Serum Resistance-Associated Protein Blocks Lysosomal Targeting of Trypanosoma brucei

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Trypanosoma brucei is the causative agent of nagana in cattle and can infect a wide range of mammals but is unable to infect humans because it is susceptible to the innate cytotoxic activity of normal human serum. A minor subfraction of human high-density lipoprotein (HDL) containing apolipoprotein A-I (apoA-I), apolipoprotein L-I (apoL-I), and haptoglobin-related protein (Hpr) provides this innate protection against T. b. brucei infection. This HDL subfraction, called trypanosome lytic factor (TLF), kills T. b. brucei following receptor binding, endocytosis, and lysosomal localization. Trypanosoma brucei rhodesiense, which is morphologically and physiologically indistinguishable from T. b. brucei, is resistant to TLF-mediated killing and causes human African sleeping sickness. Human infectivity by T. b. rhodesiense correlates with the evolution of a resistance-associated protein (SRA) that is able to ablate TLF killing. To examine the mechanism of TLF resistance, we transfected T. b. brucei with an epitope-tagged SRA gene. Transfected T. b. brucei expressed SRA mRNA at levels comparable to those in T. b. rhodesiense and was highly resistant to TLF. In the SRA-transfected cells, intracellular trafficking of TLF was altered, with TLF being mainly localized to a subset of SRA-containing cytoplasmic vesicles but not to the lysosome. These results indicate that the cellular distribution of TLF is influenced by SRA expression and may directly determine the organism's susceptibility to TLF.

Humans are resistant to infections by Trypanosoma brucei brucei, which can infect cattle and wild animals (4, 35, 48). This innate resistance to T. b. brucei is mediated by a subfraction of human high-density lipoprotein (HDL), termed trypanosome lytic factor (TLF), which represents <0.5% of the total serum HDL (21, 38, 39, 45, 46). The protein composition of TLF was recently elucidated by mass spectroscopy and biochemical reconstitution, and it was found to be composed of apolipoprotein A-I (apoA-I), apoL-I, and Hpr (13, 29, 43). In human serum, these proteins are colocalized within spherical particles containing phospholipids and cholesterol. When isolated from human HDL, both Hpr and apoL-I are toxic to T. b. brucei; however, reconstitution of these proteins into the same HDL particle increases the specific activity of trypanosome killing approximately 10-fold (43). In human serum, >99% of the trypanolytic activity is associated with native HDLs containing both Hpr and apoL-I. Furthermore, the specific activity of this HDL subclass for T. b. brucei killing is several hundredfold higher than those of HDLs containing Hpr or apoL-I alone (43). The synergism of Hpr and apoL-I may be critical for the sterile innate immunity humans have against T. b. brucei infection.

The cellular pathway for TLF killing of T. b. brucei requires high-affinity binding to cell surface receptors in the flagellar pocket, followed by endocytosis and routing to the lysosome (12, 19, 20, 26, 44). Interruption of trafficking to or acidification of the lysosome spares T. b. brucei from TLF killing. The biochemical events that occur within the lysosome, eventually leading to trypanosome lysis, may be complicated since multiple toxins are present in the TLF particle. Two potential mechanisms for TLF killing have been proposed. The first model proposes that within the acidic lysosome, TLF is able to accelerate the reduction of Fe³⁺, resulting in the formation of reactive free radicals, lipid peroxidation, and eventually, lysosomal membrane destabilization (3). The second model is based on studies with recombinant apoL-I which demonstrate that apoL-I is able to form anion channels in vitro, and when taken up by T. b. brucei, triggers depolarization of the lysosomal membrane, leading to an influx of chloride and subsequent osmotic swelling of the lysosome until the trypanosome lyses (36). The recent report that TLF can form cation-selective pores in unilamellar vesicles is consistent with a pore-forming mechanism for trypanosome killing (33). The identification of two toxins within the same native HDL raises the interesting possibility that the two proposed mechanisms may work in concert to provide the synergism observed with native and reconstituted HDL containing Hpr and apoL-I (43). Despite the uncertainties concerning the precise mechanism of killing, there is general agreement that localization of TLF to the lysosome is a prerequisite step (32, 47).

