Iron-dependent regulation of transferrin receptor expression in *Trypanosoma brucei*

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Transferrin is an essential growth factor for African trypanosomes. Here we show that expression of the trypanosomal transferrin receptor, which bears no structural similarity with mammalian transferrin receptors, is regulated by iron availability. Iron depletion of bloodstream forms of *Trypanosoma brucei* with the iron chelator deferoxamine resulted in a 3-fold up-regulation of the transferrin receptor and a 3-fold increase of the transferrin uptake rate. The abundance of expression site associated gene product 6 (ESAG6) mRNA, which encodes one of the two subunits of the trypanosome transferrin receptor, is regulated 5-fold by a post-transcriptional mechanism. In mammalian cells, the stability of transferrin receptor mRNA is controlled by iron regulatory proteins (IRPs) binding to iron-responsive elements (IREs) in the 3′-untranslated region (UTR). Therefore, the role of a *T. brucei* cytoplasmic aconitase (TbACO) that is highly related to mammalian IRP-1 was investigated. Iron regulation of the transferrin receptor was found to be unaffected in Δaco::NEO/Δaco::HYG null mutants generated by targeted disruption of the TbACO gene. Thus, the mechanism of post-transcriptional transferrin receptor regulation in trypanosomes appears to be distinct from the IRE/IRP paradigm. The transferrin uptake rate was also increased when transferrin was transferred from medium supplemented with foetal bovine serum to medium supplemented with sera from other vertebrates. Due to varying binding affinities of the trypanosomal transferrin receptor for transferrins of different species, serum change can result in iron starvation. Thus, regulation of transferrin receptor expression may be a fast compensatory mechanism upon transmission of the parasite to a new host species.

Key words: aconitase, deferoxamine, gene targeting, host range, iron regulatory protein.

INTRODUCTION

Like all living organisms, trypanosomes require iron for growth [1,2]. The delivery of iron into bloodstream forms of *Trypanosoma brucei* is mediated by host transferrin that is taken up via a unique receptor. The transferrin receptor of *T. brucei* is a heterodimeric complex encoded by two expression site associated genes, ESAG6 and ESAG7 [3–7], and shows no homology to the homodimeric mammalian transferrin receptor. ESAG6 is a heterogeneously glycosylated protein of 50–60 kDa modified by a glycosylphosphatidylinositol membrane anchor, while ESAG7 is a 42 kDa glycoprotein carrying an unmodified C-terminus [3]. Binding of one molecule of transferrin [7] requires the association of both ESAG6 and ESAG7 as shown by coexpression in heterologous systems [4–6]. Usually, ESAG5 are cotranscribed together with the variant surface glycoprotein (VSG) gene as a large polycistronic transcript from a telomeric VSG expression site. Since the trypanosome genome may contain as many as 20 different expression sites [8,9], ESAG6 and ESAG7 are multicycopy genes. However, only one expression site is actively transcribed at one time [10–12].

In mammalian cells, regulation of transferrin receptor expression by the cellular iron status is a paradigm for post-transcriptional regulation and is mediated by iron regulatory proteins (IRPs; for reviews see [13–15]). Upon iron depletion, IRPs bind to stem-loop structures referred to as iron-responsive elements (IREs) within the 3′-untranslated region (UTR) of the transferrin receptor transcript [16,17]. The binding of IRPs enhances the stability of the mRNA, which leads to increased receptor expression [18]. Iron loading of the cell abolishes the specific RNA-binding activity, thus leading to rapid endonucleolytic degradation of unprotected transferrin receptor mRNA and subsequently decreased levels of transferrin receptor [19]. Two IRP polypeptides (IRP-1 and IRP-2) have been identified in vertebrates and shown to be regulated by iron. IRP-1, also referred to as cytoplasmic aconitase, is a bifunctional protein that exhibits aconitase activity when cellular iron is abundant, and RNA-binding activity when cellular iron is scarce [20]. A labile iron-sulphur [4Fe–4S] cluster in the catalytic centre functions as the iron sensor [20]. IRP-2, which is closely related to IRP-1, lacks aconitase activity and is rapidly degraded in iron-loaded cells [21,22].

