Identification of a CD36-related Thrombospondin 1-binding Domain in HIV-1 Envelope Glycoprotein gp120: Relationship to HIV-1-specific Inhibitory Factors in Human Saliva

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Summary

Human and non–human primate salivas retard the infectivity of HIV-1 in vitro and in vivo. Because thrombospondin 1 (TSP1), a high molecular weight trimeric glycoprotein, is concentrated in saliva and can inhibit the infectivity of diverse pathogens in vitro, we sought to determine the role of TSP1 in suppression of HIV infectivity. Sequence analysis revealed a TSP1 recognition motif, previously defined for the CD36 gene family of cell adhesion receptors, in conserved regions flanking the disulfide-linked cysteine residues of the V3 loop of HIV envelope glycoprotein gp120, important for HIV binding to its high affinity cellular receptor CD4. Using solid-phase in vitro binding assays, we demonstrate direct binding of radiolabeled TSP1 to immobilized recombinant gp120. Based on peptide blocking experiments, the TSP1–gp120 interaction involves CSVTCG sequences in the type 1 properdin-like repeats of TSP1, the known binding site for CD36. TSP1 and fusion proteins derived from CD36-related TSP1-binding domains were able to compete with radiolabeled soluble CD4 binding to immobilized gp120. In parallel, purified TSP1 inhibited HIV-1 infection of peripheral blood mononuclear cells and transformed T and promonocytic cell lines. Levels of TSP1 required for both viral aggregation and direct blockade of HIV-1 infection were physiologic, and affinity depletion of salivary TSP1 abrogated >70% of the inhibitory effect of whole saliva on HIV infectivity. Characterization of TSP1–gp120 binding specificity suggests a mechanism for direct blockade of HIV infectivity that might be exploited to retard HIV transmission that occurs via mucosal routes.

Human immunodeficiency virus (HIV) can be cultured from most tissues and body fluids of infected individuals. Saliva represents a significant exception. In an early report, HIV-1 was isolated from only 1 of 71 saliva samples of HIV+ donors (1). Recent work confirmed the paucity of infectious virus in salivas (2), with a mean viral load in 25 samples of 162 genome equivalents/ml, at the limits of detection by reverse transcription PCR (3). Clinical support for the limited transmissibility of HIV by salivas includes: lack of infection following contamination of open wounds with saliva from HIV+ individuals (4); low occupational risk for HIV infection among dentists in practices with large numbers of patients at risk for HIV infection (5); and the inability to infect adult chimpanzees by direct application of HIV to intact oral mucosa (6).

Such retarded transmission is not a general characteristic of viruses which can be shed orally. The annual attack rate for hepatitis B virus among unvaccinated dentists is 2.6% (7), human T cell lymphotrophic virus type I is found in saliva (8), and the type D retrovirus etiologic in a simian immune-deficiency syndrome can be readily isolated from macaque saliva and spread by this fluid (9). The ability of saliva to suppress HIV-1 also is relatively specific. It does not alter the infectivity of Herpes simplex virus (10), and both cytomegalovirus and Epstein–Barr virus are readily shed in oral secretions of HIV seronegative (11) and seropositive persons (12, 13). Other body fluids from HIV+ individuals do contain HIV in relatively high titers, including tears (14), genital secretions (15), feces (16), and breast milk (17), and the latter three have been implicated in HIV transmission. Particulate and filterable oral secretions capable of inhibiting HIV infection represent potential explanations for the paucity of HIV in saliva. Reports from several different groups imply that two processes are involved (6, 10, 11, 18–25). Some studies found that whole saliva and submandibular secretions, but not parotid fluid, could sequester HIV virions...
(10, 11, 18, 19, 24, 25), whereas others identified soluble inhibitory factors capable of direct inhibition in secretions from all salivary glands, but only at very high concentrations (23, 25). Submandibular saliva contains sulfated polylaccharides of low (M G2) and high (M G1) molecular weights (26), with the latter forming an anionic charge barrier to binding of the high-affinity HIV receptor, CD4, to the HIV envelope glycoprotein gp120 (22). Secretory leukocyte protease inhibitor (SLPI), a 12-kD protein found in whole saliva, has an effect independent of HIV binding to CD4 (20), although its significance in vivo has been questioned (27).

Fibronectin, a matrix adhesion molecule, binds directly to gp120, but was shown to inhibit infectivity only at high concentrations (28).

