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Secretory leukocyte protease inhibitor: inhibition of human immunodeficiency virus-1 infection of monocytic THP-1 cells by a newly cloned protein

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Abstract

The ability of the salivary protein, secretory leukocyte protease inhibitor (SLPI), to inhibit human immunodeficiency virus-1 (HIV-1) infection *in vitro* has been reported previously and has led to the suggestion that SLPI may be partially responsible for the low oral transmission rate of HIV-1. However, results contradictory to these findings have also been published. These discrepancies can be attributed to a number of factors ranging from the variability of macrophage susceptibility to HIV infection to the quality of commercially available preparations of SLPI. To resolve these differences and to study further the potential anti-HIV-1 activity of SLPI, the purified and re-folded protein, expressed from a synthetic gene, was examined using human monocytic THP-1 cells. This newly cloned SLPI reduced HIV-$1_{Ba\text{-}L}$ infection in differentiated THP-1 cells, in contrast to the results observed

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when using commercially available preparations of SLPI. Interestingly, while the two proteins displayed different anti-HIV effects they had comparable anti-protease activity. The identification of the THP-1 cell line as a system that supports HIV replication, which can be inhibited by a preparation of SLPI now available in large quantities, sets the stage for a thorough investigation of the molecular and structural basis for the anti-HIV activity of SLPI.

Keywords: Secretory leukocyte protease inhibitor; Human immunodeficiency virus-1; THP-1 cell line; Synthetic gene

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Secretory leukocyte protease inhibitor (SLPI)[1] is a potent 11.7 kD serine protease inhibitor found in mucosal secretions, including bronchial and cervical mucus [1], seminal plasma [2], and secretions of the parotid and submandibular salivary glands [3,4]. SLPI inhibits two neutrophil serine proteases (neutrophil elastase and cathepsin G), the pancreatic proteases (α-chymotrypsin and trypsin) [5], as well as human mast cell chymase [6] and tryptase Clara [7]. Its presence in various mucosal secretions suggests that its primary function is to protect mucosal tissues from proteolytic degradation by neutrophil proteases released in response to inflammatory stimuli [8,9]. SLPI may also play a role in the host defense against certain bacterial and fungal infections [10,11], as well as the pneumotropic Sendai and influenza A viruses [7].

The presence of SLPI in salivary secretions has raised the possibility that it might be one of the components responsible for the anti-human immunodeficiency virus type-1 (HIV-1) activity of whole saliva and the low oral transmission rate of acquired immune deficiency syndrome (AIDS) [12–15]. However, the ability of SLPI to block HIV infection remains controversial. Two laboratories have reported that recombinant SLPI (rSLPI) inhibits HIV-1 infection of macrophages and primary T-cells [13–16,18,19], while a third group found that SLPI had no anti-HIV effect in human T-cell lines, peripheral blood lymphocytes, and primary macrophages [17].[2] We have also observed significant variability in the ability of rSLPI to inhibit HIV-1 infection

[1] *Abbreviations used:* BSA, bovine serum albumin; Cat G, human neutrophil cathepsin G; Chy, chymotrypsin; CCR5, CC chemokine receptor 5; ELISA, Enzyme-linked immunosorbent assay; ESI-MS, electrospray ionization mass spectrometry; HIV-1, human immunodeficiency virus-1; HPLC, high-pressure liquid chromatography; HS, heparan sulfate; IPTG, isopropyl-β-D-thiogalactoside; Kn, kanamycin; LB, Luria–Bertani medium; MIP, macrophage inflammatory protein; NE, human sputum elastase; PCR, polymerase chain reaction; PES, polyether sulfone; RANTES, regulated upon activation, normal T-cell-expressed, and presumably secreted; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SLPI, secretory leukocyte protease inhibitor; SP, sulfopropyl; $TCID_{50}$, tissue culture infectious dose.

[2] All of these studies have been performed using the same, commercially available, preparation of SLPI obtained from either Synergen and/or R&D Systems.

in macrophages [20]. The observed discrepancies may be due to the fact that the susceptibility of macrophages to HIV-1 infection is influenced by isolation, culture and infection conditions, donor variations, and maturation of macrophages at the time of infection [21].

Since SLPI is thought to inhibit a step beyond virus binding, but before reverse transcription [16,18], it is likely that it interacts with a cell surface molecule(s) involved in virus entry and whose expression is variable depending on the experimental conditions. It has been suggested that the anti-HIV effect of rSLPI does not depend on its anti-protease activity, but rather the interaction of SLPI with cell surface molecules other than CD4 [13,16,18,19]. Although CD4 serves as the primary receptor for HIV-1, other cofactor(s) are essential for virus-cell membrane fusion required for virus entry [22,23]. We found no correlation between the binding of rSLPI to human T-cells and the expression of the cell surface proteins CD4, CD26, and CCR5 [24]. It has been reported recently that the cell surface glycosaminoglycan, heparan sulfate (HS), may participate in HIV-cell attachment and virus entry [25–27]. The cationic SLPI forms a tight complex with the negatively charged polysulfated glycosaminoglycan, heparin [28]. Thus, binding of SLPI to HS expressed on the cell surface may affect HIV interactions with its receptors and inhibit infection. However, treatment of differentiated THP-1 cells with heparinases does not reduce SLPI binding [29].