In the present study, we show that iron availability regulates expression of the transferrin receptor in bloodstream forms of *T. brucei*. By a reverse genetic approach, we have investigated whether an IRP-1-like aconitase present in the cytoplasm of *T. brucei* is necessary for post-transcriptional regulation of the transferrin receptor.

MATERIALS AND METHODS

Reagents and chemicals

Bovine holo-transferrin, deferoxamine mesylate, leupeptin, antipain, chymostatin, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64), pepstatin A, cycloheximide, chloramphenicol, puromycin and dibutyl phthalate were purchased from Sigma (Deisenhofen, Germany). PMSF, Triton X-100 and Dulbecco’s modified Eagle’s medium were obtained from Dulbecco. By a reverse genetic approach, we have investigated whether an IRP-1-like aconitase present in the cytoplasm of *T. brucei* is necessary for post-transcriptional regulation of the transferrin receptor.
Boehringer Ingelheim (Heidelberg, Germany). N\textsubscript{-p}-tosyl-l-lysine chloromethyl ketone and paraffin (highly liquid) were from Merck (Darmstadt, Germany). Sodium borohydride (5–20 Ci/mmol), [\textsuperscript{2}P]dCTP (3000 Ci/mmol) and [\textsuperscript{32}P]UTP (3000 Ci/mmol) were purchased from Amersham (Braunschweig, Germany).

Trypanosomes

Culture-adapted bloodstream forms of \textit{T. brucei} cell line TC221 derived from stock 427 [23,24] were grown in Baltz medium supplemented with 16.7\% (v/v) heat-inactivated foetal bovine serum [25]. Monomorphic MITat1.4 bloodstream-form trypanosomes also derived from stock 427 [26] were propagated in HMI-9 medium [26] supplemented with 10\% (v/v) heat-inactivated foetal bovine serum but without Serum Plus\textsuperscript{\textregistered}. All trypanosome cultures were maintained at 37 °C in a humidified atmosphere containing 5 \% CO\textsubscript{2}.

Targeting constructs

For promoterless replacement of the \textit{ThACO} gene the following parts were assembled in a pBluescript backbone: (i) the 5\textsuperscript{-}UTR of \textit{ThACO}, (ii) a neomycin or a hygromycin phosphotransferase gene cassette flanked by the 5\textsuperscript{-}UTR of the actin gene and a truncated 3\textsuperscript{-}UTR of the procyelic acidic repetitive protein gene, and (iii) the C-terminal coding sequence plus the 3\textsuperscript{-}UTR of \textit{ThACO}. A Xbal-MluI 3\textsuperscript{-}UTR fragment of \textit{ThACO} was amplified from procyelic \textit{T. brucei} cDNA with primers 5\textsuperscript{-}CCACCAGC-TCTAGACTCGTGTGGG\textsuperscript{3\textsuperscript{'}} and 5\textsuperscript{-}GCCACAGAAGAT-CTCCGCC\textsuperscript{3\textsuperscript{'}}, (restriction sites are underlined), subcloned into the pSL301 polylinker (Invitrogen, Leek, The Netherlands) and move as an Xbal-MluI fragment into \textit{Xbal-SacI}-cut pBluescript to give pB3\textsuperscript{aco}. A SalI and blunt ended 5\textsuperscript{-}UTR fragment was amplified from cloned \textit{ThACO} with primers 5\textsuperscript{-}GCAGCA-GTGCAGCAGCAAAATATT\textsuperscript{3\textsuperscript{'}} and 5\textsuperscript{-}GCCACAGAGAT-CTCCGCC\textsuperscript{3\textsuperscript{'}}, and inserted into \textit{SalI-Snal}-cut pB3\textsuperscript{aco} to generate pB5\textsuperscript{aco}. Subsequently, a \textit{Stul}-Xbal 3\textsuperscript{-}UTR procyelic acidic repetitive protein gene fragment amplified from p\textit{UTR}220 (p\textit{A1}–220, cf. [27]) with primers 5\textsuperscript{-}CTGAA-TCTAGAAGAACTGTCAG\textsuperscript{3\textsuperscript{'}} and 5\textsuperscript{-}CACTTTCTATTTTTTTT-CAGGCCTTTTG\textsuperscript{3\textsuperscript{'}}, the \textit{BgIII-Stul} neo-cassette amplified with primers 5\textsuperscript{-}ATAGGGAGATCTAGCG\textsuperscript{3\textsuperscript{'}}, and 5\textsuperscript{-}CCCAAGG-GCTGTCAGAAG\textsuperscript{3\textsuperscript{'}}, from plasmid pLew\textsubscript{77} and the \textit{BgIII-Stul} hygro-cassette amplified with primers 5\textsuperscript{-}CTCTGTTCGC-TGGGCTCTACT\textsuperscript{3\textsuperscript{'}}, and 5\textsuperscript{-}GATGCGGATCGAG\textsuperscript{3\textsuperscript{'}}, from plasmid pLew\textsubscript{5} were fused with \textit{BgIII-Xbal}-cut pB5\textsuperscript{3\textsuperscript{ac}} in a three-component ligation to give the targeting vectors pB\textit{Aaconeo} and pB\textit{Aacohyg} (pLew\textsubscript{77} and pLew\textsubscript{5} were kindly provided by Drs. M. Engstler and G. A. M. Cross, Laboratory of Molecular Parasitology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, U.S.A.).