We have focused on thrombospondin 1 (TSP1) as a mediator of HIV inhibition. TSP1 is a trimeric sulfated glycoprotein that belongs to a family of high molecular weight extracellular matrix molecules (for review see references 29 and 30). TSP1 is implicated in suppressing the infectivity of certain bacteria and protozoa, including Staphylococcus aureus, Babesia, toxoplasma, leishmania, and the malaria sporozoite (31, 32). Unlike other multifunctional glycoproteins, TSP1 is found in very low quantities in plasma, but is stablized during reversible binding to other matrix molecules, resulting in markedly elevated levels at certain cell surfaces (33). We recently established the presence of an evolutionarily conserved TSP1-binding domain, termed CLESH-1, functional in at least two members of the CD36 gene family, cell surface adhesion receptor CD36 (34) and lysosomal integral membrane protein II (LIMPII; reference 35). A homology search revealed CLESH-1-related sequences in HIV envelope glycoprotein gp120. Therefore, we were interested in whether a TSP-mediated direct interaction with these putative binding motifs could account for anti-HIV activity in saliva. In this study, we demonstrate the ability of purified TSP1 to block HIV-1 infection of primary and transformed target cell lines. These effects involve binding of TSP1 to C2 and C3 conserved regions adjoining the V3 loop of HIV-1 envelope glycoprotein gp120. These areas are key determinants for binding of gp120 to its high affinity cellular receptor, CD4 (36). The effect of TSP1 on CD4-gp120 complex formation suggests a potential role for TSP1 in the prevention and therapeutics of HIV-1 infection.

Materials and Methods

Protein Sequence Analysis and Homology Search. Deduced amino acid sequences encoded by exon 5 of CD36 or homolog LIMPII (Fig. 1) that contain previously characterized TSP1-binding motifs (35) were used as query for a BLAST-enhanced alignment utility search (BEAUTY; reference 37), which incorporates pattern-induced multiple alignment (PIMA; reference 38) sequence family clusters, conserved cluster domains, and PROSITE annotated libraries (39). HIV-1 consensus and subtype sequence alignments were retrieved from the Los Alamos Sequence Database.

Preparation of TSP1. Human platelet-rich plasma was obtained from the NY Blood Center (New York, NY). Human thrombin was from Boehringer Mannheim (Indianapolis, IN). Purified human calcium-replete TSP1 was prepared from release of thrombin-activated washed platelets as previously described (34, 40). Dot-blot analysis with mAbs to fibronectin (FN) and vitronectin (Calbiochem, La Jolla, CA) showed no reactivity. Endotoxin content was monitored by the Limulus amebocyte lysate test, and was <1 U/μg protein. Polyclonal rabbit anti-TSP antisera and murine anti-TSP1 and anti-TSP2 mAbs 11.4 and 45.2, respectively, were prepared and characterized as previously reported (41, 42).

Purified Fusion Proteins and Peptides. Characterization of glutathione-S-transferase (GST) fusion proteins derived from CD36 or CD36 homolog LIMPII are reported elsewhere (35), and are numbered to indicate encoded amino acids. CFP86–157 and LFP97–155 contain functionally homologous minimal TSP1-binding domains (CD36 aa93–120; reference 34), whereas CFP298–439 and LFP165–243 represent downstream sequences. A truncation mutant LFP75–78 was used as an additional GST1 control. Large-scale production and purification of soluble fusion protein followed the method of Frangioni and Nef (43). For some experiments, LIMPII peptide (L75–155) was cleaved from the GST moiety with coagulation Factor X (Boehringer Mannheim), and purified by size exclusion chromatography (Centricron 10; Amicon, Beverly, MA). TSP1 synthetic peptides were from Chiron M Immunotopes (Victoria, Australia). Recombinant baculovirus-expressed HIV envelope glycoprotein gp160 (gp120 noncovalently linked to transmembrane component gp41) was derived from HIV-1 isolate IIIB (IntraCel Corp., Cambridge, MA). The following purified recombinant proteins were provided by the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Bethesda, MD): baculovirus-expressed HIV-1 gp120 derived from LAV and MN isolates, CHO cell-expressed soluble CD4, and HIV-1lnm envelope synthetic peptides.

