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The Cationic Properties of SEVI Underlie Its Ability To Enhance Human Immunodeficiency Virus Infection▽

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Human semen contains peptides capable of forming amyloid fibrils termed semen-derived enhancer of viral infection (SEVI) that can greatly increase human immunodeficiency virus (HIV) infection. While SEVI appears to enhance virion attachment to target cells, its underlying mechanism of action is unknown. We now demonstrate that the intrinsic positive charges of SEVI (pI = 10.21) facilitate virion attachment to and fusion with target cells. A mutant form of SEVI in which lysines and arginines are replaced with alanines retains the ability to form amyloid fibrils but is defective in binding virions and enhancing infection. In addition, the interaction of wild-type SEVI with virions and the ability of these fibrils to increase infection are abrogated in the presence of various polyanionic compounds. These anionic polymers also decrease the enhancement of HIV infection mediated by semen. These findings suggest that SEVI enhances viral infection by serving as a polycationic bridge that neutralizes the negative charge repulsion that exists between HIV virions and target cells. Combinations of agents that neutrale SEVI action and produce HIV virucidal effects are an attractive future direction for microbicide development.

Human immunodeficiency virus (HIV) was identified more than 25 years ago but remains a pressing threat to public health worldwide, particularly in developing nations. Sub-Saharan Africa is home to 68% of all adults and nearly 90% of all children infected with HIV; in 2007, 76% of AIDS deaths occurred in this region (UNAIDS 2007). In both developing and developed nations, semen is the principal vector mediating transmission of the virus.

Semen provides a protective environment for HIV virions that may act to enhance transmission. Seminal fluid contains basic amines such as spermine, spermidine, putrescine, and cadaverine that can protect HIV virions from inactivation by the acidic secretions present in the vaginal tract (2, 7, 14). Recently, Münch and colleagues identified a factor in semen that greatly enhances HIV infection (9). This factor, which they termed SEVI (semen-derived enhancer of viral infection), is composed of a proteolytic peptide encompassing residues 248 to 286 of prostatic acid phosphatase (PAP), a highly abundant protein in semen. Synthetic versions of SEVI enhance HIV infection up to 10^5-fold under conditions of limiting viral inoculums (9). These observations suggest that SEVI may play an important role in promoting semen-mediated transmission of HIV.

In this study, we sought to identify the mechanism by which SEVI enhances infection. SEVI exists as amyloid fibrils, and only this fibrillar form of the PAP248-286 peptide enhances HIV infection (9). In addition, we noted that PAP248-286 contains a high abundance of positively charged lysine and arginine residues. We hypothesized that SEVI enhances infection by serving as a cationic polymer that shields the electrostatic repulsion that normally occurs between the membranes of HIV virions and their target cells. To test this hypothesis, a mutant PAP248-286 peptide that lacks lysines and arginines was assessed for the ability to form amyloid fibrils and enhance HIV infection. We also tested the effects of various anionic polymers on SEVI- and semen-mediated enhancement of HIV infection. Our findings suggest that SEVI boosts viral infectivity by decreasing charge-charge repulsions between HIV and its host cells, thereby greatly increasing virion attachment to cellular targets. Compounds that neutralize SEVI activity are attractive adjunctive agents in microbicides due to their ability to impair the infectivity of HIV virions by blocking the action of potent semen-derived viral enhancement factors.

MATERIALS AND METHODS

Peptide synthesis and fibril formation. PAP248-286, PAP248-266, and PAP248-286(Ala) peptides were synthesized by 9-fluorenylmethoxy carbonyl solid-phase chemical synthesis and purified by reversed-phase high-performance liquid chromatography at the University of Utah Health Sciences Center DNA/Peptide Core Facility. For some experiments, peptides were synthesized by ViroPharmaceuticals GmbH (Hannover, Germany) or by P. Henklein (Charite, Berlin, Germany). Peptide masses were confirmed by mass spectrometry. Lyophilized peptides were resuspended in phosphate-buffered saline (PBS) at a concentration of 10 mg/ml. To promote full fibril formation, peptide solutions were agitated for 1 to 7 days at 37°C at 1,400 rpm with an Eppendorf Thermomixer.

