The Gene for a T Lymphocyte Triggering Factor from African Trypanosomes

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Summary

An early and essential event in the protective immune response against most viruses and protozoa is the production of interferon-γ (IFN-γ). In contrast, during infection with African trypanosomes, protozoan parasites that cause human sleeping sickness, the increased levels of IFN-γ do not correlate with a protective response. We showed previously that African trypanosomes express a protein called T lymphocyte triggering factor (TLTF), which triggers CD8+ T lymphocytes to proliferate and to secrete IFN-γ. Here, we isolate the gene for TLTF and demonstrate that the recombinant version of TLTF specifically induces CD8+, but not CD4+, T cells to secrete IFN-γ. Studies with TLTF fused to the green fluorescent protein show that TLTF is localized to small vesicles that are found primarily at or near the flagellar pocket, the site of secretion in trypanosomes. TLTF is likely to be only the first example of a class of proteins that we designate as trypanokines, i.e., factors secreted by trypanosomes that modulate the cytokine network of the host immune system for the benefit of the parasite.

African trypanosomiasis is a widespread, fatal disease in Africa that is commonly called sleeping sickness in humans and ngana in cattle. These diseases are caused by several different species or subspecies of Trypanosoma, which are transmitted by tsetse flies to the bloodstream of their mammalian hosts where they circulate extracellularly and can eventually affect the central nervous system. Mortality occurs from either massive parasitosis or secondary infections due to immunosuppression, an important trait of the disease.

Previously, we showed that trypanosomes release a T lymphocyte triggering factor (TLTF) that induces CD8+ T cells to secrete IFN-γ (1, 2); subsequent studies indicated that TLTF binds to the CD8 molecule on T cells (3). When rodents are infected with Trypanosoma brucei, IFN-γ production in the spleen increases markedly. Moreover, in rats depleted of CD8+ T cells by injection of anti-CD8 monoclonal antibody or in knockout mice carrying a deletion of the CD8 gene, a trypanosome infection does not induce as much IFN-γ production, the parasitemia is decreased and the infected animals survive longer (3). In pas-

Abbreviations used in this paper: ADF, adult T cell leukemia-derived factor; GFP, green fluorescent protein; GST, glutathione-S-transferase; IGIF, interferon-γ-inducing factor; THIO, thioredoxin; TLTF, T lymphocyte triggering factor.
Cleveland, OH). Homology searches of databases were performed using the BLAST algorithm (7). Protein sequence alignments were done using the University of Wisconsin GCG software package (8).

The recombinant thioredoxin (THIO) and glutathione-S-transferase (GST) fusion proteins were generated according to protocols provided by the supplier of the expression plasmids (AMRAD, Australia; Invitrogen, California). The PCR amplified fragments of the TLTF cDNA that were cloned into the expression plasmids contained the TLTF start codon and either the complete TLTF open reading frame or the first 145 codons. Bacillus subtilis topoisomerase that had been further purified by electrophoresis and dialysis (GST–TLTF) according to standard procedures (9).

Plasmid DNA containing the gene for GST–OV7 was the gift of Dr. J. Catmull (University of Iowa, Iowa City, IA).

The T. brucei expression vector pHd–GFPm3 was generated by replacing the luciferase gene of pHd1 (10) with the GFPmut3 gene (11). This mutant version of GFP gives 30- to 100-fold greater fluorescence than wild-type GFP from Aquorea victoria. To generate the GFP–TLTF fusion construct, EcoRI sites were introduced in front of the GFP stop codon and the TLTF start codon via PCR amplification of the corresponding genes using GFP- or TLTF-specific primers, which included an EcoRI restriction site. In the case of TLTF, we used the same approach to introduce a BamHI site immediately following the TLTF stop codon. The TLTF open reading frame was then cloned in frame behind the GFP gene to generate pHd–GFPm3–TLTF.