Resistance to the cytotoxic activities of normal human serum has been recognized as the key feature distinguishing T. b. brucei from the human pathogen T. b. rhodesiense. Human serum resistance in all isolates of T. b. rhodesiense coincides with the expression of the serum resistance-associated gene.
(SRA) (8, 9, 10, 31, 40, 51, 37, 49). SRA is a member of the variable surface glycoprotein (VSG) family of proteins in African trypanosomes, and despite having low sequence homology (<25%) with VSGs, it shares several sequence and structural features with VSGs (6, 9, 11). SRA is an expression site-associated gene in T. b. rhodesiense and is located up-stream of the VSG in the active telomeric expression site (51).

The role of SRA in resistance to human serum was conclusively shown in transfection studies of T. b. brucei with SRA (50, 51). These studies were extended to show that recombinant apo-l-I and SRA bind in vitro by a coiled-coil interaction between the two proteins, and this has been proposed to directly inhibit trypanosome killing by apo-l-I (47). Immunofluorescence microscopy analysis of T. b. rhodesiense indicated that apo-l-I and SRA colocalized to the lysosome in trypanosomes treated with apo-l-I. However, other studies have shown that TLF uptake and cellular localization differed in resistant and susceptible lines of T. b. rhodesiense (19). TLF accumulation was reduced approximately sixfold in resistant trypanosomes, and the cell-associated TLF was excluded from the lysosome (19). This indicated that differences in cellular trafficking of TLF might contribute to trypanosome susceptibility to TLF killing.

Here we show that transfection of SRA into three different bloodstream-stage T. b. brucei isolates expressing different VSGs was sufficient to confer high levels of resistance to TLF and human serum. Epitope-tagged SRA also conferred TLF resistance and allowed subcellular localization of SRA to non-lysosomal vesicles predominately located between the nucleus and the kinetoplast. In SRA-expressing cells, TLF was not routed to the lysosome, and colocalization of TLF and SRA was observed in small cytoplasmic vesicles. Based on these observations, we conclude that the association of SRA with TLF-containing endosomes results in rerouting of TLF, thus preventing lysosomal localization and trypanosome death.

MATERIALS AND METHODS

Trypanosome isolates and culture methods. Culture-adapted monomorphic isolates of T. b. brucei MiTat 1.2 (42/21), pleomorphic MiTat 1.1 (KETR 667), and GuTat 10.1 (TREU 927/4) cells were used throughout these studies. Bloodstream-stage cultures were grown in HMI-9 medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 10% Serum Plus (JRH BioSciences, Richmond, CA) in a 0.4-cm GenePulser cuvette (Bio-Rad 165-2088) at 1.5 kV, 25 μF, and 200 μF. Electroporated cells were allowed to recover for 24 h in HMI-9 medium before the addition of 2.5 μg/ml G418 sulfate. Drug-resistant populations were cloned, grown on agarose plates containing 2 μg/ml G418, and subsequently maintained in HMI-9 medium with constant drug selection (7).

Purification of trypanolytic HDLs and in vitro lysis assays. Normal human blood was obtained from healthy fasting donors. Lytic HDLs were purified as described previously, and aliquots were frozen at −70°C (21). One unit of lytic activity is defined as the amount of HDL needed to kill 50% of human-serum susceptible T. b. brucei (ITat 1.3) organisms in a standard 2-h lysis assay at 37°C (21). Typically, 0.01 μg of purified TLF provided a unit of trypanolytic activity in this in vitro assay (43). Lysis assays performed with cultured cells were extended to 4 h. The percentage of lysed cells was counted among 100 cells. Growth inhibition assays were performed by the addition of human serum (50%) to cultured bloodstream-stage trypanosomes. After 24 h, the percentage of cells surviving was determined by phase-contrast microscopy.