Thioflavin T and Congo red binding. Agitated peptide solutions were added to 5 μM thioflavin T (Sigma-Aldrich) in PBS at a concentration of 250 μg/ml unless otherwise indicated, and the increase in fluorescence (excitation wavelength, 440 nm; emission wavelength, 482 nm) in triplicate wells was assayed with a Perkin-Elmer Ls-5B luminescence spectrometer. For Congo red staining, peptide solu-

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tions (250 μg/ml) were mixed with a Congo red solution (Sigma-Aldrich) and incubated for 10 min at room temperature. Fibrils were then pelleted and resuspended in dimethyl sulfoxide, and the optical density of this solution was determined at 490/650 nm.

Electron microscopy. Fibrils of PAP248-286 (500 μg/ml in PBS) were prepared with Parlodion-filmed carbon-coated grids and 2% potassium phosphotungstate, pH 6.5, by the drop method (8). Samples were examined at 80 kV in a JEOL 1230 electron microscope (JEOL) and photographed with a USC1000 digital camera (Ultrascan).

Primary cells. $CD4^+$ T cells were isolated from fresh human peripheral blood mononuclear cells by negative selection with $CD14^+$ microbeads and positive selection with $CD4^+$ microbeads (Miltenyi). Cells were maintained in RPMI medium supplemented with L-glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum. $CD4^+$ T cells were isolated from human endometrial biopsy samples by treatment with collagenase IV (200 U/ml) and DNase I (35 U/ml) (both from Sigma) for 20 min at 37°C and filtered through a nylon mesh to generate a single-cell suspension. After extensive washing, $CD4^+$ T cells were positively selected with $CD4^+$ microbeads (Miltenyi) and cultured in RPMI medium supplemented as described above. $CD4^+$ "crawl-out" cells were isolated by culturing minced endometrial tissues overnight and purifying $CD4^+$ T cells from the supernatant with $CD4^+$ microbeads (Miltenyi).

Viral production. BlaM-Vpr chimeric CXCR4-tropic NL4-3 and CCR5-tropic 81A viruses were produced by calcium phosphate-mediated transfection of 293T cells with DNA proviral expression plasmids. Two days later, supernatants were clarified by sedimentation and concentrated by ultracentrifugation. Virions used for infectivity assays in TZM-bl cells were isolated by harvesting supernatants from 293T cells transfected with the CCR5-tropic plasmid pBRHIV-1NL4-3_92TH01-24 (10) without ultracentrifugation. Cyan fluorescent protein (CFP)-labeled virions used for fluorescence microscopy were generated by transfecting 293 cells with 600 ng of an HXB2 plasmid (pHXBN+GFP+, a gift from Sang Kyung Lee and Premlate Shankar, Harvard Medical School, Boston, MA) and 200 ng of HIV Gag-CFP (13). Viral titers were measured by anti-$p24^{Gag}$ enzyme-linked immunosorbent assay (ELISA; Perkin-Elmer).

Polyanion reagents. Heparin, dextran sulfate, and chondroitin sulfate were obtained from Sigma. Oversulfated heparin, de-O-sulfated heparin, and oversulfated chondroitin sulfate were obtained from Neoparin. The polyanions were stored in PBS at a concentration of 10 mg/ml at −20°C.