Transfection and gene targeting

Trypanosomes were purified from the blood of infected rats [28] and 2 × 10\textsuperscript{5} cells were electroporated with 10 \mu{g} of \textit{SalI-MluI}-cut p\textit{Baconeo} or p\textit{Bacohyg} using a BTX Electro Cell Manipulator set at 1.2 kV, 25 \mu{F} and 186 \Omega [29]. For selection, 0.5 \mu{g}/ml G418 and 0.5 \mu{g}/ml hygromycin respectively were added to the growth medium. Double-resistant cells were cloned by limiting dilution and several independent clones were analysed by Southern and Western blotting following standard procedures [30,31].

Immobilization and radioactive labelling of transferrin

Transferrin-Sepharose was prepared by coupling bovine holo-transferrin to CNBr-activated Sepharose 4B (Pharmacia, Freiburg, Germany) as described by the manufacturer. Bovine holo-transferrin was labelled with \textit{H} by reductive methylation using sodium borohydride as described previously [7]. The specific radioactivity of [\textit{H}]holo-transferrin was 50 c.p.m./ng.

Purification of the trypanosome transferrin receptor

Bloodstream-form trypanosomes were harvested by centrifugation and washed once with ice-cold SB medium (60 mM Na\textsubscript{2}HPO\textsubscript{4}, 3 mM KH\textsubscript{2}PO\textsubscript{4}, 45 mM NaCl, 50 mM glucose, pH 8.0). Cells were resuspended in ice-cold SB medium to a density of (0.35–31) × 10\textsuperscript{9}/ml in the presence of proteinase inhibitors (200 \mu{M} N\textsubscript{-}p-tosyl-l-lysine chloromethyl ketone, 400 \mu{M} PMSF, 10 \mu{M} leupeptin, 2 \mu{M} E-64, 1 \mu{M} pepstatin A). Triton X-100 was added to a final concentration of 2\% (v/v) in order to lyse the trypanosomes. Lysates were briefly sonicated for 2 min in a water bath and centrifuged at 4 °C for 60 min at 114 000 g. The transferrin receptor was precipitated from supernatants by end-over-end rotation overnight with a 25 000-fold excess of transferrin bound to Sepharose to ensure quantitative isolation of the receptor [3,7]. The beads were washed five times with PBS/0.2\% (w/v) Triton X-100 and bound proteins eluted by boiling the beads in SDS/PAGE sample buffer. The eluates were analysed by SDS/PAGE and immunoblotting as described previously [5].

Transferrin uptake experiments

Transferrin uptake experiments were performed as described previously [7]. Bloodstream-form trypanosomes ([1.15 – 2] × 10\textsuperscript{7} cells) were incubated with 10 \mu{g}/ml [\textit{H}]holo-transferrin in 1 ml of Medium [Baltz medium supplemented with 1\% (v/v) BSA] in the presence of proteinase inhibitors (50 \mu{g}/ml each of leupeptin, antipain, chymostatin and E-64) at 37 °C and in an atmosphere of 5\% CO\textsubscript{2}. After a 1 h incubation the cells were harvested by centrifugation through 100 \mu{l} of oil (95\% (v/v) dibutyl phthalate, 5\% (v/v) paraffin oil) and analysed by liquid scintillation counting.