It is possible that differences in the commercially available batches of rSLPI may also contribute to the variability. It has been suggested that both instability and susceptibility to oxidation may result in a loss of antiviral activity of rSLPI [18,19]. Studies in our laboratory, however, have eliminated degradation or oxidation of the rSLPI molecule as the cause of the variable anti-HIV effect of rSLPI [20].

To address these difficulties, we have constructed an expression system to provide large quantities of the SLPI protein, and used the THP-1 cell line as a model system for the evaluation of the anti-HIV effect of SLPI. THP-1 cells [30,31] differentiate into macrophage-like cells after treatment with phorbol 12-myristate 13-acetate (PMA) [32] and are frequently used to study HIV macrophage/monocyte interactions, as well as the relationship between cellular differentiation and virus production [33–38]. For the construction of the expression system, the human SLPI gene was synthesized by a PCR-based strategy using long oligonucleotides and cloned into an expression vector [39,40]. Subsequently, the protein was overexpressed in *Escherichia coli*, and the purified protein successfully folded as assessed by its ability to inhibit chymotrypsin, elastase, and cathepsin G [41]. This SLPI (designated sSLPI)[3] reduced the infection of differentiated THP-1 cells by HIV-$1_{Ba\text{-}L}$. The antiviral effect observed with sSLPI was not observed with a commercially available preparation (i.e., rSLPI), although both exhibited comparable anti-protease activity. The identification of a system that supports HIV replication which can be inhibited by a preparation of SLPI, coupled with the availability of an expression system for SLPI and its mutants will now enable us to investigate the biochemical and structural basis for the anti-

[3] For simplicity of the discussion, the SLPI produced by the synthetic gene will be designated sSLPI while the commercially available preparation of SLPI will be designated rSLPI.

HIV activity of SLPI. Some of these results have been presented earlier in preliminary form [42].

Experimental procedures

Materials. All reagents, buffers, and solvents were obtained from either Aldrich Chemical or Sigma Chemical, unless noted otherwise. Tryptone and yeast extract were obtained from Difco (Detroit, MI). The YM-3 ultrafiltration membranes were obtained from Millipore (Bedford, MA). Isopropyl-β-D-thiogalactoside (IPTG), thin-walled PCR tubes, and Genie Prep spin columns were obtained from Ambion (Austin, TX). Syringe filters were obtained from Nalgene (Naperville, IL). The expression vector pET24a(+) was obtained from Novagen (Madison, WI). Restriction enzymes, T4 DNA ligase, and low melting point agarose were obtained from Promega (Madison, WI). Reagents for the PCR were obtained from either Stratagene (La Jolla, CA) or F. Hoffmann–La Roche (Basel, Switzerland). Oligonucleotides were synthesized by Oligos Etc. (Wilsonville, OR). Human sputum elastase (NE) was purchased from Elastin Products (Owensville, MO) and human neutrophil cathepsin G (Cat G) was purchased from Calbiochem-Novabiochem (San Diego, CA). Sequencing grade chymotrypsin (Chy) was obtained from F. Hoffmann-La Roche. Recombinant SLPI (rSLPI) was acquired from R&D Systems (Minneapolis, MN).

Bacterial strains. *Escherichia coli* strain DH5α was used for transformation of ligated plasmids and *E. coli* strain BL21(DE3)pLysS was used for expression of the recombinant proteins. Both strains were obtained from Novagen. Cells for general cloning and expression were grown in LB media supplemented with kanamycin (50–100 μg/mL). The compositions of LB and SOC medium are described elsewhere [43].

Cells and viruses. Monocytic THP-1 cells (American Type Culture Collection; ATCC, TIB-202) were maintained at 37 °C, under 5% CO_2 in RPMI 1640 medium (Irvine Scientific, Santa Clara, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Irvine Scientific), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM) (RPMI medium/10) [30,31]. The cells, cultured as a single-cell suspension, were split 1:4 once a week to give a density of 1×10^6 cells/mL. The supernatant of chronically infected H9 HTLV-IIIB cells was used as a source of the HIV-1_{IIIB} strain. The p24 concentration of the stock was 150 ng/mL as determined by ELISA. A monocytotropic strain, HIV-$1_{Ba\text{-}L}$, was obtained from Advanced Biotechnologies. (Columbia, MD) and propagated in macrophages [44]. The p24 concentration of the stock solution was 364 ng/mL, and the $TCID_{50}$ was $\sim 1 \times 10^6$/mL.