Virus pull-down assay. NL4-3 virions (100 ng/ml) were incubated with SEVI (31.25 μg/ml) and the indicated anionic polymers (31.25 μg/ml) for 3 h at 37°C. The samples were then centrifuged at 5,000 rpm for 3 min, and HIV levels in the supernatant and pellet were assayed with an anti-$p24^{Gag}$ ELISA kit (Perkin-Elmer). To determine whether SEVI fibrils interact directly with polyanions, a two-step binding assay was performed. SEVI was preincubated with the indicated polyanionic compounds for 1 h at 37°C. Mixtures were then centrifuged at 5,000 rpm for 3 min to pellet fibrils and any interacting polyanions. Supernatants were discarded, and pellets were resuspended with virions and incubated for 1 h at 37°C. Samples were then centrifuged at 5,000 rpm for 3 min, and HIV levels in the supernatant and pellet were determined by $p24^{Gag}$ ELISA. Results are reported as the percentage of $p24^{Gag}$ in the pellet relative to the total $p24^{Gag}$ content.

To assess the infectivity of the HIV virions associated with SEVI, a two-step binding assay was carried out as described above, except pellets were resuspended in 180 μl DMEM after the second centrifugation and then diluted 1:10 before being used to infect TZM-bl cells. β-Galactosidase activity was measured 3 days postinfection with the Gal-Screen kit (Applied Biosystems).

HIV virion fusion assay. To examine HIV type 1 (HIV-1) fusion to target cells, we performed a virion fusion assay as previously described (4). BlaM-Vpr-containing NL4-3 or 81A virions were pretreated for 1 h at 37°C with fibrils (31.25 μg/ml) in the absence or presence of anionic polymers (31.25 μg/ml) and added at a final concentration of 50 ng/ml (NL4-3) or 100 ng/ml (81A) to 10^6 $CD14^-$ $CD4^+$ target cells in a final volume of 200 μl. For inhibitor studies, 2 μM AMD3100 or TAK779 was also added. In some experiments, 10^6 CD14-$CD4^+$ target cells were pretreated with fibrils (31.25 μg/ml) or 10% pooled semen (Children's Wish Center, Göttingen, Germany) in the absence or presence of polyanionic compounds (31.25 μg/ml) for 1 h at 37°C. Cells were then washed twice with medium and added to NL4-3 virions (50 ng/ml) in a final volume of 200 μl. In all experiments, fusion was allowed to occur for 4 h at 37°C, after which target cells were loaded with CCF2 and incubated overnight at room temperature. Cells were then stained with CD4-PE Cy7 and CD3-APC Cy7 antibodies (BD Biosciences), fixed with 2% paraformaldehyde, and analyzed with a BD LSRII. Data were analyzed with FlowJo software (Treestar).

Infection of TZM-bl cells. To assess the relative infectivity of SEVI versus PAP248-286(Ala) fibrils, supernatants harboring HIV virions (560 ng/ml p24) were diluted 10-fold and then preincubated for 10 min at room temperature with fibrils at the indicated concentrations. The virion-fibril mixtures were then diluted 15-fold and used to infect TZM-bl cells (10^4/well). β-Galactosidase activity in triplicate wells was measured 3 days postinfection with the Gal-Screen kit (Applied Biosystems).

To test the inhibitory activity of the anionic polymers upon pretreatment of virions, 293T supernatants containing HIV virions (560 ng/ml p24) were diluted 10-fold, treated with SEVI (37.5 μg/ml) for 10 min, and then incubated with the indicated concentrations of the anionic polymers for another 60 min. The treated HIV virions were then diluted 30-fold and used to infect TZM-bl cells (10^4/well). For target cell pretreatment, 10^4 TZM-bl cells were treated with the indicated concentrations of anionic polymers for 10 min and then infected with HIV virions in the presence of SEVI (37.5 μg/ml). β-Galactosidase activity in triplicate wells was determined 2 days postinfection with the Gal-Screen kit (Applied Biosystems).

To test the effect of polyanions on semen-mediated enhancement of HIV infection, 10^4 TZM-bl cells were treated with anionic polymers for 60 min and then infected with 293T supernatants containing HIV virions (2.7 ng/ml [final concentration] p24) treated with 10% semen or PBS. To minimize semen-induced cytotoxic effects, supernatants were removed after 2 h and 200 μl of fresh medium containing gentamicin (50 μg/ml) was added. β-Galactosidase activity in triplicate wells was determined 3 days postinfection with the Gal-Screen kit (Applied Biosystems).