Solid-Phase Binding Assays. In vitro binding experiments were performed as previously described for TSP1 binding to CD36 and LIMPII (34, 35). In brief, TSP1 (5–10 μg/ml) or fusion proteins and peptides (10–20 μg/ml) were immobilized on detachable 96-well microtiter plate strips (Immuno-4 R Emov-a-well; Dynatech Laboratories, Inc., Chantilly, VA) by overnight incubation at 4°C in carbonate buffer (100 mM NaCO3/1 mM MgCl2/0.02% NaN3, pH 9.8). Washed wells were blocked with 0.5% BSA, and then incubated in triplicate with soluble radiolabeled ligand for 2.5 h at 37°C. After extensive washing in 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Tween 20 (TBS-Tween), bound radioactivity was quantified by gamma counter. Radiolabeling was performed with Na125I (Amersham Life Science Inc., Arlington Heights, IL) using immobilized chloramine T (Iodo-beads); Pierce Chemical Co., Rockford, IL; references 44, 45). Specific activity determined for each experiment ranged from 0.06–1.0 μCi/μg. Specific binding was determined as quenchable in the presence of excess unlabeled ligand, above background binding to BSA-coated wells.

Immunohistochemistry. Thin tissue sections of oral mucosa autopsy specimens were processed as previously described (46). Formalin-fixed paraffin-embedded sections were dewaxed, deparafin and permeabilized in Triton X-100. Endogenous peroxidase activity was blocked by treatment with a 3% solution of H2O2 for 30 min. Slides were preincubated with normal hu-
man serum for 1 h at 22°C, and then incubated overnight at 4°C with 1:1000 dilutions of either polyclonal rabbit anti-TSP1 and anti-TSP2, or preimmune rabbit serum. After a brief blocking step with normal goat serum, successive rinses in PBS were performed between incubations with a 1:250 dilution of biotinylated goat anti-rabbit IgG (Dako, Carpinteria, CA), followed by avidin-biotin-peroxidase complex (Dako). Peroxidase deposition was visualized with 3,3′-diaminobenzidine tetrachloride. Samples were rinsed in distilled water, counterstained with hematoxylin, and viewed by light microscopy.

Quantitative Immunodetection of TSP1. A sandwich ELISA was used to measure levels of TSP in saliva and cell culture supernatants. Polystyrene 96-well microtiter plates (Falcon, Oxnard, CA) were coated overnight at 4°C with 50 µl/well of 5 µg/ml anti-TSP1 mAb 45.2 in carbonate buffer. Plates were extensively washed with carbonate buffer and then blocked with 1% BSA in TBS-Tween. This was followed by incubating 50 µl of culture supernatants from each well was replaced with fresh medium. HIV infection was performed using HIV-1 isolate IIIB stock virus. The PBMC medium also contained 32 U/ml IL-2.
HIV-1 gp120 Binds Thrombospondin 1

We used solid-phase binding assays to assess direct binding of TSP1 to the HIV-1 envelope complex. Fig. 2A shows concentration-dependent, saturable binding of radiolabeled soluble TSP1 to immobilized recombinant gp160. An apparent affinity of \( z \leq 250 \) nM was comparable to that demonstrated for binding of TSP1 to purified platelet CD36. Binding was effectively quenched in the presence of 10-fold molar excess unlabeled soluble TSP1 (Fig. 2B, 94 ± 6% inhibition), demonstrating specificity. In addition, a LIMPII fusion protein LFP75–155 containing the TSP-binding domain partially blocked binding (47 ± 14% inhibition), whereas control fusion protein LFP156–243 representing downstream LIMPII sequences did not, supporting the existence of a functionally similar domain in HIV-1 env.

125I-gp120 Interacts with the CD36-binding Peptide of TSP1. CSVTCG peptides found in the type 1 repeats of TSP1 are binding sites for CD36 and LIMPII (35, 52–54). Therefore, we tested whether gp120 shared this same specificity. Saturation isotherms showed significant binding of radiolabeled gp120 (lav isolate) to immobilized CSVTCG peptide (Fig. 3), with an apparent affinity of 300 nM. The activity was sequence-specific, as demonstrated by inefficient binding to scrambled control peptide VGSCCT, or to an RGD-containing peptide similar to the GRGDA cell adhesion sequence of the last TSP1 type 3 calcium-binding repeat. This is further evidence that the TSP1-gp120 interaction is mediated through a CD36/LIMPII-related structural domain.

125I-TSP1 Binds to Recombinant HIV-1 env. We used solid-phase binding assays to assess direct binding of TSP1 to the HIV-1 envelope complex. Fig. 2A shows concentration-dependent, saturable binding of radiolabeled soluble TSP1 to immobilized recombinant gp160. An apparent affinity of \( \sim 250 \) nM was comparable to that demonstrated for binding of TSP1 to purified platelet CD36. Binding was effectively quenched in the presence of 10-fold molar excess unlabeled soluble TSP1 (Fig. 2B, 94 ± 6% inhibition), demonstrating specificity. In addition, a LIMPII fusion protein LFP75–155 containing the TSP-binding domain partially blocked binding (47 ± 14% inhibition), whereas control fusion protein LFP156–243 representing downstream LIMPII sequences did not, supporting the existence of a functionally similar domain in HIV-1 env.

