time (to 17.1 ± 0.9 hr, $P < 0.005$), whereas preimmune serum had no effect (data not shown). At very high or very low concentrations of LDL, anti-LDL-receptor antibodies had no effect, compared to nonimmune serum. The reversal of inhibition of growth by antiserum in the presence of LDL at 5 times the physiological concentration suggests a competition between LDL and antibodies for the LDL receptor. Interestingly, in the presence of 120 μg of LDL per ml, the addition of 10% (vol/vol) antiserum, which inhibited cell growth by about 50% of the inhibition achieved by the removal of lipoproteins, also decreased LDL uptake by 50% (data not shown).

When cells were incubated for 2 hr at 4°C with LDL adsorbed to colloidal gold (5), the flagellar pocket was densely labeled by gold particles, both at the pocket membrane (52% of particles counted, $n = 209$) and at the membrane of the buried segment of the flagellum (26% of particles). Labeling was less intense along the flagellar membrane outside the pocket, which bound only 22% of particles, although its membrane area is roughly 17 times larger than the two other labeled membrane domains (5). No labeling was detected elsewhere at the pericellular membrane. Similarly, when cells were fixed and incubated with anti-LDL-receptor antibodies followed by protein A-gold particles, this also resulted in an abundant labeling of the flagellar pocket membrane (38% of particles counted, $n = 339$) and the membrane of the buried segment of the flagellum (36% of particles). There was also some labeling at the flagellar membrane outside the pocket (26% of particles). Gold particles never attached elsewhere on the cell surface (Fig. 4).

DISCUSSION

Bloodstream-form trypanosomes depend on host LDL as a source of cholesterol for optimal growth. We previously

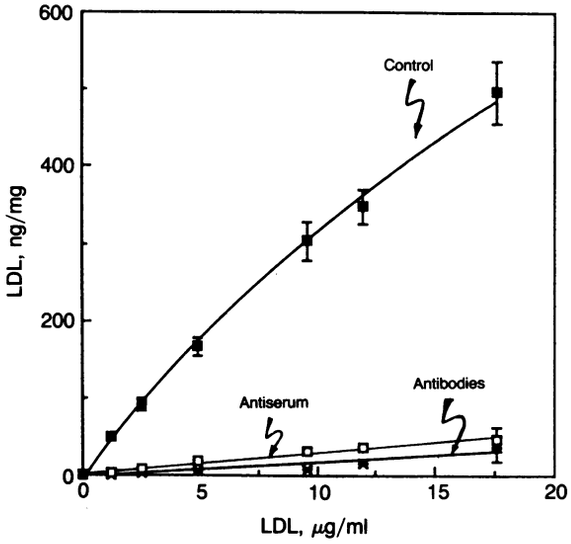

FIG. 3. Antiserum and antibodies against the 86-kDa protein inhibit the binding of LDL particles to *T. brucei*. Trypanosomes (10^8 cells per ml) were incubated as in Fig. 1, with a range of low concentrations of rabbit ^{125}I-LDL together with either 10% (vol/vol) preimmune serum (■), 10% total antiserum (□), or affinity-purified antibodies (16 μg/ml) against the 86-kDa protein (⋈). Cell-associated radioactivity was measured and expressed as ng of ^{125}I-LDL per mg of cell protein (mean ± SD, $n = 3$).

Table 2. Effect of LDL and anti-LDL-receptor antibodies on *T. brucei* growth *in vitro*

Medium conditions	Doubling time, hr (mean ± SD)	Significance*	
Control: total serum (containing LDL at 120 μg/ml)	14.4 ± 1.3	—	(n = 4)
Lipoprotein-free serum	21.1 ± 2.9	$P < 0.005$	(n = 6)
Lipoprotein-free serum supplemented with LDL at 120 μg/ml	14.8 ± 1.5	NS	(n = 4)
Total serum + antiserum (containing LDL at 120 μg/ml)	17.1 ± 0.9	$P < 0.005$	(n = 5)
Lipoprotein-free antiserum supplemented with LDL at 600 μg/ml	15.4 ± 1.1	NS	(n = 4)
Lipoprotein-free serum supplemented with LDL at 600 μg/ml	14.8 ± 1.2	NS	(n = 4)
Lipoprotein-free antiserum supplemented with LDL at 0.6 μg/ml	21.1 ± 3.6	$P < 0.005$	(n = 6)
Lipoprotein-free serum supplemented with LDL at 0.6 μg/ml	21.1 ± 2.2	$P < 0.005$	(n = 6)

*For statistical analysis, we used Student's t test for comparison of mean values between the control group and each of the other groups. NS, not significant. n = number of degrees of freedom in the evaluation of the growth curves.

showed that *T. brucei* is capable of selective uptake of various host serum proteins, including LDL, through the flagellar pocket (5) and that macromolecular material eventually ends up in the secondary lysosomes of the trypanosome (20, 21). At 120 μg of LDL per ml (i.e., close to saturation; see Fig. 1*A*), the uptake corresponded to 24 μg of LDL per mg of cell protein per hr (5). In good agreement with this value, Gillett and Owen (22) have reported that, when *T. brucei* was incubated with 400 μg of LDL per ml, the protein was degraded by the parasite after a lag time of about 30 min, at a rate close to 20 μg of LDL per mg of cell protein per hr; the degradation of LDL was associated with an accelerated esterification of cholesterol by the parasite. Thus, cholesterol taken up with LDL is made available to *T. brucei* for metabolic use. Our present experiments indicate that the uptake of LDL involves a specific receptor and that functional inhibition of this receptor leads to a significant retardation of growth. It is still unclear whether this retardation results from an increase of generation time or is due to a lag phase required for adaptation to growth under conditions of limiting LDL.