**DNA Transfections.** Prokaryotic transgenes (clone YTAT1.1, obtained from E. Ullu, Yale University, New Haven, CT) were maintained in Cunningham's SM medium (12) supplemented with 20% FCS. Cells were harvested from mid-log phase cultures (2–4 × 10⁶ cells/ml), washed once with electroporation medium (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄, 25 mM Hepes, 2 mM EDTA, 5 mM MgCl₂, pH 7.6) and resuspended in the same buffer at 1–3 × 10⁶ cells/ml. DNA (50 μg) was introduced into cells by electroporation with a BioRad Gene Pulser using two pulses of 1,500 V at 25 μF in 0.4 cm cuvettes. Transfected cells were transferred to fresh culture medium and examined for GFP expression 16–24 h after transfection. For ethidium bromide staining, cells were incubated with 2 mg/ml ethidium bromide for 15 rain, then washed twice with PBS. Cells were examined using a BioRad MRC-1024 Laser Scanning Confocal Microscope.

**IFN-γ Assays.** Purification of native TLTF, isolation of mononuclear cells and the spot assay for IFN-γ secretion were performed as described previously (1, 3, 4). The final concentration of concanavalin A in the assays was 5 μg/ml. The final concentration of monoclonal antibody MO1 was 5 μg/ml. All assays were conducted using cells from CD4⁴ CD8⁺ mice except where indicated. The origin of the CD4⁴ CD8⁺ and CD4⁴ CD8⁻ mice were described earlier (13). Most of the assays for IFN-γ secretion that involved the recombinant fusion proteins were conducted blind using protein prepared in the United States and sent in coded tubes to Sweden, and were done at least in triplicate.

**Results and Discussion**

A monoclonal antibody, called MO1, was used to affinity purify TLTF from bloodstream trypanosome extracts. Monovalent polyspecific antiserum directed against the affinity-purified protein was generated in rabbits and used to immunoscreen a cDNA expression library of total RNA isolated from bloodstream forms of T. b. rhodesiense, a subspecies that causes the human disease (6). A cDNA clone was identified that remained immunopositive through multiple rounds of screening. The nucleotide sequence of this cDNA revealed that it was a partial length clone lacking the 39 nucleotide sequence of the 5' spliced leader, a universal feature of trypanosome mRNAs. A full-length coding sequence was obtained from the product of a reverse transcriptase–PCR amplification of total trypanosome RNA using an internal 3' primer derived from the TLTF partial cDNA sequence and a 5' primer matching the 5' spliced leader sequence. Fig. 1 shows the deduced 453-amino acid sequence of the encoded protein, a 54-kD hydrophilic polypeptide.

The amino acid sequence of TLTF bears no obvious similarity to IGIF (IFN-γ-inducing factor), a protein recently identified in mouse liver that induces IFN-γ production by CD4⁺ T lymphocytes (14). The lack of similarity between these two proteins may be because IGIF is thought to target CD4⁺ cells, whereas TLTF interacts specifically with the CD8 molecule. However, computer searches did identify similarities to two other sequences that have been deposited in GenBank without further characterization. One encodes a mouse protein of 489 residues designated as a growth arrest–specific protein. The other protein is encoded by a randomly sequenced human cDNA.
A

![Graph A](image)

B

![Graph B](image)

**Figure 2.** Antisera directed against the two different forms of recombinant TLTF inhibit interfere with the activity of native TLTF. (A) Results obtained with antisera raised against THIO-TLTF. P and I denote preimmune and immune serum, respectively. α-THIO-TLTF #1 is serum directed against a fusion protein containing the entire TLTF molecule. α-THIO-TLTF #4 is serum directed against a fusion protein containing only the NH2-terminal 145 amino acids of TLTF. α-gp63 is serum directed against an unrelated Leishmania chagasi protein, gp63 (30); α-Tc is serum directed against a recombinant version of an unrelated Trypanosoma cruzi antigen, gp72 (31) (the gift of K. Otsu and L.V. Kirchhoff, University of Iowa, Iowa City, IA). The dilution of serum used in each case was 1:5,000. (B) Results obtained with α-GST-TLTF #1, a rabbit antiserum directed against a GST fusion protein containing the entire TLTF protein (1:1,000 dilution).

from a fetal lung cDNA library. Comparison of these proteins with TLTF reveals several regions of high sequence identity (Fig. 1). Although the properties and functions of these two mammalian proteins are yet to be described, their percent identity to TLTF (35% identity, 58% similarity) appears too large to be fortuitous.