To estimate the growth rates of wild-type and transfected trypanosomes, cells were inoculated at 1 × 10^6 cells/ml in HMI-9 medium and counted daily until stationary phase was reached. The growth rate was calculated using the following equation: 1/v = (log_{10} N_f − log_{10} N_i)/(t_f − t_i).

DNA and RNA analysis. Genomic DNA was isolated as described previously (30). Twenty nanograms of genomic DNA was used for PCRs to examine the presence of SRA (5′ primer, CAACCTCTAAGAATCAAATAG; 3′ primer, AATTCTGAAAATGTTGAAAG) and tubulin (5′ primer, CCTGTGCCAT ATGGCAAAG; 3′ primer, GGGGGTGCATTTTGTGC) gene sequences. Southern blots were performed according to standard protocols (42). Total RNA was isolated with Trizol reagent (Roche Biochemicals, Indianapolis, IN), and 5 μg of RNase-free RNase-treated 15% formaldehyde gels and electroblotted at 15 V in 0.5× MOPS buffered running and transfer solutions for 1.5 h, followed by 50 V for 1.5 h, onto a positively charged nylon membrane (Roche Biochemicals, Indianapolis, IN). DNA probes were labeled by the random primer method (Life Technologies-Invitrogen, Carlsbad, CA) with [32P]dCTP. The blots were hybridized in 0.9 M NaCl, 5× Denhardt’s solution, 10% dextran sulfate, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 40% formamide, 0.1 mg/ml salmon sperm DNA overnight at 42°C and subsequently washed at 60°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% SDS, and finally 0.1× SSC, 1% SDS at room temperature. Alternatively, the probes were labeled with AlkPhos Direct (Amersham Pharmacia, Piscataway, NJ), hybridized, and developed according to the manufacturer’s description.

Analysis of SRA by SDS-PAGE and Western blotting. Cultured cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH2PO4, 1.5 mM KH2PO4, 14 mM Na2HPO4, and 1.0 mM KH2PO4) and then resuspended in lysis buffer (100 mM Tris, pH 8; 10 mM EDTA; 0.5% SDS) containing a protease inhibitor cocktail (Complete Mini; Roche Biochemicals, Indianapolis, IN). Freshly prepared cell lysates (equivalent to 3 × 10^7 cells per lane) were separated in 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), run under standard conditions (1.5 kV, 25°C), and transferred to nitrocellulose membranes (0.2-μm Protran; Schleicher and Schuell, Dassel, Germany) for 45 min at 57 V. Polyclonal antibodies against VSG 221 were added at a 1:2,000 dilution, incubated for 1 h at room temperature, and then incubated overnight at a 1:5,000 dilution. Membranes were washed with PBS and Alexa Fluor 488-labeled goat anti-mouse immunoglobulin G (Molecular Probes, Eugene, OR) was added at a 1:1,000 dilution in PBS-10% FBS for 1 h at room temperature. Slides were washed with PBS, and Alexa Fluor 488-labeled goat anti-mouse immunoglobulin G (Molecular Probes, Eugene, OR) was added at 4°C. Cells were viewed with a confocal microscope (Zeiss LSM 5 PASCAL; Carl Zeiss, Jena, Germany).
ing the secondary antibody incubation.Slides were washed two times in PBS and microscopically analyzed.

To determine whether SRA colocalized with the endoplasmic reticulum (ER), fixed cells were incubated with monoclonal anti-Ty (1:50) and polyclonal anti-

Bip (1:4,000) (1). Goat anti-mouse Alexa Fluor 488-labeled and goat anti-rabbit Alexa Fluor 594-labeled antibodies were added, and slides were processed as
described above. To determine SRA localization with the lysosome, cells were 
stained with the anti-Ty antibody as described above and then incubated with a monoclonal antibody to the lysosomal marker p67 (1:1,000) conjugated to Alexa 
Fluor 594 (23).