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Peptides Define the TSP1-binding Site in gp120. Fig. 4A shows the location of a series of 20 aa synthetic peptides with respect to gp120 domain structure (mn isolate). Given the constraints of the solid phase assay, peptides were immobilized either singly or in pairs, and tested for an ability to bind radiolabeled TSP1. As shown in Fig. 4B, active peptides corresponded to regions of gp120 containing homologous CLESH-1 motifs. Peptide pairs that extended motif sequences showed augmented binding compared to either peptide alone. In addition, single peptides representing sequences outside strongly homologous split motif half-sites also showed significant TSP1 binding. Interestingly, a V3-containing peptide (No. 4, aa311–330) with TSP1-bind-
binding activity also displayed augmented binding in combination with an inactive C2 peptide within the first CLESH-1 motif (N.o. 3, aa291–310). These data suggest the presence of two competent TSP1-binding elements in the predicted regions of gp120, including V3 sequences in the first motif, with the potential for interchangeable combinations of site usage, and possible TSP-mediated structural alterations that might disrupt conformation-dependent binding of gp120 to CD4 receptor.

TSP1 Inhibits Binding of [125I]-CD4 to gp120. An obvious question is whether TSP1 could compromise the ability of gp120 to bind to CD4. Fig. 5 shows competitive inhibition of radiolabeled CD4-binding to gp120 derived from two different viral isolates (mm and lav). In the presence of 10-fold molar excess TSP1, complete inhibition of [125I]-CD4-binding to immobilized gp120 was seen. CSVTCG peptide showed a partial but significant inhibition of CD4-gp120 complex formation (53 ± 9%), confirming a TSP-specific effect, while the RGD-containing peptide had little effect (4 ± 4%), and the scrambled control actually enhanced binding. Consistent with structural homology data, TSP1-binding CD36- and LIMPII-derived fusion proteins proved strong competitors (both ~89% inhibition), whereas downstream LIMPII control protein had a minimal effect (32 ± 5%). For comparison, a 1:2 dilution of whole saliva was a potent inhibitor in this assay system. These observations support a role for salivary TSP1 as a direct inhibitor of HIV infectivity. A analysis of TSP1-mediated Inhibition of HIV-1 Infectivity

Quantitation and Localization of Salivary TSP1. As shown in Table 1, levels of TSP1 in whole saliva from either HIV+ or HIV− donors were at least 10-fold greater (1–12 μg/ml) compared to plasma. Amounts of TSP1 in parotid saliva fractions were equivalent to that of plasma, with the bulk of TSP1 found in the submandibular secretions. These values are consistent with previous reports of anti-HIV activity predominantly in submandibular and not parotid gland fluids (11, 19) although only a single saliva fractionation was performed. To document that elevated levels of TSP1 in saliva may be secondary to local production, rather than leakage and concentration from plasma, we performed immunohistochemical staining of fixed tissue from oral mucosa. Moderate to intense levels of TSP1-directed immunoreactivity were evident on gingival epithelium (Fig. 6), confirming that cell-associated TSP1 may correspond to significantly high local concentrations in the oral cavity. Inhibition of HIV-1 Infectivity by Purified TSP1. To investigate whether purified TSP1 could inhibit HIV-1 infection similarly to saliva, viral isolate III B was added to target cells after preincubation with TSP1. Many studies have shown that filtration of virus–saliva mixtures is required for maximum inhibition (10, 11, 18, 24). The physiologic equivalent of such filtration is thought to be the constant cleansing of the oral cavity by salivary flow. Therefore, in some experiments preincubated virus–TSP1 mixtures were passed through 0.2-μm filters. Different multiplicities of infectious virus per target cell were tested in both systems. At concentrations found in saliva (2–10 μg/ml), TSP1 reduced HIV-1 infection of PHA-activated donor PBMCs by >83% when prefiltered, as measured by ELISA detection of p24 viral antigen (Fig. 7). This was comparable to a 1:2 dilution of whole saliva. In contrast, fibrinogen, another high molecular weight adhesive glycoprotein in saliva, had no effect in this system. Specificity was documented by...
abrogation of the inhibitory effect in the presence of a specific anti-TSP1 polyclonal antibody (107% of control p24), but not control IgG (data not shown). Prolonged incubation of the TSP–virion mixture was unnecessary, as exposures as brief as 5 min appeared sufficient to reduce infectivity by \( \geq 50\% \) at 1 \( \mu \text{g/ml} \) TSP1 (data not shown). The TSP1 effect also was apparent for HIV-1 IIIB infection of CD4\(^+\) T-lymphoblastoid and monocytoid cell lines (SK23 > 90.7% and U937 > 83.0% inhibition, respectively). In addition, two monocytotropic strains of HIV-1, p13 and HA593, representing patient isolates obtained from the NIH AIDS Retroviral Repository (Bethesda, MD), were susceptible to inhibition by purified TSP1. At viral moi of 0.8, 100 \( \mu \text{g/ml} \) TSP1 inhibited HIV infectivity by \( >98\% \) (data not shown). In contrast to prefiltration experiments, direct addition of TSP1 was able to inhibit HIV-1 by 50–75% only when a high concentration of TSP1 and a low moi of inoculum was used (Fig. 7). In parallel assays, saliva could inhibit HIV-1 as a direct addition only when added in dilutions of <1:4. However, preincubation of virus with saliva permitted dilutions >1:10. The results again are consistent with levels of TSP1 found in these dilutions of saliva.

