Novel Function of Prothymosin Alpha as a Potent Inhibitor of Human Immunodeficiency Virus Type 1 Gene Expression in Primary Macrophages

Arevik Msoian,†‡ Avelino Teixeira,‡§ Anthony A. High,§ Robert E. Christian, Donald H. Hunt,‡¶ Jeffrey Shabanowitz,§ Xinyan Liu,‡ and Mary Klotman*‡

1. Avelino Teixeira,‡§ Anthony A. High,§ Robert E. Christian, Donald H. Hunt,‡¶ Jeffrey Shabanowitz,§ Xinyan Liu,‡ and Mary Klotman*‡

Mount Sinai School of Medicine, Department of Medicine, Division of Infectious Diseases, New York, New York 10029;
Mount Sinai School of Medicine, Department of Medicine, Division of Nephrology, New York, New York 10029;
Department of Chemistry, University of Virginia, McCormick Road, Charlottesville, Virginia 22901; and
Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

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CD8 T lymphocytes control human immunodeficiency virus type 1 (HIV-1) infection by a cytotoxic major histocompatibility complex-restricted pathway as well as by secretion of noncytotoxic soluble inhibitory factors. Several components of CD8 cell supernatants have been identified that contribute to the latter activity. In this study we report that prothymosin alpha (ProTα), a protein found in the cell culture medium of the herpesvirus saimiri-transformed CD8 T-cell line, K#1 50K, has potent HIV-1-inhibitory activity. Depletion of native ProTα from an HIV-1-inhibitory fraction of CD8 cell supernatants removes the inhibitory activity, supporting its role in inhibition via soluble mediators. ProTα is an abundant, acidic peptide that has been reported to be localized in the nucleus and associated with cell proliferation and activation of transcription. In this report we demonstrate that ProTα suppresses HIV-1 replication, its activity is target cell specific, and inhibition occurs following viral integration. Native and recombinant ProTα protein potently inhibit HIV-1 long terminal repeat (LTR)-driven gene expression in macrophages. Furthermore studies using different promoters in lentiviral vectors (cytomegalovirus and phosphoglycerate kinase) revealed that suppression of viral replication by ProTα is not HIV LTR specific.

Control of viral replication in human immunodeficiency virus type 1 (HIV-1)-infected individuals requires cooperative functioning of the innate and adaptive arms of the immune system (9, 27). CD8 T cells derived from HIV-1-positive and -negative donors are thought to contribute to both innate and adaptive immune responses to HIV. In addition to cytotoxic T-lymphocyte activity, CD8 T cells have been shown to secrete soluble molecules that inhibit HIV-1 replication in vitro (3, 16, 31, 49).

The β-chemokines RANTES, MIP-1α, and MIP-1β, which competitively inhibit HIV-1 entry of R5 viruses into susceptible cells, are present in the supernatant of cultured CD8 T cells (5). Macrophage-derived cytokine (MDC), another chemokine isolated from CD8 cell-conditioned medium, has activity against both R5 and X4 strains of HIV-1, although chemically synthesized MDC has more restricted inhibitory activity (8, 36, 38). Interleukin-16 (IL-16), as well as gamma interferon, can be found in variable amounts in CD8 T-cell supernatants and has associated anti-HIV-1 activity (1, 18, 32). However, a number of groups have shown that the anti-HIV-1 activity found in CD8 T-cell supernatants is not fully accounted for by known β-chemokines, IL-16, MDC, or gamma interferon (13, 30, 31).

The amino-terminal fragment of the urokinase-type plasminogen activator and antithrombin III, both present in CD8 T-cell-conditioned medium, have been shown to have HIV-1-inhibitory activity in tissue culture (12, 48). In this study we report that prothymosin alpha (ProTα), a protein found in the cell culture medium of the herpesvirus saimiri (HVS)-transformed CD8 T-cell-line K#1 50K, previously demonstrated to have anti-HIV activity (3), has potent HIV-1-inhibitory activity that is target cell specific and inhibits steps following viral integration. Native and recombinant ProTα proteins inhibit HIV-1 long terminal repeat (LTR)-driven gene expression in infected macrophages.