Fluorescence microscopy. Labeling of SEVI and binding of HIV to TZM-bl cells were performed as previously described (9). Briefly, cellular supernatants containing HIV Gag-CFP were incubated with 20 μg/ml fluorescein isothiocyanate (FITC)-labeled SEVI in the absence or presence of 30 μg/ml heparin for 30 min, followed by 30 min of binding to TZM-bl cells at 37°C. Cells were then fixed in paraformaldehyde, and binding of SEVI/HIV was monitored with a 60× oil objective (numerical aperture 1.4) in a Nikon TE2000 inverted wide-field microscope.

RESULTS

PAP248-286 forms amyloid fibrils that enhance HIV fusion with primary cells from peripheral blood and female genital mucosa. To generate SEVI amyloid fibrils, we chemically synthesized PAP248-286. After agitation, PAP248-286 solutions became turbid and interacted in a dose-dependent manner with thioflavin T, a dye that selectively binds amyloid fibrils (Fig. 1A). In contrast, a C-terminally truncated peptide, PAP248-266, did not form such fibrils (Table 1 and Fig. 1A). Electron microscopy confirmed the formation of branching needle-like amyloid fibrils by PAP248-286 but not by PAP248-266 (Fig. 1B and data not shown).

Next, the effect of SEVI on HIV virion entry into primary CD4 T cells was assessed with a virion-based HIV fusion assay (4). CXCR4-tropic NL4-3 and CCR5-tropic 81A virions containing β-lactamase–Vpr were preincubated with increasing concentrations of SEVI or preagitated PAP248-266 control peptide and then added to $CD4^+$ peripheral blood lymphocytes (PBLs). Fusion of NL4-3 and 81A virions was enhanced in direct proportion to the concentration of SEVI added but not by PAP248-266 (Fig. 1C). This level of fusion enhancement is consistent with that previously observed (9). Since the yield of amyloidogenic PAP fragments from seminal fluid was ~35 μg/ml (9), SEVI was used at a slightly lower concentration of 31.25 μg/ml in all subsequent virion fusion experiments. The SEVI-mediated enhancement of virion fusion did not bypass the requirement for the appropriate coreceptor, as the CXCR4 antagonist AMD3100 inhibited the fusion of CXCR4-tropic NL4-3 but not CCR5-tropic 81A virions, whereas the CCR5 antagonist TAK779 had the opposite effect (Fig. 1C).

