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Identification of Immunodominant Epitopes in *Trypanosoma cruzi* Trypomastigote Surface Antigen-1 Protein That Mask Protective Epitopes

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The gene that encodes trypomastigote surface Ag-1 (TSA-1), a major surface Ag of the bloodstream trypomastigote stage of *Trypanosoma cruzi*, was expressed in a baculovirus expression system. To determine the epitope(s) in TSA-1 that was recognized during *T. cruzi* infection and after immunization with TSA-1, subregions of the TSA-1 gene were expressed in a bacterial expression system. As seen by Western blotting, both mice and rabbits immunized with recombinant TSA-1 protein, as well as *T. cruzi*-infected mice, developed strong immune responses to the carboxyl-proximal region of TSA-1, but show no reaction to the amino-proximal portion of TSA-1. When mice were immunized with either recombinant TSA-1 protein or the carboxyl-proximal region of TSA-1, they did not survive challenge with 10^3 bloodstream trypomastigotes. However, 70% of the mice immunized with the amino-proximal portion of TSA-1 survived challenge with 10^3 bloodstream trypomastigotes. Thus, the immune responses elicited by recombinant TSA-1 or the carboxyl-proximal portion of TSA-1 are nonprotective during *T. cruzi* infection. In contrast, vaccination with the amino proximal region of TSA-1 elicits a protective immune response. These results suggest that responses to immunodominant epitope(s) within the carboxyl-proximal portion of TSA-1 mask epitopes within the amino-proximal portion that are capable of stimulating host-protective immune responses. It is suggested that immunodominant regions in surface molecules such as TSA-1 may provide a mechanism for the parasite to evade the host immune response by directing the response away from epitopes that have the potential to elicit a reaction that is damaging to the parasite.

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*Trypanosoma cruzi*, the causative agent of Chagas disease, is a parasitic protozoan endemic to Central and South American countries (1). This disease is the major cause of heart disease within endemic areas, affecting approximately 18 million people (2). In the vertebrate host, the infective trypomastigote stage of the parasite circulates in the bloodstream, invades host cells, and transforms into the replicative amastigote form. During *T. cruzi* infection, both Chagas patients and experimental animals develop strong immune reactions to parasite-derived Ags (3–6); however, these immune responses do not result in complete parasite clearance from the host.

Similar nonprotective immune responses are observed in other protozoan parasitic infections, and it has been suggested that such responses may result from immunodominant regions that direct the immune response away from epitopes that otherwise would generate an immune response harmful to the parasite (7, 8).

The major surface Ags of the trypomastigote stage, which stimulate an immune response during a natural *T. cruzi* infection, are encoded by the trypomastigote surface Ag/trans-sialidase supergene family (9–17). TSA-1^3^ was the first member of the family to be identified and characterized, and it encodes a trypomastigote-specific surface protein of approximately 85 kDa (9). The TSA-1 gene family is distinguished from other members of the supergene family by the presence of nonapeptide tandem repeats near its carboxyl-terminus (10, 12). Although the TSA-1 protein and proteins encoded by other members of

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3 Abbreviations used in this paper: TSA-1, trypomastigote surface Ag-1; rbTSA-1, recombinant trypomastigote surface Ag-1 produced in the baculovirus expression system; recTSA-1, recombinant trypomastigote surface Ag-1 produced in the pTrcHis expression system; aa, amino acid.
the superfamily elicit an immune response during infection, little is known about the nature of the response or the protective value of the response to the infected host. Therefore, we initiated studies to investigate the immune responses of mice and rabbits immunized with TSA-1 and polypeptides derived from subregions of the gene expressed in baculovirus and bacterial systems. In addition, the vaccination properties of the TSA-1 protein were investigated by immunization of mice with TSA-1 recombinant protein and subregions of the TSA-1 protein and subsequent challenge with T. cruzi trypomastigotes. Our results indicate that immunodominant region(s) in TSA-1 generate nonprotective immune responses to a natural T. cruzi infection and mask other epitopes in TSA-1 that have the potential to induce protective immune responses.