Protease substrates. The activities of chymotrypsin and cathepsin G were assayed using the protease substrate *N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide. The activity of elastase was assayed using *N*-methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide. The synthetic chromogenic substrates were obtained from Sigma Chemical.

General methods. Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere [43]. Plasmid DNA was introduced into cells by

electroporation using a Cell-Porator Electroporation System (GibcoBRL, Gaithersburg, MD). DNA sequencing was done at the University of Texas (Austin) Sequencing Facility. HPLC was performed on a Waters system using either a TSKgel phenyl 5-PW hydrophobic column or a TSKgel SP-5-PW (cation exchange) column (TosoHaas, Montgomeryville, PA). Protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing conditions on 17% gels on a vertical gel electrophoresis apparatus obtained from Bio-Rad [45]. Protein concentrations were determined using the method of Waddell [46]. Kinetic data were obtained on either a Hewlett Packard 8452A Diode Array spectrophotometer or on a V_{max} microplate reader (Molecular Devices, Sunnyvale, CA), as indicated in the text.

Synthesis of the human SLPI gene. The synthesis of the human SLPI gene was carried out by the PCR in three stages in a Perkin–Elmer DNA Thermocycler using six long oligonucleotides, two oligonucleotide primers, and the PCR reagents [39,40]. The six long oligonucleotides (~75 bases each), corresponding to segments of the full-length human SLPI gene [47], are shown in Table 1. Two oligonucleotide primers, 5′-GAGATATACATATGTCTGGTAAAAGC-3′ (primer A) and 5′-GTCGACTTATGCTTTTACCGG-3′ (primer B), were also synthesized in order to amplify the PCR-synthesized gene. Primer A consists of a *Nde*I restriction site (underlined) followed by 12 bases corresponding to the coding sequence for the SLPI gene. Primer B consists of a *Sal*I restriction site (underlined), followed by a stop codon and 12 bases corresponding to the complementary sequence of the SLPI gene.

Table 1
The sequences of the six oligonucleotides used to synthesize the SLPI gene

Segment A[a]
5′-GA GAT ATA CAT ATG TCT GGT AAA AGC TTC AAA GCT GGC GTA TGC
CCG CCG AAA AAA TCC GCG CAG TGT CTG CGG-3′
Segment B
5′-ACA ACG TTT TTT ACC CGG GCA CTG CCA GTC GGA CTG GCA TTC CGG
TTT TTT GTA CCG CAG ACA CTG CGC GGA-3′
Segment C
5′-CCG GGT AAA AAA CGT TGT TGC CCG GAC ACC TGC GGC ATC AAA
TGC
CTG GAT CCG GTT GAT ACC CCG AAC CCG-3′
Segment D
5′-CAG CAT CAG ACA CTG GCC ATA GGT TAC CGG GCA TTT ACC CGG TTT
TCG ACG AGT CGG GTT CGG GGT ATC AAC-3′
Segment E
5′-GGC CAG TGT CTG ATG CTG AAC CCG CCG AAC TTC TGC GAA ATG GAC
GGC CAG TGT AAA CGA GAT CTG AAA TGC TGT-3′
Segment F[a]
5′-G CTT GTC GAC TTA TGC TTT TAC CGG GGA AAC ACA AGA TTT GCC
GCA
CAT ACC CAT ACA GCA TTT CAG ATC TCG-3′

[a] The *Nde*I restriction site in Segment A and the *Sal*I restriction site in Segment F are underlined.

In stage 1 of the PCR, a 25-μL reaction mixture consisted of 10× PCR buffer (200 mM Tris–HCl, pH 8.8, 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100, 1 mg/mL nuclease-free BSA, 2.5 μL), dATP, dTTP, dGTP, and dCTP (1 μL each from 5 mM stock solutions), segments A–F (1 μL each from 1 μM stock solutions), primers A and B (2 μL each from 10 μM stock solutions), *Pfu* DNA polymerase (0.25 μL of a 2.5 U/μL solution), and de-ionized water (11.25 μL). The reaction mixture was contained in a thin-walled reaction tube and layered with sterile paraffin oil. The PCR protocol consisted of 25 cycles, a 2-min incubation period at 94 °C preceding the 25 cycles, and a 10-min incubation period at 72 °C following the 25 cycles. Each cycle consisted of three steps: denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 2 min. The reaction tubes were maintained at 4 °C upon completion. The amplified products were extracted from the oil [48,49], electrophoresed on a 2% agarose gel, and visualized by UV transillumination. The band corresponding to a 344 bp product was excised and extracted from the gel [49,50].