SEVI enhances HIV infection of cultured cell lines and

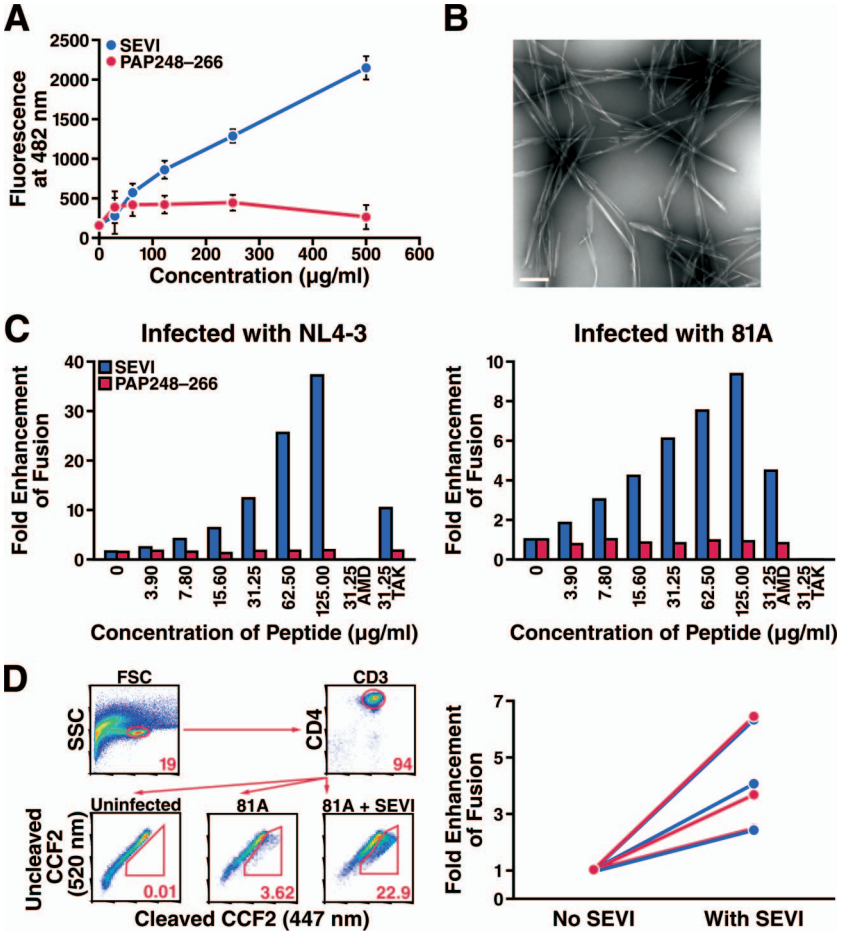

FIG. 1. PAP248-286 forms fibrils and enhances the fusion of HIV-1 to primary CD4$^+$ T cells. (A) Increasing concentrations of agitated PAP248-286 or PAP248-266 were mixed with 5 μM thioflavin T, and emission at 482 nm was recorded. Results are representative of data obtained from three independent experiments with two different batches of synthetic peptide. Shown are average values (± standard deviations) of triplicate measurements. (B) Electron micrograph of PAP248-286 fibrils (500 μg/ml). White bar = 200 nm. (C) CD14$^-$ CD4$^+$ T cells were infected with BlaM-Vpr-containing NL4-3 or 81A virions in the presence of increasing concentrations of either SEVI or agitated PAP248-266 and then analyzed for virion fusion. The entry inhibitors AMD3100 and TAK779 were added to the indicated samples. Results are reported as *n*-fold enhancement of fusion relative to the level of fusion in the absence of any added peptide. These results were confirmed in two independent experiments. (D) CD3$^+$ CD4$^+$ T cells isolated from endometrial biopsy samples were infected with BlaM-Vpr-containing 81A virions in the absence or presence of SEVI. Bottom left: fluorescence-activated cell sorter plots for the detection of virion fusion from a single donor. Values at the bottom right of plots reflect the percentages of cells that fused with virions. Right: *n*-fold enhancement of fusion in the presence of SEVI (n = 5 donors). Blue lines are from experiments with CD4$^+$ T cells purified directly from endometrial tissues. Red lines are from experiments with CD4$^+$ T cells purified from endometrial "crawl-out" cells. FSC, forward scatter; SSC, side scatter.

primary cells from peripheral blood and tonsillar tissues (9), but its ability to enhance HIV infection of target cells residing in the female genital tract has not been assessed. We investigated the ability of SEVI to enhance HIV fusion to CD4$^+$ T cells isolated from endometrial biopsy samples. In the presence of SEVI, fusion of 81A virions to CD4$^+$ T cells from multiple biopsy samples was consistently enhanced, with effects ranging between two- and sixfold (Fig. 1D).

TABLE 1. Sequences of peptides used in this study

Peptide	Sequence[a]
PAP248-286	GIH**K**Q**K**E**K**S**R**LQGGVLVNEILNHM**KR** ATQIPSY**KK**LIMY
PAP248-266	GIH**K**Q**K**E**K**S**R**LQGGVLVNE
PAP248-286(Ala)	GIH**A**Q**A**E**A**S**A**LQGGVLVNEILNHM**AA** ATQIPSY**AA**LIMY

[a] Basic residues are bold; residues mutated to alanine are underlined.