**Materials and Methods**

**Construction and expression of recombinant TSA-1 in the baculovirus expression system**

We expressed the entire TSA-1 coding region in baculovirus by cloning the 2.85-kb BamHI-Sall fragment from the \( \lambda \) clone Teg2 (12) into the shuttle vector pVL1939 (18). The BamHI site is at position 18 in the TSA sequence, whereas the SalI site is at nucleotides 3' of the translation stop signal. Insect cells were cotransfected with the recombinant shuttle vector and wild-type viral DNA. Cells infected with recombinant virus were identified by microscopic examination and purified by three rounds of plaque assays. Purified recombinant viruses were identified by Western blot analysis of media from infected cells with use of the anti-1.11 rabbit polyclonal antiserum (see below). Recombinant TSA-1 (rbTSA-1) protein was found in the media of infected cells 48 h postinfection. The level of protein increased until day 6; however, after day 4 the media began to contain increasing levels of intracellular insect cell proteins as a result of cell lysis. Therefore, media were harvested three days postinfection, diluted threefold with sterile deionized water, and passed over a 5-ml Mono-Q column (Pharmacia LKB, Piscataway, NJ) that was equilibrated with loading buffer (50 mM NaCl, 20 mM Tris, pH 7.5) at 4°C. The column was washed with 30 ml of 150 mM NaCl, 20 mM Tris, (pH 7.5), and the protein was eluted with 15 ml of 350 mM NaCl, 20 mM Tris (pH 7.5). The rbTSA-1 protein was further purified by preparative electrophoresis by use of a 7.5% SDS polyacrylamide gel in a Bio-Rad Prep Cell (Bio-Rad, Richmond, CA). Fractions containing rbvTSA-1 were identified by analytical SDS-PAGE, Western blotting, and detection with use of the anti-1.11 polyclonal antiserum. Fractions containing rbvTSA-1 protein were pooled, concentrated by using Centricon-10 concentrators (Amicon, Beverly, MA), and stored at -70°C.

**Bacterial protein isolation**

The TSA-1 gene was expressed as a fusion protein in *Escherichia coli* (recTSA-1) with the first aa of the TSA-1 portion being the valine residue at position 29, which is the predicted processing site of the signal sequence. The fusion protein was isolated by passage of a cell lysate over a ProBond resin column (Invitrogen), followed by further purification by using preparative SDS-PAGE, as described above. The NH\(_2\)-proximal region of TSA-1 in pTH1–8 also was expressed in *E. coli* and purified by the procedure described above for recTSA-1.

Production and purification of the 1.11 and 4.15 fusion proteins was as described elsewhere (20), with some modifications. *E. coli* strain DH5-\( \alpha \)-transformed with the recombinant plasmid was grown overnight at 37°C in Luria broth containing ampicillin. The overnight culture was diluted 1:10 in Luria broth and incubated for 1 h at 37°C. Protein expression was induced with isopropylthio-\( \beta \)-galactoside at a final concentration of 0.1 mM, and the culture was incubated for an additional 4 h. Induced cells were pelleted by centrifugation, the pellet was resuspended in PBS + 1% Triton X-100, and the cells were lysed by sonication. The 1.11 fusion protein was released as a soluble protein after sonication. After centrifugation to remove cellular debris, the supernatant was passed over glutathione-agarose beads (Sigma Chemical Co., St. Louis, MO). Absorbed fusion protein was eluted with 5 mM reduced glutathione/50 mM Tris, pH 8.0. The 1.11 fusion protein was further purified by preparative electrophoresis (see above). Protein used in the in vitro proliferation assays was passed over an Exscripti-Gel D column (Pierce, Rockford, IL) to remove SDS.

The procedure used to isolate the 4.15 fusion protein was similar to that described above, except that the 4.15 fusion protein was insoluble after sonication in PBS + 1% Triton X-100. The cellular lysate that contained insoluble 4.15 fusion protein was extracted with 2 M urea and further purified by preparative electrophoresis.

**Polyclonal antiserum**

Polyclonal antiserum against rbvTSA-1, 4.15, 1.11, and glutathione-S-transferase proteins was prepared by immunizing 6-mo-old female New Zealand white rabbits with 100 \( \mu \)g protein emulsified in CFA. Rabbits were boosted at 3-wk intervals with 100 \( \mu \)g Ag emulsified in IFA. Female BALB/cByJ mice were used for immunization with the above proteins and T cell proliferation experiments. Groups of 6- to 8-wk-old mice were injected i.p. with 40 \( \mu \)g protein emulsified in CFA. Mice were boosted three times at 2 wk intervals with 20 \( \mu \)g Ag emulsified in IFA.

**Parasites**

The Peru strain of *T. cruzi* was obtained from Dr. Stuart Krassner's laboratory (University of California, Irvine, CA). The characteristics of this strain have been described (21), and the parasite is maintained by passage in female BALB/cByJ mice.