To further localize SRA with the endocytic marker tomato lectin (TL; Vector Laboratories, Burlingame, CA) (34) and TLF, we directly conjugated TL and 

TLF to Alexa fluorochromes. Cells were incubated with 4 μg/ml TL and/or 10 to 

20 μg/ml TLF for 30 min at 37°C. After incubation, the cells were put on ice, 
washed, resuspended in PBSG at 4°C, smeared onto a slide, and fixed with cold methanol. For colocalization of SRA with TL or TLF, cells were stained with the anti-Ty as described above. For localization of TLF and p67, cells were incubated with 50 μM chloroquine for 30 min before adding Alexa-conjugated TL to a 
final concentration of 20 μg/ml. Cells were incubated for an additional 90 min 
before being processed and stained for p67 as described above. Trypanosomes were 
analyzed at a magnification of ×100 with a Zeiss fluorescence microscope, 
and digital images were captured with a Zeiss AxioCam video camera. The 
contrast and brightness of some images were adjusted and overlaid with Adobe 
Photoshop software.

RESULTS

Genotypic and phenotypic analysis of SRA-transfected T. b. brucei. Previous studies have shown that transfection of procyclic T. b. brucei with SRA and subsequent transmission through tsetse flies resulted in bloodstream-stage T. b. brucei organisms that were resistant to normal human serum (51). The cyclical transmission of trypanosomes through tsetse flies is difficult due to the low transmission efficiency, variability in the ability of T. b. brucei isolates to infect tsetse flies, and specific developmental changes in gene expression influencing the VSG expression sites and other genes. To circumvent these problems and to directly determine if SRA and Ty epitope-tagged SRA were sufficient to confer resistance to normal human serum, three different isolates of bloodstream-stage T. b. brucei were transfected with SRA and SRA-Ty. The location of the Ty 

epitope within the SRA gene was not expected to disrupt the proposed N-terminal alpha-helical domains implicated in having a role in SRA resistance (Fig. 1A) (6, 47). Similar transfections of bloodstream-stage T. b. brucei with the hemagglutinin (HA) epitope fused to the amino or carboxy terminus of SRA were reported previously (50). Genomic DNAs from wild-type 427, transfected 427-SRA and 427-SRA-Ty, wild-type 667, transfected 667-SRA and 667-SRA-Ty, wild-type 927, and transfected 927-SRA and 927-SRA-Ty organisms were analyzed for the presence of the SRA gene by PCR with primers complementary to the 5’ and 3’ coding sequences of SRA. A 1.4-kb SRA product was only detected in SRA- and SRA-Ty-transfected T. b. brucei lines and not in wild-type cells (Fig. 1B). Southern blotting confirmed single-copy integration of SRA in the SRA and SRA-Ty transfectants (data not shown). Control PCRs using primers specific to the α-tubulin gene produced the expected 0.75-kb product (Fig. 1B).

By light microscopy, no morphological differences were observed between SRA-transfected and wild-type cells. In addition, cells with and without SRA reached approximately the same cell densities and grew at the same rates, indicating that the expression of SRA did not influence the growth characteristics of bloodstream-stage T. b. brucei (data not shown).

Susceptibility of SRA-transfected T. b. brucei to TLF killing. In order to determine whether transfection with SRA was sufficient to confer the serum resistance phenotype, transfected cells were incubated with increasing concentrations of TLF (Fig. 2) or normal human serum (data not shown). The three wild-type lines of T. b. brucei were highly susceptible to lysis by TLF. Following SRA transfection, the three lines of T. b. brucei showed high levels of resistance to TLF (Fig. 2). The level of serum resistance in SRA-transfected T. b. brucei was similar to that observed for human infectious T. b. rhodesiense (data not shown). No difference in the level of TLF resistance was detected between cells transfected with SRA alone and those transfected with SRA-Ty (data not shown). To confirm whether
SRA-induced TLF resistance was comparable to that seen for *T. b. rhodesiense*, the survival of SRA-transfected *T. b. brucei* was examined at concentrations of TLF similar to those found in human serum (1,000 U/ml) and by incubation for 24 h under the same growth conditions in the presence of 50% human serum. The SRA-transfected *T. b. brucei* lines remained viable under these conditions (data not shown).