Northern blot analysis

Total RNA was isolated by a single-step guanidine method [31] and separated, transferred and hybridized as described previously [32]. The \textit{ESAG6}-specific \textit{32}P-labelled RNA probe spanning the 3\textsuperscript{-}terminal 195 bp of the open reading frame (ORF) plus 239 bp of the 3\textsuperscript{-}UTR was generated from plasmid pHD679 by \textit{in vitro} transcription (generous gift from Dr. C. Clayton, Zentrum für Molekulare Biologie Heidelberg, Heidelberg, Germany). Plasmids containing the coding regions of VSG 117 and \textit{\alpha}-tubulin respectively (kindly provided by Drs. M. Engstler and G. A. M. Cross, The Rockefeller University, New York, U.S.A.) were labelled by random priming. All hybridization signals were quantified using a phosphorimager.

RESULTS

Iron-dependent regulation of transferrin uptake and transferrin receptor expression

To determine whether transferrin uptake is subject to regulation by the availability of iron in bloodstream forms of \textit{T. brucei}, we
Table 1  Iron regulation of transferrin uptake in MITat 1.4 bloodstream forms of *T. brucei*.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Transferrin uptake (ng of transferrin/10^7 cells per h)</th>
<th>Factor*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>None</td>
<td>47.9†</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>25 µM deferoxamine</td>
<td>139.5†</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>25 µM Fe^3+/-deferoxamine</td>
<td>58.7†</td>
<td>2.1</td>
</tr>
<tr>
<td>Aco — / — 1</td>
<td>None</td>
<td>36.7 ± 0.6†</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>25 µM deferoxamine</td>
<td>119.7 ± 17.5†</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>25 µM Fe^3+/-deferoxamine</td>
<td>37.1 ± 5.8†</td>
<td>2.0</td>
</tr>
<tr>
<td>Aco — / — 2</td>
<td>None</td>
<td>37.7†</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>25 µM deferoxamine</td>
<td>97.5†</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>25 µM Fe^3+/-deferoxamine</td>
<td>33.1†</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Fold increase of transferrin-uptake in deferoxamine-treated to untreated cells.
† Mean of two independent experiments.
‡ Mean ± S.D. of three independent experiments.

Figure 1  Induction of the transferrin receptor by iron depletion

(A) Exponentially growing wild-type (wt) and null mutant (aco — / — 1) bloodstream forms of strain MITat 1.4 were incubated in HMI-9 medium (lanes C), in HMI-9 medium with 25 µM deferoxamine (lanes Df) or in HMI-9 medium with 25 µM iron-saturated deferoxamine (lanes FeDf). After a 20 h incubation, trypanosomes were harvested and the transferrin receptor was precipitated from cell extracts using transferrin-Sepharose. Bound proteins were eluted by boiling the beads in SDS/PAGE sample buffer and aliquots corresponding to 5 x 10^7 cell equivalents were analysed by immunoblotting using anti-(*T. brucei* transferrin receptor) antibodies [5]. The double band of ESAG6 is due to heterogeneous glycosylation [3]. The relative band intensities obtained by densitometric scanning of the immunoblots are indicated below the lanes (the control was set to 1). The molecular mass in kDa of standard proteins is indicated in the left margin.

(B) TC221 bloodstream forms were grown in the presence of protein synthesis inhibitors (25 µg/ml each of chloramphenicol and puromycin and 50 µg/ml cycloheximide [34]) in medium alone (lane C) or in medium supplemented with 50 µg/ml deferoxamine (lane Df). After a 5 h incubation, trypanosomes were harvested and the transferrin receptor was purified and analysed as described above but equivalents of 1 x 10^7 parasites were applied to each lane. The additional double band at 80 kDa corresponds to bovine transferrin released from the beads and non-specifically detected by the antiserum. The molecular mass in kDa of standard proteins is indicated in the left margin.

