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Trypanosoma brucei transferrin receptor can bind C-lobe and N-lobe fragments of transferrin

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ABSTRACT

Transferrin (Tf) is a dumbbell-shaped iron transport protein composed of two homologous lobes (C-lobe and N-lobe) and is an essential growth factor for the protozoan parasite *Trypanosoma brucei*. The trypanosomal receptor for Tf uptake (*Tb*TfR) is a heterodimeric complex that bears no structural similarity with the human Tf receptor. As a first step in identifying the region of Tf involved in binding to the *Tb*TfR, C-lobe and N-lobe fragments were assessed for their capability to interact with the receptor. Preparations of C-lobe and N-lobe fragments were obtained by digestion of iron-loaded bovine Tf with proteinase K-agarose. The individual fragments were then purified by concanavalin A affinity chromatography. Uptake experiments with bloodstream forms of *T. brucei* demonstrated that both C-lobe and N-lobe fragments were ingested by the parasites. The uptake of the isolated lobes could be inhibited by an excess of Tf and vice versa. Dot blot binding assays showed that both C-lobe and N-lobe fragments were capable of binding to the *Tb*TfR. Both isolated lobes were also able to support the growth of bloodstream forms of *T. brucei* when cultured in Tf-depleted medium. However, the C-lobe fragment was more efficiently taken up and more potent in supporting parasite growth. The results indicate that the interaction of Tf with the *Tb*TfR is different from that with the human Tf receptor. This difference may be exploited for the development of agents specifically interfering with the binding of Tf to the *Tb*TfR.

1. Introduction

Trypanosoma brucei is the causative agent of sleeping sickness in humans and nagana disease in cattle. As for most living organisms iron is essential for the viability of bloodstream forms of *T. brucei* [1,2]. Iron is provided by the host and is delivered to the circulating trypanosomes as a complex with host transferrin (Tf) [3,4]. Bloodstream forms of *T. brucei* express a unique Tf receptor (*Tb*TfR) (for a review on the *Tb*TfR see [5]) which is structurally completely different from the host Tf receptor.

*Tb*TfR is a heterodimeric protein complex encoded by two expression site-associated genes, ESAG6 and ESAG7 [6–10]. Both ESAG6 and ESAG7 are heterogeneously glycosylated proteins of 50–60 and 40–42 kDa, respectively [6,8]. The amino acid sequences of ESAG6 and ESAG7 are almost identical over their N-terminal half but show differences in the C-terminal region [11]. The hydrophobic C-terminal stretch of ESAG6 is replaced by a glycosylphosphatidylinositol (GPI) anchor which attaches the heterodimeric *Tb*TfR to the plasma membrane [6]. Binding of one molecule of Tf [10] requires association of both ESAG6 and ESAG7 as was shown by co-expression in heterologous systems [7–9]. The ligand-binding site of the *Tb*TfR has been identified within two small stretches at amino acids 205–215 and 223–238 of ESAG6 and ESAG7, respectively [12]. These two stretches form surface-exposed loops [5] as has been predicted from sequence homology between ESAG6 and ESAG7, and the N-terminal domain of variant surface glycoprotein (VSG) molecules [12,13]. Uptake of Tf by bloodstream forms of *T. brucei* occurs by a mechanism distinct from the well-established Tf cycle in mammalian cells. For *T. brucei*, Tf bound to the GPI-anchored *Tb*TfR is taken up into the cell by bulk flow endocytosis. The low pH of the endosome triggers the release of iron from Tf. At low pH, the resulting apo-Tf has low affinity for the *Tb*TfR [14] and is liberated allowing transport to lysosomes where it is proteolytically degraded [10,15]. The resulting peptide fragments are released from the trypanosomes while iron remains associated with the cell [10]. The unoccupied *Tb*TfR is recycled to the cell surface to mediate further cycles of Tf uptake [16]. Thus,

Abbreviations: Con A, concanavalin A; DAPI, 4,6-diamidino-2-phenylindole; ESAG, expression site-associated gene; hTfR, human transferrin receptor; Lf, lactoferrin; *Tb*TfR, *T. brucei* transferrin receptor; Tf, transferrin; VSG, variant surface glycoprotein; Z-FA-DMK, benzyloxycarbonyl-phenylalanyl-arginyl-diazomethyl ketone.

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the mechanism of Tf uptake in *T. brucei* resembles the uptake of low density lipoprotein and asialoglycoproteins by their specific receptors in mammalian cells.

Tf is a monomeric glycoprotein of a molecular mass of around 78 kDa which can bind two ferric iron ions in association with the binding of bicarbonate [17]. It presents a bilobal structure with the two lobes representing the C-terminal and N-terminal halves of the protein, with each lobe capable of binding one ferric iron ion [18]. The two lobes are sequentially and structurally homologous and are connected by an unstructured linker peptide. Each lobe is divided into two dissimilar domains with the iron-binding centre at the domain interface [18]. Only the C-lobe contains N-linked oligosaccharide side chains that are capable of binding to the jack bean lectin concanavalin A (Con A) [17].

The region of Tf that is recognised by the *Tb*TfR is unknown. However, as the *Tb*TfR is embedded in the surface coat of densely packed VSG dimers and as ESAG6 and ESAG7 are about 20% smaller than a VSG molecule (~50 and ~42 kDa vs ~58 kDa) but folded in a similar fashion as the N-terminal domain of the VSG [5,12], it is reasonable to assume that the *Tb*TfR is likely to be recessed into the VSG surface coat leaving only the ligand binding site accessible [5,19,20]. Such an arrangement was recently confirmed by molecular modelling of the *Tb*TfR in the VSG coat [20]. This also implies that Tf only has access to the ligand-binding site of the *Tb*TfR via one of its lobes [19]. This is supported by the observation that only anti-*Tb*TfR antibodies recognising the ligand-binding site are taken up by bloodstream forms of *T. brucei* [10]. In this paper we present the results of a study determining which lobe of Tf binds to the *Tb*TfR.

