Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 185-191

A RAPID METHOD PURIFIES A GLYCOPROTEIN OF M_r 145,000 AS THE LDL RECEPTOR OF *TRYPANOSOMA BRUCEI BRUCEI*

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Received April 19, 1991

Summary - The trypanosome LDL receptor has been isolated from bloodstream form and cultured insect-stage trypanosomes as a protein of M_r 145,000, using a rapid purification procedure in the presence of a cocktail of protease inhibitors, whereas previously a polypeptide of M_r 86,000 was purified as the LDL receptor. Both the 145,000 and the 86,000 polypeptides are glycosylated and recognized by a monospecific antibody raised against the 86,000 species. This antibody inhibits LDL binding to the intact trypanosomes, to the isolated 145,000 receptor and to the 86,000 species. Hence, the previously isolated 86,000 polypeptide is a degradation product probably representing the cleaved-off ectodomain of the trypanosome LDL receptor.

Introduction - The protozoan hemoflagellate *Trypanosoma brucei*, the causative agent of both human sleeping sickness and nagana in lifestock, is an extracellular parasite that lives in the bloodstream and other body fluids of the mammalian host. Once ingested by the tsetse fly, the bloodstream form trypanosomes differentiate into procyclic (insect) form trypanosomes. Trypanosome growth requires an efficient supply of nutrients from the blood or in vitro from the culture medium [1]. *T. brucei* is assumed to take up cholesterol from the host [2] by receptor-mediated endocytosis of LDL particles [1].

Previously [3], we have described the isolation and partial characterization of a protein of M_r 86,000 as the putative LDL receptor of *T. brucei*, after separation of trypanosomes from blood cells through a DEAE-cellulose column and in the presence of phenylmethylsulfonylfluoride (PMSF) as protease inhibitor [4,5].

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0006-291X/91 $1.50

In this paper, we report a simplified and accelerated method for the purification of the LDL receptor in the presence of an extensive cocktail of protease inhibitors. This new procedure results in a larger LDL-binding protein (M_r 145,000) from which the 86,000 polypeptide is probably derived by partial proteolysis.

Materials and Methods

Isolation and cultivation of trypanosomes - Bloodstream forms of *Trypanosoma brucei brucei* stock 427 were grown in 300-g Wistar rats [6]. Blood was withdrawn from infected rats (10^9 trypanosomes per ml of blood) by cardiac puncture under ether anesthesia. At this stage, a mixture of protease inhibitors including 1 mM PMSF, 1 µM antipain, 1 µM leupeptin, 1 µM tosyl-L-lysine chloromethylketone (TLCK), 1 µM aprotinin and 1 µM soybean trypsin inhibitor (STI) was added to the infected blood. Trypanosomes were separated from blood cells by centrifugation at 800 x *g* for 10 min at 4°C and harvested in the supernatant. They were washed twice in 65 mM phosphate buffer pH 8 containing 55 mM glucose (PSG) and recovered by centrifugation at 1,500 x *g* for 10 min at 7°C .

Cultured procyclic trypomastigotes of *T. brucei* were grown in SDM-79 medium plus 10 % foetal calf serum [7]. Before use, cultured forms were washed twice in PSG and centrifuged as bloodstream forms.

Rat-liver homogenates - Wistar rats weighing 300 g were anesthetized with ether. The livers were then removed, minced and homogenized at 4°C in 3 ml per g liver, of a buffer made of 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$ containing 1 mM PMSF.

Purification of LDL particles - Human LDL particles (density 1.019-1.055 g/ml) were prepared from freshly isolated plasma supplemented with KBr to increase plasma density [8]. Contaminating proteins were eliminated by a final centrifugation at 214,000 x *g* for 24 h at 4°C [9]. The final preparations contained a single band of M_r 550,000 on a sodium dodecylsulfate (SDS)/polyacrylamide gel (PAGE) [10]. The protein content was determined by the method of Lowry et al., using bovine serum albumin as standard [11].

Purification of LDL receptors - All procedures were carried out at 4°C. For rat liver, 1 mM PMSF was included in all buffers; for trypanosomes, 1 mM PMSF plus the cocktail of protease inhibitors described above were included in the purification buffers.

- *Solubilization* : Trypanosome pellets (10^9 cells, 10 mg of protein) or rat-liver homogenates (10 mg of protein) were solubilized in the lysis buffer containing 10 mM Tris-HCl pH 7.4, 1 % (w/v) Nonidet P-40, 150 mM NaCl, 1 mM diethylenediamine tetraacetic acid (EDTA), to a final protein concentration of 5 mg/ml. The suspension was sonicated once for 20 sec at 25 watts (Sonifier model W185, Heat-system/Ultrasonics, Plainview, NY, USA) and gently agitated for 30 min. The insoluble material was removed by centrifugation at 100,000 x *g* for 60 min.