In the second stage of PCR, the gel extract (containing the 344 bp product) was used as the template for amplification by the PCR. Three reaction mixtures were made up (25 μL each) as described above, with two modifications. The gel extract (2 μL) was used in place of the six long oligonucleotides and two mixtures contained additional quantities of $MgCl_2$ (0.5 and 1.0 μL from a 25 mM solution). The PCR protocol consisted of 25 cycles, a 3-min incubation period at 94 °C preceding the 25 cycles, and a 5-min incubation period at 72 °C following the 25 cycles. Each cycle consisted of three steps: denaturation at 94 °C for 1 min, annealing at 55 °C for 75 s, and elongation at 72 °C for 75 s. Upon completion, the reaction mixtures were treated as described above. UV transillumination of the gel showed that all three reaction mixtures generated a product consisting of ~344 bp.

In the last stage of PCR, the SLPI gene was further amplified using the gel extract containing the 344 bp product. A 200 μL-reaction mixture consisted of 10× PCR buffer (20 μL), $MgCl_2$ (4.0 μL from a 25 mM stock solution), dATP, dTTP, dGTP, and dCTP (1.6 μL each from 25 mM stock solutions), gel extract (16 μL), primers A and B (16 μL each from 10 μM stock solutions), *Pfu* DNA polymerase (2 μL of a 2.5 U/μL solution), and de-ionized water (124.4 μL). The mixture was divided into four equal portions and subjected to the PCR protocol used in stage two. The PCR product was isolated and stored [49].

The resulting PCR product and the pET24a(+) were digested with *Nde*I and *Sal*I restriction enzymes [43], purified on a 1% agarose gel, and extracted from the gel [49]. The linearized vector and the SLPI gene were ligated using T4 ligase (3 U) at 16 °C overnight [43]. The DNA was precipitated from the mixture [43] and re-suspended in sterile water (10 μL). Aliquots (2 μL) were used to transform *E. coli* strain DH5α by electroporation following the manufacturer's directions. Subsequently, an aliquot (10 μL) of the transformed cells was incubated in SOC media (1 mL) for 1 h, centrifuged for 3 min, and a portion of the supernatant (900 μL) removed. The cell pellet was re-suspended in the remaining media and plated onto an LB/Kn (100 μg/mL) agar plate and grown overnight at 37 °C. Eleven single colonies were chosen at random and screened for the presence of insert by the PCR using two primers: one that is specific for the T7 promoter region of the pET-24a(+) vector

(5′-TAATACGACTCACTATAGG-3′) and one that is specific for a sequence of the T7 terminator region of the pET-24a(+) vector (5′-TAGTTATTGCTCAGCGGT-3′). The presence of the insert is indicated by the observation of a PCR product of the appropriate size (~500 bp). Accordingly, the 11 individual colonies were treated as described elsewhere [49] and the resulting cell suspension was used as the template in the described PCR protocol [49]. The products were analyzed by agarose gel electrophoresis. Positive colonies were grown in liquid LB/Kn medium (10 mL, 100 μg/mL) overnight, and the newly constructed plasmid (designated pET24a–SLPI) was isolated [51]. The isolated plasmid was treated as described [49] and stored at −20 °C. For protein expression, an aliquot (1 μL) was introduced into *E. coli* strain BL21(DE3)pLysS by electroporation as described above.

For sequencing, the plasmid (pET24a–SLPI) was isolated from an overnight growth of *E. coli* strain DH5α cells by the alkaline lysis method [43], treated with 2.5 M ammonium acetate, precipitated with ethanol, and suspended in TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0). Subsequently, a portion of the DNA (~15 μg) was exchanged into water using an Ambion Genie Prep spin column according to the manufacturer's directions.

Expression of the SLPI gene. A single colony of the expression strain was used to inoculate 50 mL of LB/Kn (50 μg/mL). After overnight growth at 37 °C, 5 mL of the culture was used to inoculate 750 mL of LB/Kn (50 μg/mL) in a 2-L Erlenmeyer flask, giving an initial OD_{600} of ~0.05. Cultures were grown to an OD_{600} of 0.6–0.8 at 37 °C with vigorous shaking and then induced with isopropyl-β-D-thiogalactoside (IPTG) (0.5 mM final concentration). Incubation was continued for 3–4 h at 37 °C. Cultures were harvested by centrifugation (7000*g*, 12 min) and stored at −80 °C. Typically, 4.5 L of culture grown under these conditions yields ~8.5 g of cells.