Figure 2  Iron regulation of ESAG6 mRNA

Exponentially growing wild-type (wt) and null mutant (aco — / — 1 and 2) bloodstream forms of strain MITat 1.4 were incubated in HMI-9 medium (lanes C), in HMI-9 medium with 25 µM deferoxamine (lanes Df) or in HMI-9 medium with 25 µM iron-saturated deferoxamine (lanes FeDf). After a 20 h incubation, trypanosomes were harvested, and 6 µg of total RNA was size fractionated on a 1.2% (w/v) formaldehyde agarose gel. The blots were sequentially hybridized with ESAG6 (panel A) or VSG 117 (panel B) probes and an α-tubulin probe. The size of the RNA standard is given in kb. ESAG6 and VSG 117 mRNA was quantified on a phosphorimager and normalised to the α-tubulin mRNA signal. Relative values (the control was set to 1) are given below the autoradiograms. The ESAG6-specific probe hybridized to a transcript of 1.7 kb as reported previously [35,36]. Cross-hybridization to an anonymous 2.2 kb RNA transcript is indicated by an asterisk. This transcript is not regulated by iron.

first measured the uptake rate of radiolabelled transferrin under iron-depletion conditions. Exponentially growing bloodstream forms of *T. brucei* were incubated for 20 h with or without the iron chelator deferoxamine or, as a specificity control, with the chelator presaturated with iron. The uptake of labelled transferrin was increased by iron depletion approx. 3-fold relative to both controls. These measurements were performed with strain MITat 1.4 (Table 1) and with the cell line TC221 (results not shown). The transferrin uptake rates of the controls were within the range of previous results [2,5,7,33]. Since the uptake of transferrin is receptor-mediated in *T. brucei*, the expression of the transferrin receptor in iron-depleted bloodstream forms was determined. The transferrin receptor was affinity purified from the respective cultures using transferrin-Sepharose and was detected with antibodies directed against both ESAG6 and ESAG7, the two highly related subunits of the transferrin receptor. The amount of transferrin receptor was increased 3-fold in iron-depleted bloodstream forms of strain MITat 1.4 (Figure 1A) and cell line TC221 (results not shown) as quantified by densitometric scanning of the Western blots. The control with iron-presaturated deferoxamine confirmed that the effect was specifically due to iron depletion.

Mechanism of transferrin receptor regulation

To elucidate the mechanism of regulation by iron availability, possible changes in the transferrin receptor protein stability as well as changes of transferrin receptor synthesis under iron-depletion conditions were considered. First, bloodstream forms were treated with deferoxamine in the presence of a cocktail of protein synthesis inhibitors that has been shown to inhibit
overall protein synthesis by > 98% under the given conditions [34]. The protein synthesis inhibitors elicited a significant decrease in the amount of transferrin receptor, as expected (results not shown). However, no difference was noted with and without deferoxamine under these conditions (Figure 1B). This result indicated that iron depletion was not affecting the specific rate of turnover of the transferrin receptor in the absence of de novo protein synthesis. The maximally induced transferrin receptor level was reached in less than 4 h of incubation with deferoxamine (results not shown). These kinetics suggested a direct effect on transferrin receptor synthesis. Therefore, the steady state amount of ESAG6 mRNA coding for one of the two subunits of the transferrin receptor was determined by Northern blotting (Figure 2A). The hybridization probe covered the region of the gene encoding the C-terminal end of the ESAG6 subunit that is not present in the otherwise highly homologous ESAG7 subunit. The 1.7 kb ESAG6 transcript was induced approx. 3-fold in deferoxamine-treated bloodstream forms relative to the untreated control. In the presence of chelator presaturated with iron, the ESAG6 transcript was repressed 2-fold relative to the untreated control. This effect was attributed to some free iron added with the saturated chelator. Together, the abundance of the ESAG6 transcript was regulated more than 5-fold by the availability of iron. To distinguish between transcriptional and post-transcriptional regulation of ESAG6 mRNA by iron, the VSG 117 mRNA was quantitated in the same set of samples. The VSG is cotranscribed with ESAG6 and ESAG7 as a large polycistronic precursor RNA from the same promoter located in the telomeric VSG expression site [8,36–38]. Since the amount of VSG mRNA did not change significantly upon iron depletion (Figure 2B), the mRNA coding for ESAG6 is unlikely to be regulated at the level of transcription initiation. Together, our results indicate that ESAG6 mRNA is regulated by a post-transcriptional mechanism and that the degree of regulation of the mRNA is sufficient to explain the observed induction of transferrin receptor expression as well as the observed changes in the transferrin uptake rate.