MATERIALS AND METHODS

Isolation of HIV-1-suppressive factors from HVS-transformed CD8+ cell lines. K#1 50K CD8+ cells (3) were cultured at a concentration of 1 × 106 to 1.5 × 106 cells/ml in phenol red-free RPMI 1640 (Life Technologies, Rockville, MD) supplemented with IL-2 (50 U/ml), 100 U/ml of penicillin, 10 µg/ml of streptomycin, and 2 mM l-glutamine without serum for 48 h. The cells were removed by centrifugation, and the supernatant was clarified by filtration through 0.45-µm cellulose acetate filters. A final volume of 1.3 liters of clarified cell-conditioned medium was applied to the QXL resin, the flow was paused, the resin was allowed to sediment, and the adaptor of the Streamline 25 column was lowered to the top of the sedimented bed. The flow rate used was sufficient to expand the sedimented bed to 1.5 times its sedimented volume. When the diluted cell-conditioned medium had all been applied to the QXL resin, the flow was paused, the resin was allowed to sediment, and the adaptor of the Streamline 25 column was lowered to the top of the sedimented bed. The flow was then reversed and the resin washed with 10 resin volumes of Tris, pH 8.0, followed by a linear gradient of 0 to 1 M NaCl with peak capture. Peaks that showed...
HIV-1-suppressive activity were brought up to 1.8 M (NH₄)₂SO₄. These were applied to a Resource phenyl Sepharose hydrophobic interaction column (Amersham Biosciences, Piscataway, NJ) at 1 ml/min. Bound material was eluted with a linear gradient of 1.8 M to 0 M (NH₄)₂SO₄ with peak capture.

HIV-suppressive peaks from the hydrophobic interaction chromatography step were further fractionated using a Superdex Peptide H 10/30 gel filtration column (Amersham Biosciences, Piscataway, NJ). The peaks were analyzed without any chemical treatment by using an LCQ ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA) fitted with a custom-built nanoflow-high-pressure liquid chromatography microelectrospray ionization source (43a). Tandem mass spectrometry spectra were searched against the human nonredundant database (NCBI, Bethesda, MD), de novo sequenced, and manually validated.

Reagents. Synthetic thymosin alpha 1 (T1α) was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY), and biological activity (in vivo mouse protection assay against Candida albicans) of the peptide was tested and provided in the data sheet by the company. Recombinant human ProTα and antibody to ProTα were purchased from Alexios Biosciences (San Diego, CA). Antibodies against STAT1 and P-STAT1 were purchased from Cell Signaling Technology (Beverly, MA). The integrase inhibitor L-731988 was a gift from Merck (19).

Cells. CD8⁺ K#1 50K cells were derived from an adult healthy donor and transformed by HIV-1 BaL as described previously (31). HeLa CD4 cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Primary macrophages were isolated by adherence from peripheral blood mononuclear cells after 10 to 14 days in culture with Dulbecco modified Eagle medium containing 20% fetal bovine serum. Macrophages were isolated from peripheral blood mononuclear cells after 10 to 14 days in culture with Dulbecco modified Eagle medium containing 20% fetal bovine serum. Primary macrophage cultures were infected with recombinant ProTα/H9251 at an MOI of 0.01 or 0.1. Virus was washed off at 2 hours followed by the addition of fresh medium.

Viruses. HIV-1 BaL and HIV-1 JRFL were purchased from ABI (Columbia, Maryland). HIV-1 primary isolates 92BR028 and 92BR080-R5 and 92RW099-R5/X4 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The viral construct mC2,C3 BaL (kindly provided by Dr. D. Trono) was used for these studies. Equivalent fractions from successive gel filtration chromatography steps were pooled. Aliquots of each pool were digested with trypsin and analyzed by using an LCQ ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA) fitted with a custom-built nanoflow-high-pressure liquid chromatography microelectrospray ionization source (43a). Tandem mass spectrometry spectra were searched against the human nonredundant database (NCBI, Bethesda, MD), de novo sequenced, and manually validated.