Early studies suggested that the VSG expressed on the surfaces of trypanosomes might influence their susceptibility to normal human serum. Each of the *T. b. brucei* lines used in the current studies expresses different VSG genes producing serologically distinct VSG coats. Based on the *T. b. brucei* isolates tested, it appears that resistance to TLF is highly dependent on SRA expression but independent of the VSG expressed on the trypanosome surface. The transfection of bloodstream-stage lines of *T. b. brucei* directly demonstrated that expression of the SRA gene is sufficient to confer resistance to TLF and may lead to human infectivity.

**Expression of SRA mRNA in transfected cells.** SRA mRNA is an abundant transcript in *T. b. rhodesiense*, representing as much as 10% of the total cellular RNA (10, 18, 31). In order to determine the level of SRA mRNA in transfected cells and to compare this level with that in human infectious *T. b. rhodesiense*, total cellular RNA was isolated from *T. b. brucei* transfected with either vector alone (427) or SRA (427-SRA) and from a human-serum-resistant line of *T. b. rhodesiense*. Blots were hybridized with probes specific for the VSG expressed by *T. b. rhodesiense* (VSG-R) and the VSG-221 expressed with vector alone (427) or with SRA (427-SRA-Ty). The Western blot was probed with antibodies to the Ty epitope, and proteins of approximately 59 and 65 kDa were detected in extracts from *T. b. brucei* 427-SRA-Ty (arrows).

While the transgenic SRA gene is expressed from the ribosomal locus in *T. b. brucei*, the expression level of the gene is similar to the endogenous expression level of SRA in human-serum-resistant *T. b. rhodesiense* strains.

**Expression of SRA in transfected *T. b. brucei*.** Despite high levels of SRA mRNA, the detection of SRA protein in *T. b. rhodesiense* and in SRA-transfected *T. b. brucei* has been problematic in some cases (8, 9, 51). However, visualization of SRA has been reported for *T. b. rhodesiense* cells by use of a polyclonal mouse antiserum raised against recombinant SRA (31). In addition, endogenous SRA in *T. b. rhodesiense* as well as recombinant SRA (rSRA) expressed in *T. b. brucei* was detected using antibodies raised against an SRA polypeptide (47). To examine SRA expression in transfected *T. b. brucei*, total cell lysates from *T. b. brucei* transfected with vector alone (427) or with SRA (427-SRA-Ty) were separated by 10% SDS-PAGE and analyzed by Coomassie staining and Western blotting with monoclonal antibodies specific to the Ty tag inserted into the SRA gene. Epitope-tagged proteins, migrating at approximately 59 and 65 kDa, were detected in Western blots of cell lysates of both 427-SRA and 667-SRA but not in lysates of wild-type 427 and 667 (Fig. 3B: data for 667 and 667-SRA are not shown). The predicted size of mature SRA is approximately 38,000 Da. While the nature of the doublet is unknown, it may be the result of differences in posttranslational modification of SRA. The staining patterns observed are similar to previous Western blot results obtained with *T. b. rhodesiense* extracts analyzed with anti-rSRA and SRA-HA-transfected *T. b. brucei* probed with anti-HA (31, 50). As reported by others, we found that the detection of SRA
within the ER or targeting to the lysosome for degradation. T. b. brucei is mainly localized to intracellular compartments.