It is noteworthy that iron availability regulates both the T. brucei transferrin receptor and the mammalian transferrin receptor by a post-transcriptional mechanism, although the receptors are structurally unrelated. The stability of mammalian transferrin receptor mRNA is regulated by the iron-dependent interaction of a member of the IRP family with IREs in its 3'-UTR. Recently, we have identified a T. brucei aconitase which is highly related to mammalian cytoplasmic IRP-1 but more distantly related to mitochondrial aconitases (J. Saas and M. Boshart, unpublished work). This protein is present in the cytoplasm of bloodstream forms. This striking analogy prompted us to test whether this IRP-1-related protein plays a role in iron-dependent transferrin receptor regulation in trypanosomes. To this end, both alleles of the single copy TbACO gene were deleted by targeted gene replacement (see the Materials and methods section and Figure 3A for details of the targeting strategy). Figure 3B shows that TbACO was successfully deleted in three independent MITat 1.4 bloodstream-form clones. The size of the DNA fragments detected with neomycin- and hygromycin-specific probes respectively was as expected (Figure 3B) and thus indicated correct homologous recombination events. In accordance with this result, the TbACO protein was absent in the three knock-out clones (Figure 3C). Since disruption of TbACO in bloodstream forms did not result in an altered growth phenotype in culture, we were able to analyse the effect of iron depletion on ESAG6 mRNA abundance, transferrin receptor

Figure 3 Gene disruption of TbACO

(A) Schematic drawing of targeting constructs and targeting strategy. (B) Southern blot analysis of SacI-digested genomic DNA (1 µg per lane) from MITat 1.4 wild-type trypanosomes (lanes wt) and three G418 and hygromycin double resistant cell lines (lanes aco−/− 1, 2 and 3). The specific probes are indicated below the autoradiograms. Size markers are given in kb pairs. (C) Western blot analysis of transfectants from the TbACO gene disruption experiment. Whole cell lysates (2 × 10⁶ cells per lane) of MITat 1.4 wild-type (lane wt) and three mutant cell lines (lanes aco−/− 1, 2 and 3) were separated on an SDS-10% (w/v) polyacrylamide gel and probed with anti-TbACO serum. The blot was reprobed with an antibody detecting the mitochondrial protein HSP60 (purchased from StressGen) as an internal control. The molecular mass of TbACO is given in kDa.

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expression and transferrin uptake in the Δaco::NEO/Δaco::HYG deletion clones. In all experiments the effect of iron depletion by deferoxamine treatment was very similar in wildtype and Δaco::NEO/Δaco::HYG cells. The results presented in Table 1, Figures 1A and 2 clearly show that the IRP-1-related TbACO is not required for post-transcriptional regulation of ESAG6 mRNA by iron.

### Physiological role of transferrin receptor regulation

Trypanosomes infect a variety of mammalian hosts. Mammalian transferrins show quite large sequence diversity [39] and thus have different affinities for a given trypanosomal transferrin receptor [6,7,40]. Therefore, culture in serum of certain hosts can lead to limiting iron supply and poor growth of the parasite [40].

Up-regulation of the transferrin receptor could help trypanosomes to satisfy their iron requirement in different host environments. To test this hypothesis, foetal calf serum-adapted TC221 bloodstream-form trypanosomes were incubated in medium supplemented with serum from horse, pig, or chicken, and after 15 h the transferrin uptake rate was measured. The uptake of transferrin was increased approx. 2-fold in medium supplemented with these sera relative to the uptake rate of parasites grown in foetal calf serum-supplemented medium (Table 2). Addition of bovine holo-transferrin abolished the effect (Table 2) indicating that the increase in transferrin uptake was only due to iron deficiency.