Purification and folding of SLPI. SLPI was purified by a modification of the procedure of Kohno et al. [41]. In a typical procedure, the cells (~8.5 g) were thawed and suspended in a volume of 50 mM sodium acetate buffer (20 mL, pH 6.0, Buffer A) that was 2.5 times their weight. The cells were disrupted at 4 °C by sonication with one pulse (1 min) from a Heat Systems W-385 sonicator equipped with a 0.5-in. tapped horn delivering approximately 330 W/pulse. The solution was made 0.5–1.0 mM in phenylmethylsulfonyl fluoride and 6-aminocaproic acid, and 0.1 mg/mL in DNase. Sonication was continued for three 5-min intervals, which were spaced 5 min apart. Each interval consisted of 2.5-s pulses spaced at 2.5-s intervals. After centrifugation (35,000*g*, 30 min), the supernatant was made 1 M in ammonium sulfate and stirred for 1 h at 4 °C. Subsequently, the solution was centrifuged (27,000*g*, 30 min), and the supernatant was injected in two portions (~10 mL each) into a Phenyl 5-PW column (150 × 21.5 mm) attached to a Waters HPLC system. The column had previously been equilibrated with 50 mM sodium acetate buffer, pH 6.0, containing 1 M ammonium sulfate (Buffer B) at a flow rate of 5 mL/min. The column was washed with Buffer B for 10 min followed by a decreasing ammonium sulfate gradient (1.0–0.0 M) in 50 min. At the end of the gradient, the column was washed with Buffer A for 10 min. The eluent was collected in 10-mL fractions. The protein eluted from 0.3 to 0.1 M ammonium sulfate as determined by the distinctive orange/brown color of the fractions and confirmed by SDS–PAGE. The appropriate fractions were collected, pooled (final volume ~125 mL), and diluted with an equal

volume of 6 M guanidine hydrochloride at 4 °C. After stirring for 1 h, the mixture was concentrated by ultrafiltration (Amicon YM-3 membrane) to about 34 mL. Subsequently, SLPI was folded using a protocol described elsewhere, although sodium acetate buffer was used in place of Tris buffer [41]. The diluted mixture containing the refolded SLPI was filtered through a 0.2 μM syringe filter to remove the cloudy white precipitate, and concentrated by ultrafiltration to a final volume of 14 mL.

The concentrate was then injected in two portions (7 mL each) into the TSKgel SP 5-PW column (150 × 21.5 mm) attached to a Waters HPLC system. The column had previously been equilibrated with Buffer A, at a flow rate of 5 mL/min. The column was washed with the equilibration buffer for 10 min followed by an increasing NaCl gradient (0.0–1.0 M) in 60 min. The eluent was monitored at 254 nm and collected in 10-mL fractions. SLPI eluted from 0.5 to 0.6 M NaCl as determined by SDS–PAGE. The most pure fractions were collected, pooled, and concentrated by ultrafiltration. The concentrate was loaded onto a Sephadex G-75 column (100 × 2.5 cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.4) at a flow rate of ~0.5 mL/min. Fractions (10 mL) were collected, analyzed by SDS–PAGE, and the purest fractions were combined (>95% as assessed by SDS–PAGE) and concentrated. This procedure yielded about 6 mg of the purified and folded SLPI. For the biological studies, the SLPI preparation was exchanged into 10 mM Hepes (pH 7.4) containing 100 mM NaCl.

Mass spectrometry. The mass of the purified SLPI was determined by electrospray ionization mass spectrometry (ESI-MS) using an LCQ Finnigan octapole electrospray mass spectrometer. The samples for ESI-MS were prepared and analyzed as previously described [52].

Assays for the anti-protease activity of SLPI. The ability of SLPI to inhibit the activities of three proteases (NE, Chy, and Cat G) was determined using a fixed concentration of protease in the presence and absence of different concentrations of SLPI. The assay mixture consisted of 50 mM Hepes buffer (pH 7.4) containing 100 mM NaCl, protease (final concentration, NE = 20 nM, Chy = 10 nM, and Cat G = 60 nM), and SLPI. For the chymotrypsin assay, 10 mM $CaCl_2$ was included. For NE, nine concentrations of sSLPI (8–40 nM) and seven concentrations (8–32 nM) of rSLPI were used. For Chy, 11 concentrations (2–18 nM) of both preparations were used. For Cat G, eight concentrations (10–100 nM) of both preparations were used. After 30 min, the Chy substrate (33 μM), the NE substrate (66 μM), or the Cat G substrate (66 μM) was added. Stock solutions of substrate were made up in dimethyl sulfoxide. Activity was monitored by following the rate of the appearance of *p*-nitroaniline at 405 nm using a V_{max} microplate reader. The chromophore is generated by the protease-catalyzed hydrolysis of the appropriate substrate. Assays were carried out in 96-well microplates at 25 °C. The ratio of the reaction rate in the presence of inhibitor to that in the absence of inhibitor, a, was determined by fitting the inhibition data to Eq. (1):