Mice immunized with the baculovirus or *E. coli* produced TSA-1 proteins were challenged by s.c. infection with 10\(^7\) bloodstream trypomastigotes (22).

**ELISA**

Recombinant TSA-1 protein was added to Corning ELISA plates (Corning Glass Works, Corning, NY) at a concentration of 20 \( \mu \)g/ml in PBS, pH 7.5, and incubated overnight at room temperature. Wells were blocked with blocking buffer (PBS containing 0.25% BSA and 0.05% Tween-20). Abs were diluted in blocking buffer and incubated for 4 h at room temperature. After being washed with deionized water, bound Ab was detected with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse (Sigma Chemical Co.). Wells were incubated at room temperature for 2 h, washed, and reacted with 3 mM p-nitrophenol. Plates that contained the substrate were incubated for 1 h in the dark and read at 405 nm by using an automated ELISA plate reader.

To determine whether Abs from immunized mice reacted with rbvTSA after reduction with DTT, ELISAs were performed with use of rbvTSA-1 protein before and after reduction and alkylation. Reduced and alkylated rbvTSA-1 protein was prepared by using the method of Reynolds et al. (23).
IMMUNODOMINANT EPITOPES IN T. cruzi TSA-1 PROTEIN

FIGURE 1. Maps of rbvTSA-1, recTSA-1, 4.15, and 1.11 constructs. Maps of the four constructs used in this study are shown with numbers that correspond to the published nucleotide sequence of TSA-1 (12). Also shown are relevant restriction enzyme sites, the position of TGA stop codons, putative poly(A) addition sites (AATAAA), and the TSA-1 nonapeptide repeat region (dotted lines). Both 4.15 and 1.11 are fusion proteins with glutathione-S-transferase (GST). RecTSA-1 is expressed in the pTrcHis bacterial expression system as a fusion protein with the histidine-containing domain (HIS).

Immunoblotting

The recombinant TSA-1 proteins were tested for immunoreactivity by using previously described procedures (24). Briefly, proteins were separated by one-dimensional PAGE and transferred to nitrocellulose using a transblot cell (Bio-Rad) overnight at 150 V. Blots were probed with polyclonal sera and detected using the ECL Western blotting detection system (Amersham, Arlington Heights, IL).

T cell proliferation assays

Mice were immunized with proteins as described above. Fourteen days after the last injection, spleens were removed and single-cell suspensions were prepared in DMEM supplemented with 25 mM HEPES buffer, 1 mM sodium pyruvate, 5 × 10^{-3} M 2-ME, 50 U/ml penicillin, and 50 μg streptomycin sulfate. Cell suspensions were enriched for T cells by passage over nylon wool columns (25). Accessory cells for Ag presentation were prepared by irradiation (3000 rad of 37Cs) of syngeneic spleen cells. T cell-enriched preparations were cultured at 2 × 10^5 cells/well in 96-well plates with 4 × 10^5 irradiated feeder cells/well in DMEM, supplemented as described above, plus 10% FCS. Cells were cultured with and without Ag for 3 days at 37°C in an atmosphere of 5% CO₂. After 3 days, 1 μCi [3H]Tdr was added to each well and incubated overnight. Cells were harvested onto filters using a cell harvester (Skatron Instruments, Inc., Sterling, VA), dried, and counted in a liquid scintillation counter.

Results

Expression and isolation of TSA-1 protein and 4.15 and 1.11 fusion proteins

The full-length TSA-1 gene was cloned and expressed in the baculovirus expression system (Fig. 1). As shown in Figure 2, the expressed protein migrates as a broad band with an apparent m.w. of 110,000 to 120,000, similar to that observed for the native form of the protein (10). Direct aa sequence analysis shows that the amino-terminus is the valine residue at position 29 of the predicted sequence (12), which indicates that cleavage of the signal sequence likely has occurred. The predicted m.w. of TSA-1 protein after removal of the signal sequence is 87,485. The larger apparent m.w. of the expressed protein likely is caused by glycosylation, as evidenced by its ability to bind wheat germ agglutinin (data not shown).

The TSA-1 gene (aa 29 to 835) was expressed in E. coli as a fusion protein containing 31 aa of bacterial protein on the amino-terminus. The expressed fusion protein migrated with an apparent m.w. of 95,000 (see below), which is similar to the predicted m.w. of 90,852.