### DISCUSSION

This report shows that expression of the transferrin receptor and hence transferrin uptake in bloodstream forms of T. brucei are regulated by iron availability. The increase in the transferrin receptor level in iron-depleted trypanosomes was 3-fold and similar to the extent of regulation of the transferrin receptor upon iron depletion in human K562 cells [41,42]. The abundance of ESAG6 mRNA, encoding one of the two subunits of the T. brucei transferrin receptor, was increased in iron-depleted trypanosomes via a post-transcriptional mechanism.

In mammalian cells, the transferrin receptor mRNA is stabilized in iron-depleted cells due to binding of IRPs to specific IREs in the 3′-UTR of transferrin receptor mRNA [43–45]. In analogy to this paradigm for post-transcriptional control of gene expression, we have investigated the role in ESAG6 regulation of an aconitase highly related to mammalian IRP-1 and present in the cytoplasm of T. brucei. Targeted deletion of the TbACO gene showed that this IRP-1-related aconitase is not essential for iron regulation of ESAG6 mRNA. In mammalian cells the closely related IRP-2 can independently mediate the iron status via IREs [46]. However, in trypanosomes the presence of an additional IRP-related protein seems very unlikely for the following reasons: (i) low-stringency Southern hybridizations did not reveal any TbACO-related gene (J. Saas and M. Boshart, unpublished work), (ii) several highly-degenerated PCR primer combinations did not amplify any other IRP- or aconitase-related sequence (J. Saas and M. Boshart, unpublished work), (iii) four rat or rabbit anti-TbACO sera did not detect any band in the 98–101 kDa range in Western blots with extracts from procyclic Δaco::NEO/Δaco::HYG null mutant lines (results not shown), and (iv) the same null mutant lines were devoid of any detectable aconitase activity (results not shown). In summary, the available evidence that the T. brucei genome contains only one IRP-related gene suggests that a different mechanism and a different type of trans-acting factor are responsible for iron sensing and regulation of transferrin receptor mRNA in this protozoan.

We cannot by any means exclude the possibility that the IRP-1-related TbACO has a function in the cytoplasm of trypanosomes, e.g. in translational control of other target genes. However, our results suggest that trypanosomes have developed a distinct mechanism to transmit the low iron signal to ESAG6 mRNA. This corresponds to the complete structural dissimilarity of the mammalian and trypanosome transferrin receptors [3–7]. Both subunits of the trypanosome transferrin receptor, ESAG6 and ESAG7, are structurally related to the N-terminal domain of the VSG suggesting an evolutionary and structural relationship between these proteins [47]. Thus, the evolutionary origin of the trypanosome transferrin receptor is certainly distinct from the origin of the mammalian transferrin receptor. In addition, trypanosomes evolved both antigenic variation and the ability to use mammalian transferrin for iron supply when they acquired a parasitic life style.

The amount of transferrin in host blood is always in excess of the parasite’s needs [2,7]. Why do trypanosomes then need a mechanism to up-regulate the transferrin receptor in response to iron depletion? Antibodies against ESAG6/7 can inhibit transferrin uptake of trypanosomes in vitro [6,7], but significant iron starvation is unlikely to result in vivo from host antibodies produced during the infection [7]. The 20 different VSG expression sites encode slightly different ESAG6/7 heterodimers, only one of which is expressed at one time. These transferrin receptor variants have widely different affinities for transferrin of a given mammalian host. The dissociation constants for bovine transferrin vary between 2 nM and 1 μM [6,7,40,47]. The affinity of a given trypanosome transferrin receptor variant for transferrin of different host species also varies [40]. Thus, the parasite will encounter limiting iron supply during natural transmissions from one host species to another when a low affinity transferrin receptor is expressed. Up-regulation of the transferrin receptor may allow immediate adaptation to a new host environment upon transmission, eventually followed by selection of an appropriate VSG expression site coding for a high affinity variant transferrin receptor [40]. Regulation of transferrin receptor expression thus may be one aspect of the parasite’s ability to adapt to a variety of mammalian hosts.
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REFERENCES


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