Inhibition of HIV-1 replication by ProTα. To screen for inhibitors that worked after viral entry, the purified macrophages were first incubated with the R5 isolate of HIV-1 BaL for 2 h at a multiplicity of infection (MOI) of either 0.1 or 0.01 and then washed and subsequently cultured in the presence of CD8⁺ cell-conditioned medium (10% by volume) or different fractions derived from serum-free conditioned medium. The medium was changed every 3 to 4 days, and fresh medium with 10% conditioned medium or fractions were added. HIV-1 replication was monitored by measuring HIV-1 p24 antigen in cell supernatants using an enzyme-linked immunosorbent assay (ELISA) (SAIC Frederick, MD). 10⁻⁷ to 10⁻⁸ primary macrophages and primary CD8⁺ cells isolated from human peripheral blood were used for these studies. Fresh medium with 10% conditioned medium or fractions were added. HIV-1 replication was monitored by measuring HIV-1 p24 antigen in cell supernatants using an enzyme-linked immunosorbent assay (ELISA) (SAIC Frederick, MD). Recombinant ProTα was also tested against primary macrophages infected with HIV-1 BaL in a dose-dependent manner, reducing viral p24 by >80% at a concentration of 2 ng/ml (equal to the protein concentration of the fraction in Fig. 1A) (Fig. 1C). To limit the assay to a single round of viral infection, replication-defective JRFL envelope-pseudotyped HIV-1 (HIV-1JRFL) containing a luciferase gene under the control of the HIV-1LTR was used to infect primary macrophages. ProTα inhibited the expression of the luciferase reporter gene in a dose-dependent manner (Fig. 1D).

To determine the spectrum of anti-HIV-1 activity of ProTα, we tested the antiviral effect of recombinant ProTα in primary macrophages infected with HIV-1 primary isolates. ProTα (200 ng/ml) inhibited replication of an R5 as well as an X4/R5 HIV-1 primary isolate (Fig. 1E) in primary macrophages. In contrast, ProTα did not exhibit anti-HIV-1JRFL activity in HeLa CD4⁺/CCR5⁺ cells (Fig. 1F) or HuT CD4⁺/CCR5⁺ cells challenged with the same virus (data not presented). However, the unfractonated CD8⁺ T-cell supernatant of K#1 50K did inhibit HIV-1JRFL in HeLa CD4⁺/CCR5⁺ cells (Fig. 1F), indicating the presence of additional antiviral factors in the whole unfractonated supernatant.

In contrast to macrophages, recombinant ProTα demonstrated little to no anti-HIV-1 activity in transformed and pri-
FIG. 1. Effects of ProTα-containing chromatographic fraction 29 and recombinant ProTα on HIV-1 replication. In each panel the indicated virus at the indicated MOI was incubated with cells. Unbound virus was washed out, and the cells were incubated with 2 ng/ml of protein from fraction 29 containing ProTα or recombinant ProTα protein (at the indicated concentration). The results (means ± standard deviations for triplicate wells) for HIV-1 Gag p24 protein are from a single experiment at day 14 postinfection. (A) Primary macrophages treated with fraction 29 as described above after incubation with HIV-1BaL (MOI of 0.01) for 2 h. (B) PHA-activated primary CD4+ T cells treated with fraction 29 after 1 h of incubation with HIV-1Ral (MOI of 0.01) or HIV-1mb (MOI of 0.01). (C) Primary macrophages infected with HIV-1jal at an MOI of 0.01 and treated with different concentrations of recombinant ProTα. (D) Primary macrophages were incubated with HIV-1REFL for 2 h and treated with indicated concentrations of recombinant ProTα; the luciferase assay was performed 48 h posttreatment. (E) Primary macrophages were incubated with R5 and R5/X4 HIV-1 primary isolates at an MOI of 0.1 as described above and treated with 200 ng/ml of recombinant ProTα. The p24 ELISA was done on day 14 postinfection. (F) HeLa CD4+/CCR5+ cells were incubated with HIV-1REFL for 2 h and treated with 200 ng/ml of ProTα or 20% CD8+ T-cell supernatant from the K#1 50K cell line after residual virus was washed out; the luciferase assay was performed 48 h posttreatment. Similar results were obtained in three independent experiments (*, P < 0.01 compared to medium result). med, medium; RLU, relative light units.