Depletion of Salivary TSP1 Abrogates the Anti-HIV Effect. To determine the extent of TSP1 contribution to saliva inhibition of HIV infectivity, clarified saliva samples were passed over affinity columns of immobilized TSP-binding domain of CD36 (CFP, aa 67–157) or LIMPII (aa 75–155), downstream fusion protein LFP156–243, or an equal volume of saliva (final twofold dilution). Samples were incubated and bound TSP1 was measured as in Fig. 2. Shown is a single data set of triplicate samples (n = 3, error as SD).

| Sample Source | HIV Status | TSP1.2 \( \mu \text{g/ml} \) (range) | n |
|---------------|------------|--------------------------------|---|
| Plasma        | negative   | 0.25 (0.1–0.34)                 | 8 |
| Whole saliva  | negative   | 4.1 (1.1–12.8)                  | 6 |
| Parotid       | negative   | 0.1                              | 2 |
| Submandibular| negative   | 2.5                              | 1 |

TSP-binding Peptide Suppresses TSP1 Anti-HIV Activity. To further delineate a TSP-specific effect, LIMPII TSP-binding peptide L75–155 (10-kD product purified after removal of GST moiety by proteolytic cleavage) was included in HIV–TSP1 preincubation mixtures as a competitor. Fig. 8 shows that 1 \( \mu \text{M} \) LIMPII peptide abrogated the inhibitory effect of even high concentrations of TSP1 (50–100 \( \mu \text{g/ml} \)) by 83–90%. Incubation of virus in the presence of a specific anti-TSP1 polyclonal antibody (107% of control p24), but not control IgG (data not shown). Prolonged incubation of the TSP–virion mixture was unnecessary, as exposures as brief as 5 min appeared sufficient to reduce infectivity by \( \geq 50\% \) at 1 \( \mu \text{g/ml} \) TSP1 (data not shown). The TSP1 effect also was apparent for HIV-1 IIIB infection of CD4\(^+\) T-lymphoblastoid and monocytoid cell lines (SK23 > 90.7% and U937 > 83.0% inhibition, respectively). In addition, two monocytotropic strains of HIV-1, p13 and HA593, representing patient isolates obtained from the NIH AIDS Retroviral Repository (Bethesda, MD), were susceptible to inhibition by purified TSP1. At viral moi of 0.8, 100 \( \mu \text{g/ml} \) TSP1 inhibited HIV infectivity by \( >98\% \) (data not shown). In contrast to prefiltration experiments, direct addition of TSP1 was able to inhibit HIV-1 by 50–75% only when a high concentration of TSP1 and a low moi of inoculum was used (Fig. 7). In parallel assays, saliva could inhibit HIV-1 as a direct addition only when added in dilutions of <1:4. However, preincubation of virus with saliva permitted dilutions >1:10. The results again are consistent with levels of TSP1 found in these dilutions of saliva.

Depletion of Salivary TSP1 Abrogates the Anti-HIV Effect. To determine the extent of TSP1 contribution to saliva inhibition of HIV infectivity, clarified saliva samples were passed over affinity columns of immobilized TSP-binding LIMPII fusion protein LFP75–155 before virus preincubation. Adsorption removed \(~95\%\) of TSP (Table 2), as assessed by sandwich ELISA, whereas the decrease in total protein was substantially less (\(~15\%\)). TSP depletion correlated with \( >70\% \) reduction in anti-viral activity, in contrast to saliva adsorbed using a control fusion protein affinity column (GST-1). The data suggest that TSP may account for a major proportion of HIV-specific inhibitory activity in saliva.