To determine whether SRA was located in endosomes or the distinctive ER staining pattern with the BiP antibody (red), T. b. brucei was double stained for SRA (anti-Ty) and the ER (anti-BiP), endocytic compartments (Alexa-conjugated TL (red)), or the lysosome (anti-p67). Double labeling of SRA-Ty with anti-BiP and anti-Ty revealed a distinctive ER staining pattern with the BiP antibody (red), with little colocalization with SRA-Ty (green; Fig. 4A to D). To determine whether SRA was located in endosomes or the lysosome, T. b. brucei 427-SRA-Ty cells were incubated with Alexa Fluor 594-labeled TL (red) for 30 min at 37°C to allow uptake and lysosomal targeting of the ligand. Cells were then fixed and incubated with anti-Ty (green). Under these conditions, TL accumulated largely in the trypanosome lysosome (Fig. 4F), and to a limited extent, in endosomes located nearer the flagellar pocket (green; Fig. 5C and G). While SRA-Ty localized to the region between the kinetoplast and the nucleus, SRA-Ty staining did not overlap with TL staining (Fig. 4E to H). To directly address lysosomal localization, we stained cells with an antibody to p67 (red) and found that SRA-containing vesicles (green) did not significantly overlap with localization of the lysosomal membrane protein p67 (Fig. 4I to L). This pattern suggests that SRA-Ty is predominately nonlysosomal.

TLF killing of T. b. brucei requires lysosomal localization and subsequent acidification (20, 26). T. b. brucei 427 was incubated with TLF conjugated with Alexa Fluor 594 (red) and TL conjugated with Alexa Fluor 488 (green) for 30 min at 37°C. Under these conditions, both TLF and TL colocalized mainly to the lysosomes of wild-type cells (Fig. 5A to D). These results are consistent with previous studies that localized TLF to lysosomes of other isolates of T. b. brucei (20, 44). In contrast, TLF did not colocalize with the endocytic marker TL in T. b. brucei 427-SRA-Ty cells (Fig. 5E to H). After 30 min, small TLF-containing vesicles were seen close to the flagellar pocket (Fig. 5F).

Once we saw that TLF did not seem to be trafficking by its normal endocytic pathway in the SRA-expressing cells, we asked whether TLF could be concentrated in the lysosome in the presence of chloroquine. Previous studies have shown that chloroquine blocks lysosome acidification and prevents the degradation of endocytosed proteins (44). Cells were treated with chloroquine for 30 min before incubation with TLF for 90 min in an attempt to force TLF into the lysosome. As expected, in wild-type T. b. brucei, TLF accumulated and colocalized with

by Western blot analysis was variable, suggesting that SRA may be highly susceptible to proteolytic degradation while cell extracts are prepared for analysis.

**Cellular localization of SRA and TLF in T. b. brucei 427-SRA-Ty.** We determined the cellular localization of SRA in T. b. brucei 427-SRA-Ty by immunofluorescence microscopy, using a monoclonal antibody against the Ty epitope. This antibody is highly specific for the Ty epitope and does not react with wild-type T. b. brucei 427 proteins by either Western blotting or immunofluorescence analysis. T. b. brucei 427-SRA-Ty cells were fixed, permeabilized, and incubated with anti-Ty. SRA-Ty was visible within cytoplasmic vesicles located mainly between the kinetoplasts and the nuclei of T. b. brucei 427-SRA-Ty cells (Fig. 4C, G, and K). Only minor cell surface labeling was observed, indicating that SRA in transfected T. b. brucei is mainly localized to intracellular compartments.