$$a = 1 - \frac{([E_o] + [I_o] + K_i^{app}) - \{([E_o] + [I_o] + K_i^{app})^2 - 4[E_o][I_o]\}^{1/2}}{(2[E_o])}, \quad (1)$$

where $[E_o]$ and $[I_o]$ are the total concentrations of protease and SLPI, respectively, and K_i^{app} is the apparent inhibition constant [53,54]. Subsequently, the inhibition

constant, $K_{i,}$ was calculated by correcting the K_i^{app} values for the effect of substrate concentration using Eq. (2):

$$K_i^{app} = K_i(1 + [S_o]/K_m), \tag{2}$$

where $[S_o]$ is the substrate concentration, and K_m is the Michaelis constant. The K_m values were determined separately using the Michaelis–Menten equation and found to be 90 μM (Chy), 140 μM (NE), and 120 μM (Cat G). The data from all experiments were fitted by non-linear least-squares fit using the Enzfitter software (Elsevier Science Publishers, Amsterdam).

Infection of PMA-treated THP-1 cells with HIV-1$_{Ba\text{-}L}$. THP-1 cells, growing as a single-cell suspension, were differentiated by treatment with phorbol 12-myristate 13-acetate (PMA). Cells were plated at 1×10^6 cells/mL in 48-well plates and treated with a 160 nM (~100 ng/mL) solution of PMA for 24 h at 37 °C. After treatment with PMA the cells adhered and became flat and amoeboid in shape. Seven days after PMA treatment, THP-1 (THP-1/PMA) cells were pre-incubated with either rSLPI or sSLPI for 2 h at 37 °C and infected with HIV-1$_{Ba\text{-}L}$. SLPI remained present during the 2-h infection period at 37 °C. Control cells were treated similarly but without the SLPI. After infection, the viral supernatant was removed, the cells were washed three times and cultured in RPMI medium/10 (1 mL). Infection was monitored by the viral p24 level in harvested culture supernatants using ELISA plates obtained from the AIDS Vaccine Program (NCI-Frederick Cancer Research and Development Center; Frederick, MD).

Results

Synthesis of the human SLPI gene. SLPI consists of 107 amino acids and is encoded by a 321 bp gene. In the PCR-based gene synthesis strategy, long oligonucleotides having overlapping bases are joined together using the short overlapping bases as primers to extend the long nucleotides [39,40]. In the same reaction vessel, the PCR-generated DNA fragment can then be amplified using two short terminal primers. Accordingly, the full-length SLPI gene was divided into six long oligonucleotides of about equal length (~75 bases each) with overlaps of 18–21 bases between the oligonucleotides (Table 1). The oligonucleotides were designed on the basis of the published DNA sequence for the human SLPI gene [47]. Segment A contains an *Nde*I restriction site (underlined) followed by 60 bases corresponding to the sequence of the SLPI gene. These bases encode amino acids Ser-2–Arg-21. Segment B contains 72 bases corresponding to the complementary sequence of the SLPI gene, which codes for the amino acids Ser-16–Cys-39. Segment C contains 72 bases corresponding to the sequence of the SLPI gene and encodes amino acids Pro-34–Pro-57. Segment D contains 72 bases corresponding to the complementary sequence of the SLPI gene, which codes for the amino acids Val-52–Lys-75. Segment E contains 75 bases corresponding to the sequence of the SLPI gene and encodes Gly-70–Cys-94. Finally, segment F contains a *Sal*I restriction site, a stop codon, and 60 bases corresponding to the complementary sequence of the SLPI gene. This sequence

Table 2
Inhibition of proteases by sSLPI and rSLPI

Inhibitor	K_i (nM)		
	Chy	Cat G	NE
sSLPI	0.21 ± 0.03	5.3 ± 0.6	0.13 ± 0.02
rSLPI	0.10 ± 0.02	6.7 ± 0.7	0.55 ± 0.05

encodes amino acids Arg-89–Ala-108.[4] Using these six segments, the two short PCR primers, and the PCR reagents, the SLPI gene was constructed and amplified in sufficient quantities for subsequent manipulations after three separate rounds of the PCR. The sequence of the synthetic gene was confirmed by DNA sequencing.

Cloning, expression, purification, and folding of the sSLPI. The PCR fragment containing the synthetic gene was cloned into a pET24a(+) vector using standard techniques and expressed in *E. coli* strain BL21 (DE3)pLysS. The soluble portion of the expressed SLPI was partially purified by hydrophobic interaction chromatography and subjected to a previously reported folding protocol [41]. The refolded SLPI was then purified to homogeneity (as assessed by SDS–PAGE) by cation-exchange and size exclusion chromatography. The molecular mass of sSLPI was determined by ESI-MS and found to be 11,711 Da, which is within experimental error of the calculated mass (11,726 Da). Typically, this procedure yielded about 1.3 mg of pure, folded SLPI per liter of cell culture.