Mutant PAP248-286(Ala) forms amyloid fibrils but is deficient in enhancing HIV infection. Next, the mechanism by which SEVI enhances HIV fusion was explored. We noted that PAP248-286 contains many basic residues (Table 1) and consequently displays a much higher isoelectric point than full-length PAP (pI 10.21 versus 5.83). We hypothesized that fibrillar PAP248-286 enhances HIV infection by providing a dense aggregate of positive charges that shields the charge repulsion between virions and target cells. To test this hypothesis, we chemically synthesized and analyzed PAP248-286(Ala), a mutant peptide in which the eight lysines and arginines in PAP248-286 were replaced with alanines (Table 1).

It was first important to determine whether PAP248-286(Ala) forms amyloid fibrils. After agitation, PAP248-

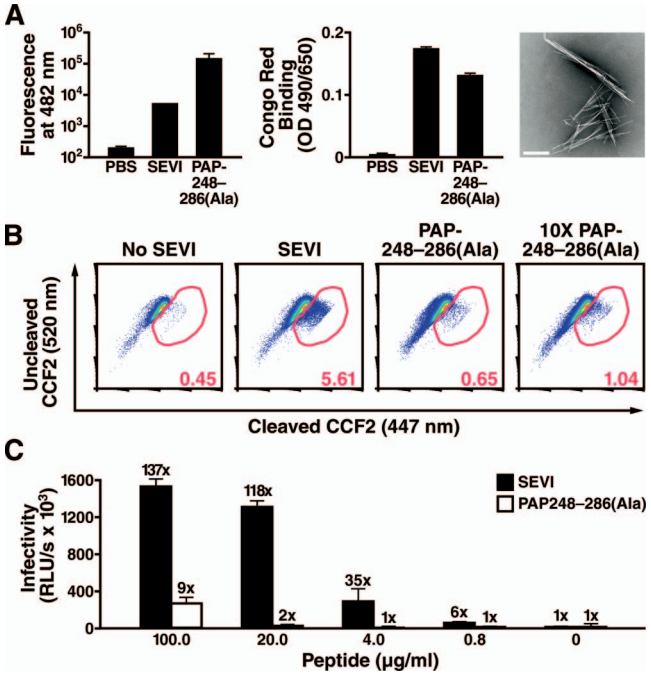

FIG. 2. PAP248-286(Ala) forms fibrils but is deficient in enhancing HIV infection. (A) Solutions containing 250 μg/ml agitated PAP248-286 (SEVI) and PAP248-286(Ala) were monitored by thioflavin T fluorescence (left panel) or Congo red binding (center panel). The right panel shows an electron micrograph of PAP248-286(Ala) fibrils (500 μg/ml). White bar = 200 nm. (B) $CD14^-$ $CD4^+$ lymphocytes were infected with BlaM-Vpr-containing NL4-3 in the presence of SEVI, agitated PAP248-286(Ala), or 10-fold more agitated PAP248-286(Ala) than SEVI. Values at the bottom right of the fluorescence-activated cell sorter plots reflect the percentages of cells that fused with virions. Results are gated on $CD3^+$ $CD4^+$ cells. The experiment was repeated four times with similar results. (C) TZM-bl cells were infected with CCR5-tropic HIV virions in the presence of SEVI or agitated PAP248-286(Ala). Cells were assayed for β-galactosidase activity 3 days postinfection. The values indicate the *n*-fold infectivity enhancement relative to the infectivity in the absence of peptide. Results are representative of data from three separate experiments. OD, optical density; RLU, relative light units.