The TSA-1 sequence was divided into two regions, the 5' portion, which encodes the amino-proximal portion of TSA-1 (aa 78 to 619), was designated 4.15; and the 3' portion, which encodes the carboxyl-proximal portion of TSA-1 (aa 618 to 835), was designated 1.11 (Fig. 1). Both regions were expressed as fusion proteins with glutathione-S-transferase (GST). The expressed 4.15 protein migrated with an apparent m.w. of 65,000, which is approximately 15,000 less than the predicted m.w. This aberrant m.w. may be the result of clustering of proline residues within the TSA-1 portion of the fusion protein. The 1.11 protein migrates with an apparent m.w. of 43,000, which is slightly less than the expected m.w. of 49,000.
FIGURE 2. Purification of rbvTSA-1, 4.15, and 1.11 proteins. Coomassie blue-stained 10% SDS-PAGE of rbvTSA-1 (lane 1), 4.15 (lane 2), and 1.11 (lane 3).

Immunoreactivity of TSA-1

As shown in the Western blot in Figure 3B, antisera from T. cruzi-infected mice immunologically recognize the rbvTSA-1 protein, which indicates that the TSA-1 protein is accessible to the mouse immune system during a T. cruzi infection. The anti-T. cruzi Abs also react with the carboxyl-proximal 1.11 fusion protein; however, no reaction was observed with the anti-T. cruzi sera and the amino-proximal 4.15 protein. Therefore, it seems that, although the 4.15 protein represents approximately 66% of the total TSA-1 protein, the B cell response to the TSA-1 surface Ag during the course of a T. cruzi infection is directed primarily against the carboxyl-proximal portion of the molecule.

To investigate whether this restricted B cell response to the TSA-1 protein occurs in the absence of an active T. cruzi infection, immunologic responses generated in rabbits and mice immunized with rbvTSA-1 were determined. As shown in Figure 3A, sera from mice immunized with rbvTSA-1 reacted with both rbvTSA-1 protein and 1.11 protein on immunoblots. In contrast, the mouse anti-TSA-1 serum does not recognize the 4.15 protein. Identical results were obtained in rabbits immunized with rbvTSA-1 protein (data not shown). Thus, as seen in the T. cruzi-infected mice, the B cell response to TSA-1 is directed primarily to the carboxyl-proximal portion of the molecule, which suggests that the specificity of the response is an inherent property of the protein and is not dependent on events that occur during the course of a T. cruzi infection.

To determine whether B cell epitopes that are sensitive to reduction were overlooked by the Western blot assay used above, both rbvTSA-1 protein and rbvTSA-1 protein, which was reduced by DTT and subsequently alkylated, were tested for recognition. As shown in Table I, both reduced and nonreduced rbvTSA-1 proteins were recognized equally by sera from mice immunized with rbvTSA-1 protein and mice chronically infected with T. cruzi, thus indicating the absence of B cell epitopes that are dependent on the oxidation state of the protein.

As shown in Figure 3, C and D, immunization of mice with either the 4.15 or the 1.11 fusion protein generates Abs that recognize both the fusion protein used for immunization and rbvTSA-1 protein on immunoblots. Thus,
when mice are immunized with the amino-proximal regions of TSA-1, a B cell response is generated to both the amino-proximal fusion protein and TSA-1 protein. Identical results were obtained when mice were immunized with the amino-proximal region expressed in the pTrcHis vector, thereby indicating that production of Abs against 4.15 is not dependent on the glutathione-S-transferase portion of the fusion protein (data not shown).

Because TSA-1 produced by both the parasite and the baculovirus is glycosylated, whereas the 4.15 fusion protein produced in E. coli lacks glycosylation, it is theoretically possible that the production of Abs by immunization with 4.15 is the result of availability of new T or B cell epitopes that are masked by glycosylation in both the parasitic and baculoviral forms of the TSA-1 protein. To investigate this possibility, mice were immunized with TSA-1 produced in E. coli (Fig. 4C). As shown in Figure 4, Abs generated against the nonglycosylated form of TSA-1 recognized recTSA-1, rbvTSA-1, and 1.11, whereas no reaction was seen with the 4.15 protein. Thus, glycosylation of TSA-1 does not seem to be the basis for lack of Ab production against the amino-proximal region of the protein.