Inhibition of proteases by SLPI. The inhibitory activities of the sSLPI and rSLPI on chymotrypsin, human sputum elastase, and human neutrophil cathepsin G were determined by assuming that SLPI acts as a tight binding inhibitor, as described previously [53,54]. The results are summarized in Table 2. The values are comparable and consistent with those reported [47,55]. The results indicate that sSLPI is a more potent inhibitor of elastase and chymotrypsin than of cathepsin G.

Effect of SLPI on HIV-1 infection of PMA-treated THP-1 cells. The ability of SLPI (10 μg/mL) to inhibit infection of PMA-treated THP-1 cells by HIV-$1_{Ba\text{-}L}$ was investigated. At the same concentration of rSLPI, McNeely et al. [16] observed ~80% inhibition of p24 production in primary macrophages infected with HIV-$1_{Ba\text{-}L}$; this potent inhibition of virus production was sustained for 3 weeks of culture. PMA-treated THP-1 cells were infected efficiently with the macrophage-tropic (M-tropic) HIV-$1_{Ba\text{-}L}$ isolate (Fig. 1), while a very low level or complete lack of viral replication was observed with the T-cell tropic (T-tropic) HIV-1_{IIIB} isolate (data not shown). Under our experimental conditions, sSLPI reduced significantly HIV-$1_{Ba\text{-}L}$ infection, as assessed by viral p24 production. Ninety percent inhibition of viral replication was observed at 5 days post-infection and ~80% inhibition at days 7 and 11 post-infection (Fig. 1). In contrast, commercially available rSLPI did not inhibit HIV-1 infection in these cells.

[4] SLPI consists of 107 amino acids. The numbering system (1–108) includes the initiating *N*-formylmethionine, which is removed in *E. coli*. Thus, the sequence of the purified protein is identical to that reported for the human SLPI [47].

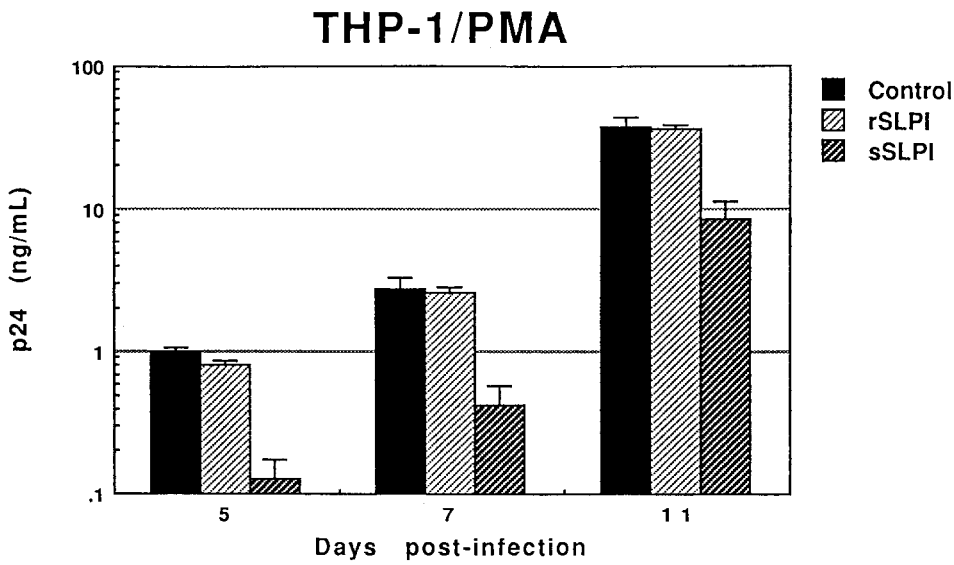

Fig. 1. The effect of sSLPI and rSLPI on p24 production in PMA-treated THP-1 cells. Seven days post-differentiation, THP-1 cells in 48-well plates, were exposed to sSLPI or rSLPI, at 10 μg/mL, as described in the Experimental procedures. Viral p24 antigen production was determined on days 5, 7, and 11. Data represent the mean and standard deviations obtained from triplicate wells, and are representative of three independent experiments.

Discussion

In this study, we have addressed two issues hampering efforts to understand SLPI's role in the prevention of HIV-1 infection. We have developed an expression system from which active SLPI can be obtained in sufficient quantities for future mechanistic and structural experiments. We have also determined that our sSLPI inhibits HIV infection of PMA-treated THP-1 cells, whereas commercial preparations of SLPI (i.e., rSLPI) do not, although both have comparable anti-protease activity. These results raise the question as to why these two preparations of SLPI exhibit different anti-HIV effects. The availability of our sSLPI will enable us to investigate the biochemical and structural basis for the anti-HIV activity of SLPI.