**Figure 5.** Competitive inhibition of \(^{125}I\)-CD4 binding to gp120. A fixed concentration of \(^{125}I\)-labeled soluble recombinant CD4 (50 nM) was added to immobilized rgp160 (solid bars, MN; hatched bars, LAV isolate-derived) in the absence or presence of 10-fold molar excess (0.5 \( \mu \text{M} \)) of unlabeled TSP1, TSP1-derived peptides CSVTCG or GRGDS, scrambled control peptide VGSCCT, fusion proteins containing the TSP1-binding domain of CD36 (CFP, aa 67–157) or LIMPII (aa 75–155), downstream fusion protein LFP156–243, or an equal volume of saliva (final twofold dilution). Samples were incubated and bound TSP1 was measured as in Fig. 2. Shown is a single data set of triplicate samples (n = 3, error as SD).

**Figure 6.** Immunohistochemical detection of cell-associated TSP1 in human gingival mucosa. Fixed oral epithelial tissue thin section was incubated with polyclonal antiserum reactive against both TSP1 and 2 (lower panel), or with preimmune serum (upper panel), followed by biotinylated second antibody, and developed using avidin-conjugated peroxidase. Brown deposits indicate sites of thrombospondin reactivity. Original magnification, \( \times 200 \).
ence of LIMPII peptide alone resulted in minimal decrease of HIV-1 infectivity (~9%), suggesting that the amounts of peptide able to block the TSP1 antiviral effect were not sufficient to compete for HIV-1 envelope binding sites on PBMC target cells. The ability of the LIMPII peptide to restore infectivity supports a direct role for a CD36/LIMPII-related TSP1-binding domain on HIV-1 gp120, and provides further evidence of a common binding site on TSP1.

Effect of TSP1 on CD4 Expression. Another mechanism to explain TSP-mediated blockade to HIV infection would be a direct effect on target cells, whereby alterations in cell function would decrease the capacity to support productive infection. To address whether exogenous purified TSP1 induced downmodulation of the high affinity HIV receptor, we monitored CD4+ Jurkat and SK23 T cell lines, as well as PHA-activated PBMCs, for differences in CD4 surface expression after culture for 3 d in the absence or presence of 100 μg/ml TSP1. By flow cytometric analysis of cells stained with fluorescein-conjugated anti-CD4 IgG, no change in relative fluorescence intensity or percentage of CD4+ cells was detected (data not shown). Thus, TSP1 likely does not reduce cell susceptibility to HIV-1 infection.

Effect of Acute and Chronic HIV-1 infection on Production of TSP1. To examine whether HIV-1 infection modulates TSP1 expression, TSP1 secretion was monitored in three groups of cells: an uninfected line of U937 promonocytic cells; U1.1 cells representing chronically infected U937 containing two stably integrated copies of HIV-1/LAI; and U937 acutely infected with HIV-1 to high copy number (1000 proviral copies/cell; reference 49). Cells were pre-exposed to buffer or PMA for 1 h at 37°C, cultured for 24 h in RPMI 1640 with 5% FCS, and then cultured for 18 h in serum-free RPMI 1640 with 0.25% BSA. TSP levels were measured by sandwich ELISA. LAI and IIIB indicate HIV-1 isolate subtypes.

Table 2. Effect of TSP1 Depletion on Salivary Inhibition of HIV-1

| Saliva Adsorption | Protein | TSP1,2 | % Inhibition |
|-------------------|---------|--------|-------------|
| None (preadsorbed) | 8.16    | 1.20   | 99.8        |
| TSP-affinity      | 6.20 (76.0%) | 0.08 (6.3%) | 31.1        |
| (LFP75–155)       |         |        |             |
| GST control       | 7.52 (92.2%) | 1.18 (98.3%) | 99.7        |

HIV-Iib (0.15 moi) was admixed with clarified whole saliva that was untreated or first adsorbed with immobilized fusion proteins for 1 h at 37°C (final 1:4 dilute). Preincubated and filtered virus-saliva mixtures then were used to infect PHA-activated PBMCs. TSP-1 activity was determined on day 7 after inoculation as in Fig. 7. Percentages in parenthesis are relative to preadsorbed concentrations. Anti-HIV-1 inhibitory effect is expressed as percentage of decrease of maximum HIV-1 p24 antigen in the absence of saliva.