With an apparent molecular mass of 86 kDa, the LDL receptor of trypanosomes is likely to be distinctly smaller than that of human cells. The human receptor consists of 839 amino acids, which predicts a 95.4-kDa protein (23). However, owing to a high degree of glycosylation, the mobility in $NaDodSO_4$/polyacrylamide gels differs considerably from this value, as illustrated by an apparent molecular mass of 164 kDa for the bovine receptor (11) and 132 kDa for the human receptors on hepatocytes and fibroblasts (24). Like the bovine receptor (pI 4.6; ref. 11), the trypanosome receptor is acidic (pI 5.9). Its affinity and pool size can also be compared with the values reported for various mammalian receptors, whose affinity (K_d) values range from nanomolar for human fibroblasts (25) to micromolar for human erythrocytes (26) and whose number per cell varies from 75,000 for human fibroblasts (27) to only 200 for human erythroblasts (26). Our binding data suggest that the bloodstream-form trypanosome exposes two classes of LDL receptors: a high-affinity class at 1800 copies per cell and a low-affinity class at 52,000 copies per cell. After LDL-affinity chromatography, purification of the LDL receptor was close to 1000-fold. This value is in good agreement with the presence of about 50,000 receptors (of 86 kDa) per cell, indicating that, in the living parasite, most of the LDL receptors may be exposed at the cell surface.

Whether localized by ligand or localized by antibody labeling, LDL receptors of trypanosomes appeared to be distributed at both the flagellar pocket and the flagellar membrane. We can estimate that the 52,000 low-affinity receptors with a surface area of 22 nm^2 per subunit for an 86-kDa protein (17) would cover a surface of at least 1.1 μm^2. Since the membrane surface of the flagellar pocket itself is only 1 μm^2 (5), there would be no place left for any other protein, including the variant surface glycoproteins (28). Thus, whereas the 1800 high-affinity receptors could easily be accommodated on the pocket membrane, the low-affinity receptors must (also) be found on the flagellum, where they could be involved in the uptake of circulating LDL particles and the subsequent transport of these particles into the flagellar pocket. It is tempting to speculate here that the two affinity classes reflect two localizations of the same receptor (29). The less numerous, high-affinity sites could be localized to the flagellar pocket.

It is intriguing that such a primitive organism as a protozoan has a receptor for a host protein that presumably evolved much later during evolution. Several possibilities could account for this fact. (*i*) An LDL receptor, or receptor-like protein, could have been present before the Trypanoso-

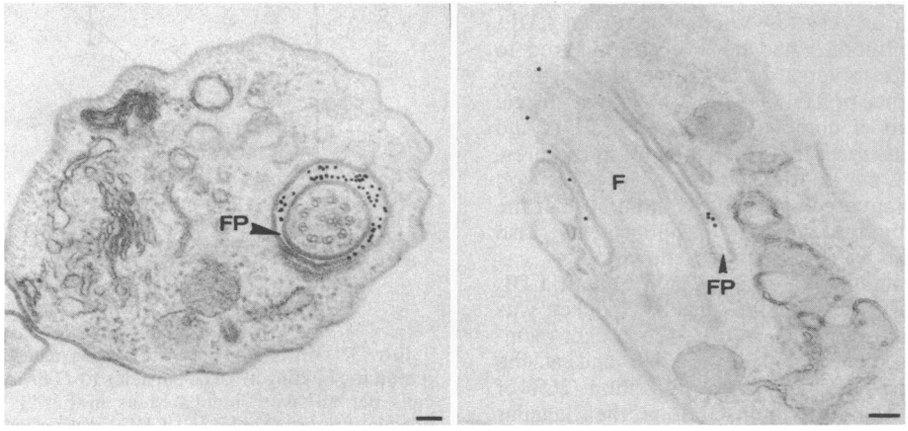

FIG. 4. Immunogold labeling of LDL receptors of *T. brucei*. Trypanosomes were incubated with anti-LDL-receptor antiserum and labeled with protein A–gold conjugates. Control experiments with preimmune serum, under the same conditions, showed no gold particles associated with the trypanosomes (data not shown). F, flagellum; FP, flagellar pocket. (Bar = 0.1 μm.)

matidae branched off from the main line of eukaryote evolution (30). (*ii*) Such a receptor could have been developed by a process of convergent evolution, as an adaptation to a parasitic way of life in the bloodstream of a mammalian host. (*iii*) The parasite could have acquired the receptor gene horizontally from its host. (*iv*) Even direct acquisition of the host receptor from host cells is conceivable, but this does not seem likely in view of the difference in molecular size between the two proteins.

This is not the first report of receptors for host proteins in protozoa. Receptors for LDL have been described in *Trichomonas vaginalis* (ref. 31; the function of these receptors remains to be elucidated), receptors for high density lipoprotein in *Trypanosoma cruzi* (32), and receptors for transferrin in *Plasmodium falciparum* (33). Moreover, in addition to a LDL receptor in *T. brucei*, we have also reported suggestive evidence for the presence of transferrin receptors in the same organism (5, 21, 34). The demonstration that the surface of the bloodstream-form trypanosome has receptors that are both essential for optimal growth and accessible to such large particles as LDL may be of great practical importance. Until now, it was generally assumed that the entire surface of the bloodstream form is covered with a densely packed, monomolecular layer of proteins, the variant surface glycoproteins (28, 35), believed to be the only proteins recognized by the immune system of the host and to be responsible for the antigenic variations of the parasite. The identification, on the plasma membrane of trypanosomes, of distinct and stable proteins such as receptors, even secluded in the flagellar pocket, could provide better tools for immunological intervention against the fatal disease sleeping sickness.

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