The expression system for SLPI was created by synthesizing the human gene and cloning the synthetic gene into a T7 expression system. In addition to generating a source for SLPI and a template for mutants, we pursued this strategy because the T7 expression system is amenable to isotope labeling in a MOPS-buffered minimal medium for structural studies [56]. The relatively small size of SLPI (107 amino acids) and the fact that it does not have post-translational modifications (e.g., glycosylation) make it feasible to perform a structural analysis of the parent protein and mutants by NMR spectroscopy. These experiments require significant quantities of isotopically labeled protein (^{15}N and ^{13}C), which are now achievable using this expression system.

Structural analysis of SLPI is a prerequisite to in-depth mechanistic experiments. This protein is rich in cysteine residues that form a total of eight disulfide bonds [57]. One obvious concern for preparations of SLPI is whether the protein is properly folded with the eight correctly paired disulfide linkages. Different conformations of SLPI, a result of incorrect formation of disulfide bonds, may be one explanation for the variability in experiments examining the anti-HIV activity of SLPI.

The anti-HIV activity of rSLPI was reported by two laboratories [13–15,18,19], while this effect was not confirmed by others [17], and variable results have been observed in our laboratory [20]. All these studies have been performed using the same, commercially available, preparation of rSLPI obtained from Synergen and/or R&D Systems. Comparison of the anti-HIV effect of rSLPI obtained by different laboratories is complicated by the fact that macrophages were cultured for different time periods before infection. McNeely et al. [16,18] cultured monocytes on glass for 10–14 days before infection with HIV-$1_{Ba\text{-}L}$. Shugars et al. [19] used 5-day old macrophages cultured on plastic, while Turpin et al. [17] cultured monocytes on plastic for 7–10 days prior to infection with HIV-1_{ADA}.

The susceptibility of macrophages to HIV-1 infection is greatly influenced by isolation, culture and infection conditions, donor variations, and macrophage maturation at the time of infection [21,58]. Freshly isolated monocytes are resistant to HIV-1 infection, and the susceptibility of macrophages to HIV-1 infection is greatly influenced by their stage of differentiation at the time of infection [59]. The receptor for the β-chemokines RANTES, MIP-1α, and MIP-1β, CCR5, was identified as the major co-receptor for the entry of macrophage tropic (M-tropic) strains of HIV-1 [58,60]. The expression of CCR5 on the cell surface significantly increases as monocytes mature to macrophages over 1–7 days of adherence, and then decreases to lower levels between days 10 and 14 [61]. These changes in surface expression of CCR5 correlate with CCR5 mRNA and the susceptibility of macrophages to productive infection [61–63].

The established monocytic THP cell line [30,31] has been used as an alternative to primary macrophages, because, after stimulation with PMA, the cells differentiate into adherent macrophage-like cells, mimicking the monocyte-to-macrophage transformation [32]. More than 80% of PMA-treated THP-1 cells change morphologically and adhere to the substratum. Undifferentiated THP-1 cells are susceptible to infection by T-cell tropic (T-tropic) HIV-1 isolates [34,37] and become susceptible to M-tropic isolates after treatment with differentiating agents [38]. We have characterized thoroughly the cell surface expression of the HIV receptor CD4 and the chemokine receptors CXCR4 and CCR5, both during propagation and following differentiation [64]. Cell surface CD4 plays a primary role in determining how efficiently THP-1 cells can be infected with X4 HIV-1_{IIIB} and R5 HIV-$1_{Ba\text{-}L}$ isolates. PMA-induced downregulation of CD4 significantly increases infection with the R5 virus, HIV-$1_{Ba\text{-}L}$, and differentiated THP-1 cells are infected efficiently with HIV-$1_{Ba\text{-}L}$. Therefore, differentiated THP-1 cells and the R5, HIV-$1_{Ba\text{-}L}$ virus were used in our study. In this study, we have shown that sSLPI inhibits infection of PMA-treated THP-1 cells by HIV-$1_{Ba\text{-}L}$.

Having established a system to study the anti-HIV effect of SLPI, experiments to identify specific cell surface protein(s) to which SLPI binds can be pursued. Using competitive binding studies, McNeely et al. [18] suggested that the anti-HIV activity of SLPI does not involve inhibition of cell-surface serine proteases. A putative receptor for SLPI was also identified. However, these binding studies were performed using freshly isolated monocytes while the infectivity studies were performed using 10-day-old adherent macrophages. Cell surface proteins are differentially expressed during the transition from monocyte to macrophage and freshly isolated monocytes are essentially refractory to HIV-1 infection [65]. Hence, the results of the competitive binding assays may have to be performed directly with PMA-treated THP-1 cells, the same cells that are used to show the anti-HIV activity of SLPI.

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