We were concerned that the expression of SRA-Ty from the ribosomal locus in T. b. brucei might result in aggregation within the ER or targeting to the lysosome for degradation. T. b. brucei 427-SRA-Ty cells were double stained for SRA (anti-Ty) and the ER (anti-BiP), endocytic compartments (Alexa-conjugated TL), or the lysosome (anti-p67). Double labeling of T. b. brucei 427-SRA-Ty with anti-BiP and anti-Ty revealed a distinctive ER staining pattern with the BiP antibody (red), with little colocalization with SRA-Ty (green; Fig. 4A to D). To determine whether SRA was located in endosomes or the lysosome, T. b. brucei 427-SRA-Ty cells were incubated with Alexa Fluor 594-labeled TL (red) for 30 min at 37°C to allow uptake and lysosomal targeting of the ligand. Cells were then fixed and incubated with anti-Ty (green). Under these conditions, TL accumulated largely in the trypanosome lysosome (Fig. 4F), and to a limited extent, in endosomes located nearer the flagellar pocket (green; Fig. 5C and G). While SRA-Ty localized to the region between the kinetoplast and the nucleus, SRA-Ty staining did not overlap with TL staining (Fig. 4E to H). To directly address lysosomal localization, we stained cells with an antibody to p67 (red) and found that SRA-containing vesicles (green) did not significantly overlap with localization of the lysosomal membrane protein p67 (Fig. 4I to L). This pattern suggests that SRA-Ty is predominately nonlysosomal.

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DISCUSSION

Initial studies using subtractive hybridization methods suggested that human-serum-resistant and -sensitive African trypanosomes might differ by the expression of a single gene (27). This possibility was tested in an elegant series of transfection experiments, in which the SRA gene from *T. b. rhodesiense* was introduced into a human-serum-susceptible line of *T. b. brucei* and was found to be sufficient to confer resistance (51). These studies relied on transfection of an insect-borne developmental stage of *T. b. brucei* and subsequent transmission through the tsetse fly prior to analysis of transfectants. This strategy, though certainly successful, is both cumbersome and potentially prone to artifacts due to changes in gene expression unrelated to the transgene. In this paper and elsewhere, direct transfection of bloodstream-stage *T. b. brucei* with SRA was shown to render the parasites resistant to both TLF and normal human serum (50). Transfected *T. b. brucei* cells express the SRA transgene at high levels, resulting in steady-state amounts of the SRA mRNA that are similar to those detected in human infectious *T. b. rhodesiense*. Using antibodies against the Ty epitope, we examined the intracellular distribution of SRA in *T. b. brucei* 427-SRA-Ty and found it to be largely localized to small cytoplasmic vesicles between the flagellar pocket and the nucleus. The incubation of *T. b. brucei* 427-SRA-Ty with Alexa Fluor 594-conjugated TLF and subsequently stained the cells for SRA using the Ty antibody. Fluorescence microscopy showed that TLF (red) and SRA-Ty (green) overlap in their subcellular distributions (Fig. 7A to H). TLF and SRA colocalized to a subpopulation of small cytosolic vesicles in SRA-transfected *T. b. brucei*, but neither was found in the lysosome. We observed many SRA-containing vesicles without TLF, but few vesicles containing TLF alone, without SRA, were detected (Fig. 7D and H). These findings are consistent with a proposed mechanism of resistance to TLF resulting from reduced targeting of TLF to the lysosome and with the idea that the association of SRA and TLF is required for resistance (19).
trypanosomes. It is possible that the cell surface reactivity was simply a consequence of cross-reactivity of the polyclonal mouse antisera against SRA with the VSG. This is a particular concern since SRA is a member of the VSG gene family and shares characteristics with other trypanosome cell surface proteins (6, 31). Alternatively, the amounts of SRA present on the cell surface in different trypanosome lines may vary. Regardless, the significance of the cell surface-associated SRA is questionable, since little or no SRA was found on the surfaces of T. b. brucei 427-SRA-Ty cells yet they were highly resistant to both TLF-1 and human serum. Other labs have also shown that the localization of endogenous SRA in T. b. rhodesiense is primarily intracellular (47). In this paper, we showed that SRA-Ty localizes to an intracellular, nonlysosomal, vesicular location in transfected T. b. brucei.