FIG. 2. Effect of recombinant ProTα on HIV-1 replication in CD4+ T cells. The indicated CD4+ T cells were infected with the indicated virus. Unbound virus was washed out, and cells were incubated with either medium alone (black bars) or 200 ng/ml of ProTα (gray bars). The amounts of extracellular HIV-1 Gag p24 protein expressed as means ± standard deviations for triplicate wells are from a single experiment at 7 days postinfection. H9 and CEM cells were infected with HIV-1IIIB (MOI of 0.1) and treated with recombinant ProTα. PHA-activated primary CD4+ cells were treated with recombinant ProTα after infection with HIV-1mb and HIV-1inal at an MOI of 0.001 or the HIV-1 X4- or X4/R5-tropic primary isolate at an MOI of 0.001.

Depletion of ProTα from the chromatographic fraction leads to partial removal of the anti-HIV-1 activity. To ensure that ProTα contributed to the anti-HIV-1 activity of CD8+ cell supernatant, a sample of the chromatographic fraction from which ProTα was identified was applied to an anti-ProTα antibody affinity column. The eluate had anti-HIV-1 activity in a single-cycle infection assay similar to that of the parent fraction while the nonbinding flowthrough had lost 80% of the activity (Fig. 3A). Using a similar approach, suppression of HIV-1jal in primary macrophages was seen only with the bound eluate of recombinant ProTα (Fig. 3B). These results further indicate that ProTα is a major component of this active fraction derived from CD8+ cell supernatants and contributes to the anti-HIV activity.

Tα1 has no anti-HIV-1 activity. Tα1 is a peptide derived from the natural proteolytic cleavage of the 28 N-terminal amino acids of ProTα and can be detected in human plasma (43). Circulating levels of this peptide have been reported to be increased in HIV-1-positive individuals (40). Furthermore, it activates dendritic cells through toll-like receptor signaling (39). To determine whether this peptide is responsible for the anti-HIV-1 activity of ProTα, we used chemically synthesized Tα1 to assay for the ability to suppress HIV-1REFL-infected macrophages. Tα1 up to 1 µg/ml had no inhibitory activity while ProTα suppressed luciferase gene expression >80% at a concentration of 20 ng/ml (Fig. 3C).

ProTα inhibits HIV-1 LTR promoter-mediated gene expression. In each of the above assays, ProTα was added following viral infection, suggesting that the effect was on steps in the virus life cycle following entry. To determine the step(s) of the viral life cycle inhibited in macrophages by ProTα, timed experiments were performed comparing ProTα to azidothymidine (AZT; HIV-1 reverse transcription inhibitor) and the integrase inhibitor L-731988. Macrophages were infected with HIV-1VSV for 2, 24, and 48 h. At the end of each incubation period the virus was washed out and the cells were treated with ProTα, AZT, or integrase inhibitor. ProTα inhibited HIV-1 gene
expression when added 2, 24, and 48 h postinfection while AZT had no effect when added at the later time points of 24 and 48 h (Fig. 4A). This suggested that ProT/H9251 inhibited HIV-1 gene expression after reverse transcription, which in macrophages is completed by 26 to 48 h (34). Moreover ProT/H9251 inhibited HIV-1 gene expression up to 48 h postinfection following completion of integration of HIV-1 in macrophages as indicated by the loss of activity of the integrase inhibitor (Fig. 4B).

To determine the effect of ProT on HIV-1 RNA production, total RNA was extracted from primary macrophages infected with HIV-1VSV and treated 72 h postinfection with ProT (200 ng/ml) or medium for 24 h and analyzed by reverse transcription-PCR. Expression of a luciferase reporter gene mRNA was reduced by 82% compared to the medium alone in primary macrophages (data not shown).