- *LDL-Sepharose affinity chromatography* : Soluble trypanosome or liver extracts were put into contact with Sepharose beads to which LDL particles had been coupled according to the manufacturer's instructions [12], using a ratio of 20 mg of LDL per g of dry gel. Binding was performed batch-wise, in a buffer made of 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 3.6 mM $CaCl_2$ and 0.8 mM $MgSO_4$ pH 7, at a concentration of 2 mg of cellular protein per ml of gel, using end-over-end agitation for 12 h. Unbound material was removed by four washes in 15 ml of 10 mM Tris-HCl buffer pH 7.4 containing 0.1 % Nonidet P-40, per ml of gel, followed by centrifugation at 800 x *g* for 5 min. Bound receptors were eluted with 60 mM KH_2PO_4/Na_2HPO_4 buffer pH 6 containing 5 mM suramin, using end-over-end agitation for 60 min, followed by centrifugation as above. At this stage, a partially purified LDL receptor preparation was obtained and a second cycle of LDL-Sepharose affinity chromatography yielded pure LDL receptors. Receptors were quickly frozen at -80°C in presence of the mixture of the protease inhibitors and stored for 2 weeks without detectable proteolysis.

SDS-PAGE and Western blotting - Purity of the LDL receptor was assessed by silver staining on a SDS-PAGE in a 5-10 % linear gradient [13]. The specificity of the LDL receptor was determined by ligand blotting, using human LDL particles (50 µg/ml) detected by incubation with antibodies against apoprotein B (Sigma Chemicals Co., St Louis, Mo, USA) [14,15]. For immunoblotting, affinity-purified antibodies, eluted from the 86,000 protein adsorbed on nylon filter [1], were used at a RIA titer of 1:800. For glycoconjugate detection, digoxigenin-labeled lectin concanavalin A (10 µg/ml) was applied on protein bound to nitrocellulose. Blots were then incubated with anti-digoxigenin-peroxidase and stained with 4-chloro-1-naphtol as chromogen.

Results and discussion - Previously, trypanosomes were separated from blood cells by passage through a DEAE-cellulose column at pH 8, and LDL receptors were isolated by the classical procedure involving two chromatographic steps, first on DEAE-cellulose at pH 6, followed by affinity chromatography on a LDL-Sepharose column [5]. This yielded a single polypeptide of M_r 86,000 (Fig. 1, lane 4). Since affinity-purified antibodies raised against this protein strongly inhibited LDL binding, it was tentatively identified as the trypanosome LDL receptor [1]. However, when trypanosomes were taken directly from the buffy coat of centrifuged blood and the receptors isolated as quickly as possible as described in Materials and Methods, the relative molecular mass of the protein so obtained increased from 86,000 to 145,000 on SDS-gels under non reductive conditions (Fig. 1, lane 1). A relative mass of 155,000 was observed under reductive conditions (not shown).

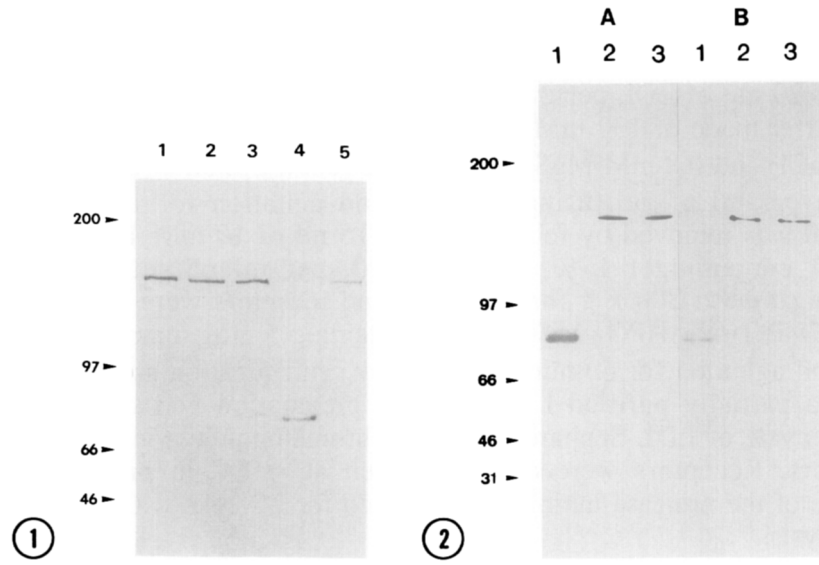

Fig. 1.
SDS gel electrophoresis of purified LDL receptors (0.2 μg).
lane 1 : bloodstream forms of *T. brucei* (buffy coat + protease inhibitors mixture)
lane 2 : cultured procyclic trypomastigotes of *T. brucei* (+ protease inhibitors mixture)
lane 3 : bloodstream forms of *T. brucei* (DEAE-cellulose column + protease inhibitors mixture)
lane 4 : bloodstream forms of *T. brucei* (DEAE-cellulose column + 1 mM PMSF alone)
lane 5 : rat liver cells (+ 1 mM PMSF alone)
Positions of Mr standards (in kDa) are indicated at left.