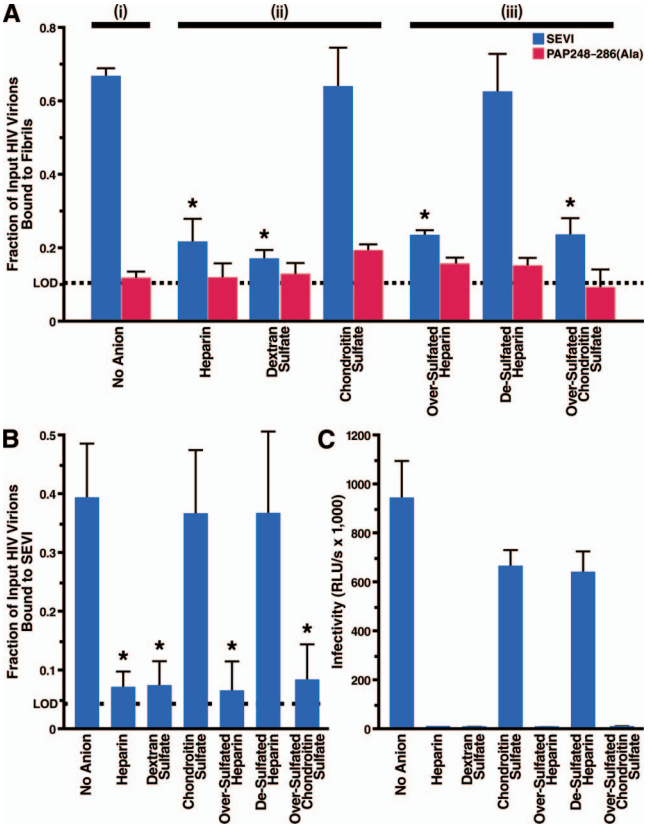

FIG. 3. SEVI fibrils, unlike PAP248-286(Ala) fibrils, efficiently bind HIV virions in a manner that can be blocked by some anionic polymers. (A) SEVI or PAP248-286(Ala) fibrils were incubated with NL4-3 virions in the presence of the indicated anionic polymers and centrifuged. The absolute amounts of $p24^{Gag}$ in the pellet and supernatant were determined by ELISA. Values are the mean ± standard deviation from one of four experiments that yielded similar results. *, $P < 0.01$ versus SEVI in the absence of anionic compound (two-tailed *t* test). LOD, limit of detection determined by the $p24^{Gag}$ signal in the absence of peptide. (B) SEVI was pretreated with the indicated anionic polymers and then centrifuged to pellet fibrils and any interacting polymers. Pellets were then incubated with HIV, and the proportion of virions bound to fibrils was determined as for panel A. Values are the mean ± standard deviation of three experiments. *, $P < 0.01$ versus SEVI in the absence of anion (two-tailed *t* test). LOD, limit of detection determined by the $p24^{Gag}$ signal in the absence of peptide. (C) TZM-bl cells were incubated with virion-exposed SEVI fibrils that were treated as described in Fig. 3B. β-Galactosidase activity was measured 3 days postinfection.

286(Ala) solutions became turbid and the peptide interacted with thioflavin T even more efficiently than SEVI (Fig. 2A, left), possibly because SEVI and thioflavin T are both positively charged and may electrostatically repel one another. The PAP248-286(Ala) solutions also interacted strongly with Congo red, another dye that binds amyloid fibrils (Fig. 2A, center). Lastly, PAP248-286(Ala) fibrils were also detected by electron microscopy (Fig. 2A, right).

Next, the ability of PAP248-286(Ala) fibrils to enhance HIV virion fusion was assessed. In contrast to the effects of SEVI, pretreatment of NL4-3 virions with PAP248-286(Ala) fibrils did not efficiently enhance fusion to $CD4^+$ PBLs when added at an equimolar or a 10-fold higher level relative to SEVI (Fig. 2B). In addition, PAP248-286(Ala) fibrils, in contrast to SEVI, did not greatly enhance HIV infection of TZM-bl reporter cells (Fig. 2C). Thus, although the absence of basic residues in SEVI does not prevent fibril formation in vitro, the resultant fibrils are deficient in enhancing HIV infection.