ProT does not induce phosphorylation of STAT1 in primary macrophages and HeLa CD4+ cells. We have shown that activation of the signal transducer and activator of transcription 1 protein (STAT1) is necessary for inhibition of LTR activation and HIV-1 gene expression by unfractionated conditioned media of the HVS/CD8/H11001 T-cell line K#1 50K (4) in certain cell lines. To establish whether ProT also induces STAT1 activation, primary macrophages and HeLa CD4+ cells were briefly (15 min) incubated with the protein at 1/9262 g/ml or with 10% K#1 50K cell-conditioned medium after incubation for 2 h in serum-free medium to reduce baseline phosphorylation. A Western blot of the electrophoresed total lysate of the treated and untreated cells was probed with an antibody to the phosphorylated tyrosine 701 of STAT1 (Fig. 5). These results indicate that in contrast to the unfractionated conditioned medium, ProT suppression of LTR-driven HIV-1 gene expression is not associated with STAT1 phosphorylation in primary macrophages.

Inhibition of HIV-1 gene expression by ProT is not selective for the HIV-1 LTR promoter. To determine whether the effect of ProT on HIV-1 gene expression is specific for the LTR promoter, we used virus generated by two other HIV-derived lentiviral vectors carrying a luciferase reporter gene under the phosphoglycerate kinase (PGK) promoter pVVPW/LBE or the cytomegalovirus (CMV) promoter pVVCW/LBE. Macrophages were infected with HIV-1VSV, carrying the lucif-
phospho-STAT1 antibody, and the blots were subsequently stripped of sample loading buffer. Western blotting analysis was done using anti-ProT/H11001 and anti-ProT/H9251. 200 ng/ml was introduced. ProT/H9251 HIV-1VSV carrying a luciferase reporter gene under the LTR (A), ProT/H9251. Macrophages were infected with HIV-1VSV and medium with 4 mM sodium butyrate, or ProT/H9251, and medium with 4 µM sodium butyrate was added to the cells. Luciferase activity (relative light units [RLU]) was measured 24 h postinfection. Primary macrophages and HeLa cells were induced by CD8/T, and medium with 4 mM sodium butyrate was added to the cells. ProT/H9251 is not selective for the HIV-1 LTR. However, ProT/H9251 had no effect on the level of expression of β-actin, tubulin, or ribosomal protein s11, suggesting that there is selectivity to the inhibition of transcription (data not shown).

**Inhibition of gene expression by ProT/H9251 involves HDAC(s) without a direct effect on enzymatic activity of HATs.** Histone acetylation mediated by histone acetyltransferases (HATs) results in accessibility of the promoter region of the integrated HIV-1 provirus to transcriptional factors and is one of the mechanisms by which HIV-1 transcription is regulated (2, 29). ProT/H9251 has been shown to associate with the HAT p300 and influence transcription (15, 35, 46). Based on several reports indicating nuclear localization of ProT/H9251 and its association with histones and HATs, we tested whether HATs are targeted by ProT/H9251 and whether a histone deacetylase (HDAC) inhibitor can rescue HIV-1 gene expression in the presence of ProT/H9251 (15, 23, 50).

Primary macrophages were infected with vesicular stomatitis virus (VSV) envelope-pseudotyped HIV-1, and 48 h postinfection the cells were treated with ProT/H9251 alone or ProT/H9251 with sodium butyrate (a known inhibitor of histone deacetylases) (Fig. 6A). The same experiments were done in macrophages infected with VSV envelope-pseudotyped lentiviruses carrying the reporter gene luciferase under different promoters (PGK and CMV) as well (Fig. 6B and C). ProT/H9251 inhibited LTR-, PGK-, and CMV-driven gene expression in primary macrophages, and this inhibition was completely reversed by the histone deacetylase inhibitor sodium butyrate in the case of LTR and PGK promoter-driven transcription (Fig. 6A and B) and partially reversed in the case of CMV promoter-driven transcription (Fig. 6C).

To further investigate the mechanism of ProT/H9251 activity, we tested whether binding of ProT/H9251 to p300 reduced the histone acetylation function of this enzyme. We used a nonradioactive HAT assay kit (Upstate Biotechnology, Lake Placid, NY). Our results indicated no reduction of the in vitro enzymatic activity of p300 in the presence of different concentrations of recombinant ProT/H9251 (data not shown).