The trypanosome cDNA was cloned into two different bacterial expression systems in which the NH2-terminal fusion partner is either GST or THIO. In each case, a PCR-derived fragment of the TLTF cDNA was cloned such that the methionine start codon is in frame with the last amino acid codon in the GST or THIO gene. In the THIO case, an additional clone was constructed in which the TLTF segment terminates at codon 145. The recombinant proteins were used to generate polyclonal sera in rabbits and mice. Each serum was then tested for its inhibition of the ability of native TLTF to induce T cells to secrete IFN-γ. Fig. 2 A shows that rabbit antisera directed against either of the two versions of THIO-TLTF (#1 and #4) reduced the activity of native TLTF to the background levels seen in the absence of TLTF. Similarly, mouse antiserum raised against a separate aliquot of THIO-TLTF #1 also reduced the activity of native TLTF to background levels. The presence of the corresponding preimmune serum did not significantly diminish TLTF activity. In control experiments, rabbit antiserum against Leishmania chagasi gp63 and mouse antiserum against Trypanosoma cruzi gp72 (each of which is a surface protein of its respective parasite) had no effect on the biological activity of native TLTF. Fig. 2 B shows that similar results were obtained in parallel experiments using rabbit antiserum directed against the GST-TLTF fusion protein.

The THIO and GST fusion proteins were also tested for TLTF activity, i.e., the ability to induce T cells to secrete IFN-γ. Fig. 3 A shows that GST-TLTF induces IFN-γ secretion from CD8+ cells, but not CD4+ cells. The splenocytes used in these assays were obtained from gene knockout mice that lacked either the CD4 gene or the CD8 gene (13). Similar to native TLTF, the GST–TLTF fusion protein induced IFN-γ secretion only from the population containing CD8+ cells and not from the cells derived from CD8 knockout mice. Control experiments in which concanavalin A was used as a mitogen demonstrated that both populations of cells were intrinsically capable of secreting IFN-γ in response to an appropriate signal. Fig. 3 B demonstrates in a separate series of experiments that the ability of GST–TLTF to induce IFN-γ secretion is specifically due to the TLTF portion of the molecule. Neither GST alone nor another unrelated GST fusion protein, GST–OVT, possessed an activity significantly greater than the background level. In similar experiments not shown, THIO–TLTF, also possessed a similar level of TLTF activity.

Next, we examined whether antibodies that specifically recognize the native TLTF molecule can inhibit the activity of recombinant TLTF. We previously demonstrated that the monoclonal antibody MO1 reduces the ability of native TLTF to induce IFN-γ secretion from CD8+ cells (4). Fig. 4 shows the results obtained when the activity of GST–TLTF was measured in the presence of MO1. Under the conditions of the assay, the activity of native TLTF was reduced by 62% in the presence of MO1. Consistent with the interpretation that GST–TLTF is the recombinant version of this protein, a concomitant decrease of 60% in the activity of GST–TLTF occurred in the presence of MO1. In the case of the unrelated GST–OVT, the number of IFN-γ-secreting cells detected in the assay was unaffected by the presence or absence of MO1.

We previously showed that TLTF is released as a soluble
factor from *T. brucei* into the culture medium (15) and yet the amino acid sequence of TLTF (see Fig. 1) does not contain a classical NH$_2$-terminal signal sequence similar to those found in most secreted eukaryotic proteins (16). Because secretion of TLTF is likely essential for its immunomodulatory function, we investigated how it is targeted to the exterior of the cell. This is an especially relevant question in trypanosomatids, because the entire surface of these organisms is covered by a very dense protein coat that restricts possible sites of endocytosis and secretion to the flagellar pocket (17). This organelle derives from a specialized invagination of the cell membrane that forms at the site where the flagellum emerges from the cell and is relatively protected from the assaults of the immune system of the host. The kinetoplast of *T. brucei* is also located near the base of the flagellum and serves as a marker for the subcellular location of the flagellar pocket.