2. Materials and methods

2.1. Reagents

Alkaline phosphatase-conjugated goat anti-rabbit IgGs, ammonium iron(III) citrate, bovine lactoferrin (Lf), bovine Tf, Con A-agarose, Con A peroxidase conjugate, dimethyl pimelimidate dihydrochloride, 6-[fluorescein-5(6)-carboxamido]hexanoic acid N-hydroxysuccinimide ester, methyl-α-D-mannopyranoside, protein G Sepharose and proteinase K-agarose were obtained from Sigma–Aldrich (Dorset, England). Rabbit anti-bovine Tf antibody was obtained from Fitzgerald (Acton, MA, USA).

2.2. Preparation of C-lobe and N-lobe fragments

The commercially available bovine Tf was purified by Con A affinity chromatography to remove species of Tf that do not bind to Con A [21]. Prior to affinity chromatography, Tf was saturated with iron by dissolving 50 mg of Tf in 1 ml iron-loading buffer (100 mM $NaHCO_3$, pH 8.5, 10 mg/ml ammonium iron(III) citrate). After incubation at 4 °C over night, the solution was passed over a PD-10 column equilibrated with Con A buffer (50 mM Na-acetate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 6.9) and fractions containing Tf were collected. Then, 1 ml containing 25 mg Tf was applied to a column containing 5 ml Con A-agarose. The column was washed with 15 ml Con A buffer and bound Tf eluted with 200 mM methyl-α-D-mannopyranoside in Con A buffer. Eluates containing Tf were concentrated by ultrafiltration (Amicon Ultra 50K device) and then passed over a PD-10 column equilibrated with reaction buffer (100 mM Tris, 25 mM $CaCl_2$, pH 8.2). Fractions containing Tf were collected and stored at 4 °C.

For preparation of individual lobes, 7.7 mg Tf in 2 ml reaction buffer was treated with 40.5 mg of proteinase K-agarose (0.2 units per mg Tf) at room temperature for 49 h. The mixture was passed over a PD-10 column equilibrated with Con A buffer and then applied to a column containing 5 ml Con A-agarose. The column was washed with 15 ml Con A buffer and N-lobe containing fractions were collected. The bound C-lobe was eluted with 200 mM methyl-α-D-mannopyranoside in Con A buffer. After concentration by ultrafiltration (Amicon Ultra 10K device), both fragments were passed over a PD-10 column equilibrated with PBS. Fractions containing C-lobe and N-lobe fragments, respectively, were collected and stored at 4 °C.

2.3. Labelling of proteins with fluorescein

Bovine Tf, C-lobe fragments, N-lobe fragments and bovine Lf were labelled at a molar ratio of approximately 1:22 with 6-[fluorescein-5(6)-carboxamido]hexanoic acid N-hydroxysuccinimide ester [10]. Unreacted reagent was removed by passing the mixture over a PD-10 column equilibrated with PBS. Fractions containing fluorescein-labelled proteins were collected and stored at −20 °C.

2.4. Tf depletion of culture medium

Anti-bovine Tf antibodies bound to protein G-Sepharose were cross-linked to the beads using dimethyl pimelimidate dihydrochloride as described previously [6]. Next, 2 ml of Baltz medium [22] supplemented with 16.7% (v/v) heat-inactivated foetal bovine serum (medium B) was applied to a column containing 1.3 ml of cross-linked beads equilibrated with PBS. The first 1.5 ml fraction was discarded while the next 0.5 ml fraction (first medium containing fraction) was collected. To regenerate the beads, the column was washed with 2 ml PBS followed by three cycles with glycine/NaCl buffer (50 mM glycine, 500 mM NaCl, pH 2.7) and PBS. The regenerated beads were used to obtain another 0.5 ml fraction of Tf-depleted medium B. This procedure was repeated until enough Tf-depleted medium B was collected.

2.5. Trypanosome cultures

Bloodstream forms of *T. brucei* clone 427–221a [23] were grown in medium B. The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

For growth experiments, trypanosomes were washed three times with Baltz medium supplemented with 2% BSA and incubated in 200 μl of Tf-depleted medium B in the absence or presence of 25 μg/ml C-lobe or N-lobe fragments in flat-bottom 96-well plates.

2.6. Fluorescence microscopy and flow cytometry

Bloodstream-form trypanosomes (1×10^7/ml) were incubated with fluorescein-labelled bovine Tf, C-lobe fragments, N-lobe fragments, or bovine Lf in Baltz medium supplemented with 2% BSA and 100 μM benzyloxycarbonyl-phenylalanyl-arginyl-diazomethyl ketone (Z-FA-DMK) in the presence or absence of competing, unlabelled bovine Tf, C-lobe fragments, or N-lobe fragments. After 2 h incubation, cells were washed two times with PBS/1% glucose and fixed with 2% formaldehyde/0.05% glutaraldehyde in PBS.

For fluorescence microscopy, cells were applied to poly-L-lysine-coated microscope slides, and treated with 0.0001% 4,6-diamidino-2-phenylindole (DAPI) in PBS. Slides were mounted in Vectashield mounting medium (Vector Laboratories, Peterborough) and imaged with a Zeiss Axioplan 2 fluorescence microscope using a Plan-Apochromat 100×/1.4 oil objective.

For quantitation of fluorescence, cells were analysed by flow cytometry using a BD Accuri C6 flow cytometer. The excitation wavelength was 488 nm and the filter set was 530/30 nm. Gates