Table 3. Production of TSP by Monocytoid Cells in the Presence or Absence of HIV-1 Infection

| Cell line | Infection status | PM A | TSP,2 |
|-----------|------------------|------|-------|
| U 937     | Parental, uninfected | 5 ng/ml | <5 nm/ml |
|           | promonocytic line |       | 51     |
| U 1.1     | U 937, chronic    |       | 50     |
|           | 2 stable copies LAI | +      | 125    |
| U 937/HIV | acute, high copy  |       | 180    |
|           | IIIB (0.02 moi)   | +      | 135    |

Cells were exposed to buffer or PM A for 1 h at 37°C, cultured for 24 h in RPMI 1640 with 5% FCS, and then cultured for 18 h in serum-free RPMI 1640 with 0.25% BSA. TSP levels were measured by sandwich ELISA. LAI and IIIB indicate HIV-1 isolate subtypes.
incubated for 1 h in the presence or absence of PMA, a mitogenic inducer of HIV from chronically infected cells (49), which has also been shown to stimulate TSP1 expression in cell lines (55). After 24 h in culture medium, cells were switched to serum-free medium for an additional 18 h and then supernatants were collected for TSP1 quantitation by ELISA. As shown in Table 3, HIV-1 infection did not diminish TSP1 production, although acute infection blunted the response to PMA induction of TSP1.

**Discussion**

We report the identification of TSP1-binding sites in the C2 and C3 regions of gp120, conserved areas of the HIV envelope that are important in binding to CD4, and demonstrate direct interaction with a specific cell adhesion sequence found in the type I repeat of TSP1. Characterization by in vitro binding and competition studies substantiates that these C D 3 6 / L I M P I I - r e l a t e d C L E S H - 1 m o t i f s in gp120 represent authentic TSP1-binding domains. The physiological significance of TSP1–gp120 complex formation is supported by observations that salivary inhibition of HIV-1 infectivity was markedly reduced by affinity depletion of TSP1; saliva samples that block infection after filtration contained levels of TSP1 correlating with inhibitory concentrations of purified TSP1; and higher amounts of TSP1 required to block HIV-1 infectivity in vitro are comparable to the greater quantities of saliva required to obtain an antiviral effect (19, 23, 25). Our findings establish a distinct mechanism to explain HIV-specific blockade of transmission via saliva.

The likelihood that HIV inhibitors in saliva identified in vitro are active in vivo is bolstered by two lines of evidence. First, in a study of 48 HIV+ patients, 88% of PBMC samples, but no saliva samples, were positive for replication-competent HIV− (56). Second, recovery rates for HIV in salivas do not differ before and after dental procedures accompanied by bleeding into the oral cavity (57), indicating that free virus from blood was removed or inactivated. The fact that HIV is not found within salivary acinar and ductal elements (58) implies that although virions and infected cells may traffic into the salivary glands, they cannot establish a productive infection.

TSP1 is synthesized in low amounts by monocytes and macrophages, epithelial cells, fibroblasts, smooth muscle cells, pneumocytes, and endothelial cells, and in larger quantities by platelets (30). However, HIV may be exposed to levels of TSP1 over two log higher on surfaces in the oral cavity. The fluid distribution of TSP1, with very low concentrations in plasma, sweat, tears, and urine, reflects the relative frequency with which HIV can be isolated from these secretions, but not from saliva. Breast secretions present another issue. HIV can be cultured from some samples of breast milk, which has been implicated in HIV transmission. Colostrum often contains high concentrations of TSP1 (>145 μg/ml), whereas lower, more variable amounts (to <1 μg/ml) have been measured in other breast secretions (59). However, HIV has not been recovered from breast milk devoid of cells (60), and breast milk contains factors that inhibit HIV infection (17), one of which may be TSP.

The concept that an extracellular matrix molecule may serve as an inhibitor of microbial pathogens is not new. For example, FN binds free gp120 (28), and might thereby sequester HIV virions. The compartmentalization of FN in gingival crevicular fluid and whole and submandibular saliva, but not parotid fluid (61), parallels our findings with TSP. However, concentrations required to inhibit HIV infectivity in vitro exceed those found in saliva by 10-fold. Although high levels of FN are present in plasma (~300 μg/ml; reference 62), recoverable infectious HIV is present as well. FN actually may facilitate HIV-mediated syncytium formation (63), and promote the growth of AIDS-Kaposi sarcoma-related cells constitutively expressing high levels of FN receptor (64), bringing further into question the physiological significance of FN–gp120 binding. In contrast, TSP1 proteolytic fragments and peptides show opposite effects, inhibiting Kaposi sarcoma and endothelial cell proliferation (33, 65).