Fig. 2.
Blots of purified LDL receptors (0.5 μg).
A. Immunoblots revealed by anti-86,000 antibodies at a RIA titer of 1:800
B. Ligand blots revealed by anti-apoprotein B antibodies
lanes 1 : 86,000 fragment from bloodstream forms of *T. brucei*
lanes 2 : 145,000 protein of bloodstream forms of *T. brucei*
lanes 3 : 145,000 protein of cultured procyclic forms of *T. brucei*
Positions of Mr standards (in kDa) are indicated at left.

When the trypanosome LDL receptor was purified after the isolation of trypanosomes by the slower DEAE-column procedure, but in the presence of the cocktail of protease inhibitors, the 145,000 band was the major polypeptide but a 86,000 species was also visible as the major break-down product (Fig. 1, lane 3). Evidence that the 86,000 polypeptide is a proteolytic product derived from the 145,000 protein comes from the fact that the 145,000 band is recognized by the antibodies directed against the 86,000 species (Fig. 2A). In contrast, when the LDL receptor was purified from rat liver in the presence of 1 mM PMSF alone, a single polypeptide

was obtained with a relative molecular mass of 145,000 (Fig. 1, lane 5) as reported in the literature [16] and no proteolysis was detected.

Using the same procedure on cultured procyclic trypomastigotes, we obtained a single polypeptide of M_r 145,000, identical in size to the receptor of the bloodstream form (Fig. 1, lane 2). The presence of a LDL receptor in procyclic forms was unexpected because of the absence of cholesterol in the glossine fluids and because the procyclic forms are reported to synthesize their own sterols [17]. However, the usual culture medium for the optimal in vitro growth of procyclic forms includes 10 % serum and thus contains LDL. Apparently, under the present culture conditions, receptor-mediated endocytosis of LDL particles remains expressed in the cultured procyclic forms, and so allows trypanosomes to use cholesterol from the medium for membrane synthesis.

Antibodies directed against the 86,000 polypeptide from the bloodstream form exhibited an immunological cross-reactivity with the procyclic receptors. Ligand blotting showed that the 86,000 fragment from the bloodstream form receptor contains a similar ligand-binding domain as the intact LDL receptor from both bloodstream and procyclic trypomastigote forms (Fig. 2B).

Binding of concanavalin A (Fig. 3) to the 86,000 and 145,000 polypeptides indicates that the trypanosome LDL receptor is a glycoprotein similar to the mammalian LDL receptor [18]. The glycosylated nature of the 86,000 polypeptide and its ability to bind LDL strongly suggest, by

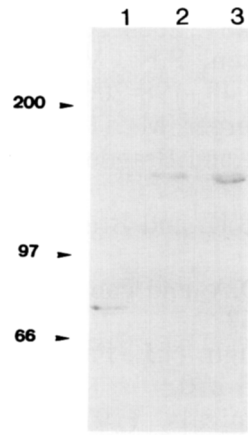

Fig. 3.
Western blots of purified LDL receptors (0.5 µg) revealed by lectin concanavalin A.
lane 1 : 86,000 fragment from bloodstream forms of *T. brucei*
lane 2 : 145,000 protein of bloodstream forms of *T. brucei*
lane 3 : 145,000 protein of cultured procyclic forms of *T. brucei*
Positions of Mr standards (in kDa) are indicated at left.

structural analogy with the mammalian receptor [19], that this fragment represents the major part of the ectodomain of the LDL receptor.

Immunization trials using the entire LDL receptor and fragments thereof are in progress to test the possibility of immunological intervention against trypanosomiasis [20].

Acknowledgments

We thank J. Van Roy and D. Cottem for the isolation and the cultivation of trypanosomes. This work has been financially supported by the UNDP/World bank/WHO Special Programme for Research and Training in Tropical Diseases, by grant of the belgian Fonds National de la Recherche Scientifique at which IC is Senior Research Assistant and PJC Senior Research Associate, and by a fellowship of the belgian Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (to PhB).

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