To examine the cellular fate of TLTF, we fused it to GFP from *Aquorea victoria* (11). Constructs encoding either this fusion protein, or GFP alone, were expressed in transiently transfected trypanosomes, which were examined by laser scanning confocal microscopy (Fig. 5). Because Northern blot analysis indicated that TLTF is expressed in both procyclic and bloodstream form trypanosomes (data not shown), we used the more readily cultured procyclic forms for these transfection experiments. Western blots using an α-GFP polyclonal antiserum (Clonetech, Palo Alto, CA) confirmed that transfected trypanosomes were producing a protein of the expected size (data not shown). Cells in the bottom panel of Fig. 5 were also stained with ethidium bromide to visualize the nucleus and kinetoplast (red staining). As can be seen in the top panel of Fig. 5, GFP alone is expressed evenly throughout the entire cell. The GFP-TLTF fusion protein, on the other hand, is restricted to vesicles that are usually, but not exclusively, located at or near the trypanosome flagellar pocket (Fig. 5, bottom). These results are consistent with TLTF being a secreted protein, as shown previously (15), and demonstrate that signals for TLTF targeting are located within the protein itself, even though it does not contain a hydrophobic NH$_2$-terminal signal sequence. Thus, TLTF is added to the growing list of secreted eukaryotic proteins for which the amino acid sequence gives no indication of a readily identifiable targeting signal (18). Interestingly, many such proteins play a role in immune system function (e.g., IL-1β and adult T cell leukemia-derived factor [ADF]). IL-1β is a monocyte-derived, proinflammatory cytokine that stimulates a broad range of cell types. ADF mediates upregulation of IL-2 receptor expression and may be involved in the abnormal proliferation of T cells observed in some types of leukemogenesis (19). Although some features of export may be shared between leaderless secreted proteins, the mechanisms responsible are unknown. Recent data indicate that multiple mechanisms exist (20) and that two dif-
different proteins may utilize partially, but not completely, overlapping pathways (21).

TLTF is likely to be the first confirmed member of a cabal of trypanosome-derived, immunomodulatory factors for which we suggest the name trypanokines. Trypanosomal cysteine protease, which is found in the serum of infected animals (22–24), is another potential trypanokine, because cysteine proteases of other microorganisms have been shown to modulate cytokine activity either directly or indirectly (25, 26). Still other unknown mitogenic trypanosome molecules have been proposed previously to be the cause of the massive nonspecific polyclonal activation of B cells during acute trypanosomiasis (27). Parallels have already been drawn between gene rearrangements that generate the antibody repertoires in vertebrates and those responsible for the perpetual antigenic variation in trypanosomes (28). Trypanokines represent another example of the ability of the trypanosome to mimic a normal host function; in this case, the synthesis of cytokines that modulate the immune response. Recent experiments with viruses and infectious bacteria indicate that this strategy may also be commonly used by other microbial pathogens (25, 29). In this regard, the availability of the TLTF gene should facilitate novel approaches towards achieving protection against trypanosomiasis. In addition, recombinant TLTF could find applications in the treatment of other infectious agents, such as *Leishmania* and viruses, for which IFN-γ production is among the first events of a protective response.

We would like to thank D. Russell for conducting preliminary immunogold localization of native TLTF. We are extremely grateful to E. Ullu for the YTAT1.1 trypanosome clone and for her assistance in our efforts to express GFP in *T. brucei*. We would also like to thank B. Cormack and S. Falkow for providing the GFPmut3 cDNA.

This research was funded in part by a grant from the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Disease to M. Bakhiet, T. Olsson, and K. Kristensson.

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Received for publication 9 April 1997 and in revised form 27 May 1997.
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