**DISCUSSION**

We identified ProT/H9251 as an active anti-HIV-1 component of one of the fractions derived from the supernatant of HSV-transformed CD8+ T cells. ProT/H9251 is a small acidic protein (12 kDa, 109 amino acids) that has been implicated in a number of diverse biologic activities (17). Known mostly for its association with cell proliferation, it has also been reported to have immunomodulatory activities (6, 44, 47), including chemotaxis. Several of the immunomodulatory activities have been reported with ProT/H9251 applied extracellularly; however, it is unclear whether the biologic activity is secondary to uptake or mediated via cell surface signaling. Regulation of ProT/H9251 has been reported to occur at the level of translation and intracellular localization (25). ProT/H9251 has a well-defined nuclear localization signal sequence, and it has been identified in the nucleus of a number of cells (11, 50, 51). While this protein has no secretory signal sequence, it has, nonetheless, been detected in human serum and the supernatants of cultured lymphocytes (10, 37), and in this report we have demonstrated its presence in supernatants of the HSV-transformed CD8+ cell line K#1 50K. Furthermore, a number of cell surface binding proteins have been identified as candidate receptors for extracellular ProT/H9251. Regarding the anti-HIV effect of ProT/H9251 that we described here for macrophages, it remains to be determined if this is mediated via uptake of the exogenously added protein or via the induction of a signaling pathway (42).

Our screening assay was set up to specifically focus on postentry events of the HIV-1 life cycle in macrophages. While the HIV-1-suppressive activity of ProT/H9251 was significant in mac-
rothrophages, there was little activity against HIV in T cells. Interestingly, infection of human T cells with HIV-1 is associated with a reduction in detectable amounts of ProTα mRNA (14, 41). Understanding the mechanism of the inhibitory activity of ProTα in the macrophage as well as control of expression in T cells may provide insights into cell-specific control of viral gene expression.

Several reports demonstrate that CD8+ T-cell supernatants inhibit HIV-1 LTR-mediated gene transcription (4, 7, 28). While ProTα appears to inhibit LTR-driven luciferase gene expression predominantly in macrophages and HeLa CD4+ cells. Differential requirements for transcriptional regulators of HIV in cells of monocytic compared to lymphocytic origin have been described elsewhere (20). Several reports demonstrate that CCAAT binding proteins (C/EBP) are necessary for HIV replication in primary macrophages but not in lymphocytes (20, 21, 26). We examined whether CCAAT box binding transcriptional factors are involved in ProTα suppression. Wild-type HIV BaL and an HIV BaL mutant, mc2,C3-BaL, which contains point mutations in two high-affinity C/EBP binding sites of the CCAAT box of LTR, were used for these experiments. Inhibition of wild-type HIV-1 replication by ProTα was observed as expected (97% ± 3%); however, mutated virus replication was suppressed to a lesser extent (63% ± 10%; data not presented). These results suggest that CCAAT box binding transcriptional factors may be involved in but not completely responsible for ProTα-mediated suppression of HIV LTR.

In HIV-1-infected cells the integrated proviral genome is tightly packaged by chromatin and is silent in the absence of stimulation. Acetylation of the associated histone proteins can stimulate transcription from the integrated HIV-1 proviral genome. Another role ascribed to ProTα is involvement in chromatin remodeling, and in this context it has been reported to interact with a number of HDACs (24) while under other conditions it has been found in association with the HAT p300/CBP (15, 45). While we were not able to demonstrate direct inhibition of p300-associated HAT in an in vitro assay system, ProTα cell-associated suppression of gene expression was reversed by the HDAC inhibitor sodium butyrate (22, 45). This is consistent with, but not definitive proof of, the idea that HDACs are involved in the ProTα-mediated transcriptional inhibition in macrophages.

ProTα is a nonspecific inhibitor of HIV-1 gene expression in macrophages; however, it is not the only postintegration inhibitory factor secreted by the CD8+ cell line K#1 50K, as our data demonstrate. CD8+ cell supernatants make a number of factors that inhibit HIV-1 specifically, as in the case of beta-chemokines, and nonspecifically, as in the case of ProTα. There are additional molecules yet to be identified that contribute to the overall broad HIV suppression seen with unfractionated CD8+ T-cell supernatants. Additional questions remain regarding the role of ProTα in vivo control of replication and the mechanism of postintegration suppression of gene expression as well as whether it can be exploited for the development of novel therapeutics.

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