We were concerned that high-level expression of SRA might result in aberrant intracellular localization. Transgenes are often expressed at abnormal levels due to the use of heterologous promoters. In our construct, SRA4 expression is driven from a constitutively active rRNA polymerase I promoter known to mediate high levels of mRNA synthesis in African trypanosomes (25, 41). The 5’ and 3’ untranslated regions were derived from tubulin sequences, which contribute posttranscriptionally to the stability of mRNAs in both bloodstream and procyclic forms. Previous studies have shown that SRA mRNA is an abundant transcript in both wild-type T. b. rhodesiense and transfected T. b. brucei, representing as much as 5 to 10% of the total mRNA (31, 51). This is comparable to the levels of VSG mRNA in African trypanosomes. We found that the amount of SRA mRNA in our transfectants was similar to the level of SRA mRNA expression in T. b. rhodesiense. Since SRA in T. b. brucei 427-SRA-Ty confers resistance to human serum and the amount of SRA mRNA is similar to that in wild-type T. b. rhodesiense, it seems likely that the SRA-containing cytosolic vesicles revealed by immunofluorescence microscopy are the primary sites of SRA activity.

The subcellular localization of SRA and how it prevents TLF from reaching the lysosome may provide a better understanding of both the function of SRA and the endocytic pathways of trypanosomes. This is in contrast to the proposed site of SRA activity being at the lysosome, where it interacts with apol-1 (47). However, an important difference between these studies is the use of purified human HDLs containing both Hpr and apol-1 in our studies rather than purified and recombinant apol-1. The uptake and trafficking of native TLF and its interaction with SRA may differ from that of recombinant apol-1. Endocytosis in trypanosomes shares many characteristics with endocytosis in other eukaryotic organisms but also exhibits several unique features, including developmental regulation, selective trafficking of proteins from the flagellar pocket, and the potential routing of resident lysosomal proteins through the flagellar pocket prior to localization to the lysosome (35). Vesicular trafficking between organelles occurs through the fusion of donor and specific acceptor membranes. This process is highly regulated and ensures proper directionality in protein sorting and packaging. Monomeric GTPases of the Rab family play a pivotal role in the control of membrane fusion and vesicle trafficking. Several T. brucei Rab proteins have now been identified that localize to specific subcellular compartments (14–17, 28, 35). The availability of antibodies to the T. b. brucei Rab proteins makes it possible to determine whether the cytosolic vesicles containing SRA and TLF are part of a vesicle recycling pathway.

Although we have shown that SRA confers human serum resistance when transfected into bloodstream-stage T. b. brucei and that SRA and TLF colocalize within cytoplasmic vesicles, the mechanism of SRA function is still unknown. One proposed mechanism is that inhibition may depend on interactions between SRA and apol-1 in the lysosome (47, 48). However, we previously showed that TLF was not targeted to the lysosome in T. b. rhodesiense, whereas a naturally occurring human-serum-sensitive variant of T. b. rhodesiense transported TLF to the lysosome prior to cell lysis (19). The distributions of TLF in T. b. rhodesiense and in T. b. brucei 427-SRA-Ty appear to be somewhat different. In contrast to the localization of TLF to small cytoplasmic vesicles in T. b. brucei 427-SRA-Ty, TLF was largely localized at or near the flagellar pocket of T. b. rhodesiense (19). Therefore, it remains possible that SRA can influence TLF uptake and lysosomal trafficking at either the flagellar pocket, where receptor-mediated endocytosis initiates, or at later steps in the endocytic pathway.

Regardless of the precise role of SRA, we have shown that SRA expression is sufficient to confer resistance to TLF and normal human serum in T. b. brucei bloodstream-stage trypanosomes. Furthermore, we have shown that SRA is responsible for the rerouting of TLF within the trypanosome endocytic pathway, with the majority of the TLF becoming associated with nonlysosomal, SRA-containing vesicles. Future experiments will address whether SRA alters TLF localization by either recycling it out of the cell or directing it to a cellular compartment where TLF degradation is accelerated. The elucidation of mechanisms of TLF resistance could lead to the development of inhibitors of the SRA-mediated resistance pathway, thereby increasing the susceptibility of T. b. rhodesiense-mediated human sleeping sickness trypanosomes to TLF and thus leading to novel treatment of the disease.

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