These results suggest that the 1.11 region of TSA-1 suppresses Ab production against epitopes present in the 4.15 region of TSA-1. To determine whether this phenomenon requires direct physical linkage of the two regions of the protein, mice were immunized with an approximately equimolar mixture of 4.15 and 1.11. As shown in Figure 5, immunization with a mixture of the two polypeptides generates a strong humoral immune response against both regions of the protein, implying that suppression of an Ab response against 4.15 necessitates physical linkage of the two regions of the TSA-1 protein.

**T cell proliferative responses to TSA-1**

To investigate the responses of T cells in mice immunized with rbvTSA-1 protein, splenic T cells were isolated by passage over nylon wool, and the proliferative responses of nylon wool nonadherent cells to rbvTSA-1, 4.15, and 1.11 proteins were tested in vitro. As shown in Figure 6, splenic T cells from rbvTSA-1-immunized mice proliferate in response to rbvTSA-1 and 4.15. In addition, there is a slight proliferative response of the splenic T cells to 1.11 fusion protein. Therefore, although the Ab responses of TSA-1-immunized mice are directed primarily at the carboxyl-proximal portion of the protein, T cells from rbvTSA-1-immunized mice develop strong proliferative responses to the amino-proximal portion of TSA-1. In this regard, it is important to note that the rbvTSA-1 protein and the 4.15 protein were produced in eukaryotic and prokaryotic expression systems, respectively, which minimizes the possibility that the T cell response from TSA-1-immunized mice to the 4.15 protein might be a result of common host cell contaminants in the two protein preparations. Splenic T cells from control unimmunized mice were also tested and showed no proliferative response (data not shown).
portion of TSA-1 does induce responses that provide significant survival in mice challenged with an otherwise lethal inoculum of trypomastigotes. However, immunization with the 4.15 amino-proximal region of the TSA-1 protein does not induce immune responses needed to survive challenge with an otherwise lethal inoculum of T. cruzi trypomastigotes. Mice that received no immunogen or only Freund’s adjuvant developed fatal infections. Thus, it seems that immunization with the entire TSA-1 protein or the 1.11 carboxyl-proximal portion of TSA-1 does not stimulate an immune response that would damage the parasite. Interestingly, two other trypomastigote surface proteins, shed acute phase antigen (SAPA)/trans-sialidase and cruzipain, contain immunodominant epitopes that direct the host immune response to an enzymatically inactive region of the molecule (5). Although it is not known whether the immunodominant regions mask other epitopes that have the potential to generate host protective immune responses, as observed for the TSA-1 protein, the similarity between the immune response to these proteins and that observed to TSA-1 invites the speculation that strategic placement of immunodominant epitopes in surface proteins may represent one mechanism by which the parasite evades a lethal host immune response.

At present, we do not know the sequence of events that generates the immunodominant effect of the 1.11 region of TSA-1. However, the observation that immunization with TSA-1 generates Abs against 1.11, but not against 4.15, whereas immunization with a mixture of 1.11 and 4.15 generates Abs against both polypeptides, indicates that the dominance of the 1.11 sequence is functional only in cis. This finding implies that the 1.11 region may directly affect the ability of the adjacent 4.15 region to be recognized by B cells, possibly as a consequence of the secondary folding of the protein, because the NH2-proximal region is strongly hydrophobic, whereas the COOH-proximal region is extensively hydrophilic (12). Alternatively, the cis-dominance may result from the inability of B cells that recognize the 4.15 region to be expanded, possibly as a result of differential Th cell commitment, which is mediated by paratope involvement in the processing of TSA-1 by B cells that recognize either the NH2-proximal or COOH-proximal region of TSA-1 (26, 27).

These results suggest that the epitope(s) in the amino-proximal region of TSA-1, which does not stimulate an immune response during a natural T. cruzi infection, can provide significant protection provided that the host is first vaccinated with this region of the protein. Therefore, it seems that one function of the immunodominant epitope(s) in the carboxyl region of the TSA-1 protein may be to direct the immune response away from epitopes in distal regions of the protein, which otherwise could stimulate an immune response that would damage the parasite. Interestingly, two other trypomastigote surface proteins, shed acute phase antigen (SAPA)/trans-sialidase and cruzipain, contain immunodominant epitopes that direct the host immune response to an enzymatically inactive region of the molecule (5). Although it is not known whether the immunodominant regions mask other epitopes that have the potential to generate host protective immune responses, as observed for the TSA-1 protein, the similarity between the immune response to these proteins and that observed to TSA-1 invites the speculation that strategic placement of immunodominant epitopes in surface proteins may represent one mechanism by which the parasite evades a lethal host immune response.

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