Defining the relationship between salivary inhibitors of HIV in vivo versus in vitro model systems is important. Concentrations of TSP1 required to inhibit HIV infectivity by >50% after direct addition were equivalent to those found in dilutions of saliva used in HIV inhibition experiments by other investigators (10, 11, 18, 24). Much lower doses of TSP1 were required to abrogate HIV infectivity when HIV–TSP mixtures were prefiltered, suggesting aggregation of virion–TSP1 complexes as a potential mechanism of inhibition. The requirement for prefiltration may have its in vivo counterpart in the continued cleansing of oral surfaces by salivary flow, with elimination of enmeshed viral particles from potential attachment sites. Experiments testing direct inhibition by whole saliva are complicated by the fact that additional salivary components may contribute to the anti-HIV effect. Indeed, TSP1 affinity depletion removed only ~70% of the HIV-inhibitory activity. In addition, nonspecific antiviral phenomena may occur with salivary dilutions of 1:1 to 1:4 (3). For example, highly charged sulfated polysaccharides such as dextran sulfate and salivary mucins present nonspecific anionic charge barriers to CD4-gp120 interactions at high concentrations (22, 66).

The presence of properdin-related sequences and properdin-binding activity described for gp120 and gp41 (67) support the functional significance of our sequence search results. The CSVTCG properdin motif is found in two of the three type 1 properdin/malaria-like repeats of TSP1. Consistent with homologies detected between C D 3 6 / L I M P I I TSP1-binding domains and conserved sequences surrounding the V3 region of gp120, in vitro binding and peptide inhibition data indicate a highly specific gp120 interaction mediated through this C L E S H - 1 site. We initially envisioned a high affinity TSP1-binding site on gp120 traversing the cysteine bridge of the V3 loop. However, envelope peptide mapping data suggested the presence of two full-length functional sites, with potential for direct involvement of the V3 loop, which presents a more complex
model in which multiple or sequential site use is possible. Discontinuity created by intervening residues between the first and second motif half-sites could induce conformational strain to distort or physically disrupt V3 loop integrity, with profound negative effects on gp120–CD4 association. Binding of TSP to the first highly homologous half-site in the C2 domain might lead to subsequent binding to weakly homologous downstream residues extending into the V3 loop, freeing the second full-length TSP-binding site in the C3 domain. The second motif encompasses a serine pair (KQSS) shown to be critical for CD4 binding (68). Mechanistically, TSP1 could compete directly with CD4, occupying an identical or overlapping site. Alternatively, the bulky TSP1 trimer may sterically block access to CD4, occupying an identical or overlapping site. This model could explain why only partial inhibition is seen using CSVTCG peptides. Elucidation of the molecular basis for TSP1 interference of CD4–gp120 association awaits additional structural studies.

Anti-HIV activities vary quantitatively among individual saliva donors. There also are differences in whether filtration is required to detect an inhibitory effect (69). However, in general titers of salivary inhibitory factors decline with disease progression, in parallel with decreased total protein concentrations (70). There is a greater chance of HIV recovery from saliva with advancing clinical stage, although the rate is still low in comparison with other body fluids (57). This raises the possibility that HIV may directly suppress production of saliva inhibitory factors, or elicit blocking molecules. TSP1 production is downregulated by DNA viruses (71), and production of FN is depressed by retroviral infection (72). However, production of TSP1 by PMN-activated monocytes was not affected by HIV infection in our system, and acute or chronic HIV infection actually upregulated TSP1 production by these cells, although to levels lower than those that have been shown to affect HIV infectivity (≤2 ng/ml). Regardless of normal levels of TSP1 production in the oral cavity, mechanical alterations also may contribute to decreased saliva inhibitory activity in vivo, as decreased salivary flow rates and buffering capacity correlate with advanced HIV disease (73).

The location of TSP1-binding motifs in highly conserved HIV domains makes these sites attractive targets for blocking agents that would be broadly reactive to HIV-1 subtypes. The ability of TSP1 to block CD4–gp120 complex formation suggests the potential use of this matrix molecule in the development of nontoxic natural inhibitors of local transmission of HIV-1, perhaps as a candidate topical adjuvant that could serve as a preventive physical barrier for recto-genital and gastrointestinal tract mucosa.

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