SEVI directly binds HIV through electrostatic contacts. The inability of PAP248-286(Ala) fibrils to enhance HIV fusion could reflect a failure to interact effectively with HIV virions and to bring them into close proximity to target cells. To directly test whether HIV virions bind PAP248-286(Ala) less efficiently than SEVI, we incubated SEVI or PAP248-286(Ala) fibrils with HIV virions, pelleted the virus-exposed fibrils by low-speed centrifugation, and measured HIV levels in the pellet and supernatant by a $p24^{Gag}$ ELISA. SEVI fibrils bound more than 60% of the input virus, whereas PAP248-286(Ala) fibrils bound less than 10% (Fig. 3A, panel i). These findings indicate that SEVI binds efficiently to HIV virions while PAP248-286(Ala) fibrils do not.

One concern was that the morphological differences in the SEVI and PAP248-286(Ala) fibrils might contribute to the their contrasting abilities to bind virions. We therefore sought an alternative approach to study whether the cationic properties of SEVI mediate its binding to HIV virions. If SEVI indeed interacts with HIV through electrostatic contacts, shielding the positive charges of SEVI with anionic polymers should nullify its ability to bind HIV during centrifugation. We therefore tested the ability of three naturally occurring polyanionic compounds, heparin, dextran sulfate, and chondroitin sulfate, to interfere with the interaction between SEVI and virions. When added concurrently with HIV to SEVI, heparin and dextran sulfate each markedly decreased this interaction; however, chondroitin sulfate did not effectively interfere with SEVI binding to HIV virions (Fig. 3A, panel ii).

The differences in the effects of the three polyanions might reflect differences in their relative anionic strengths, which are predominantly determined by the degree of sulfation. To test this possibility, we assessed the effects of anionic polymers that were subjected to chemical desulfation or oversulfation. Oversulfated chondroitin sulfate inhibited the binding of SEVI to HIV, as did oversulfated heparin, but desulfated heparin did not (Fig. 3A, panel iii). Together, these results suggest that highly sulfated polyanions inhibit the binding of SEVI to HIV virions.

To more closely examine the mechanism by which these anionic polymers inhibit the binding of SEVI to the virions, we determined whether preincubation of SEVI with polyanions blocks subsequent virus binding. We incubated SEVI with each of the polyanions and then pelleted the fibrils by low-speed centrifugation. If the polyanions directly bound to SEVI, then they should be associated with the pelleted fibrils. The pelleted, polyanion-treated SEVI was then resuspended in buffer and incubated with HIV to allow for virion binding. Fibrils were then centrifuged again, and the fraction of pelleted virions relative to the input was measured by a $p24^{Gag}$ ELISA. SEVI treated with heparin, dextran sulfate, oversulfated heparin, and oversulfated chondroitin sulfate blocked the binding of virions, suggesting that these polyanions had efficiently bound to SEVI during the preincubation. In contrast, mock-treated SEVI, as well as chondroitin sulfate-treated and desulfated heparin-treated SEVI, effectively pelleted HIV virions (Fig. 3B). These SEVI-associated virions retained the ability to infect cells as they readily induced β-galactosidase activity in TZM-bl reporter cells (Fig. 3C). These results suggest that highly sulfated polyanionic compounds can bind directly to SEVI and that this binding inhibits the ability of SEVI to subsequently bind to HIV.

Anionic polymers inhibit SEVI-mediated enhancement of HIV attachment to and fusion with cells. To determine if the polyanionic compounds that interfered with the binding of virions to SEVI also inhibited SEVI-mediated enhancement of viral fusion, we infected $CD4^+$ PBLs with virions that had been pretreated with SEVI in the presence of the various polyanionic compounds. Those agents that failed to inhibit the binding of SEVI to HIV (chondroitin sulfate and desulfated heparin) also failed to inhibit SEVI-mediated enhancement of fusion (Fig. 4A). In contrast, heparin, dextran sulfate, oversulfated heparin, and oversulfated chondroitin sulfate inhibited

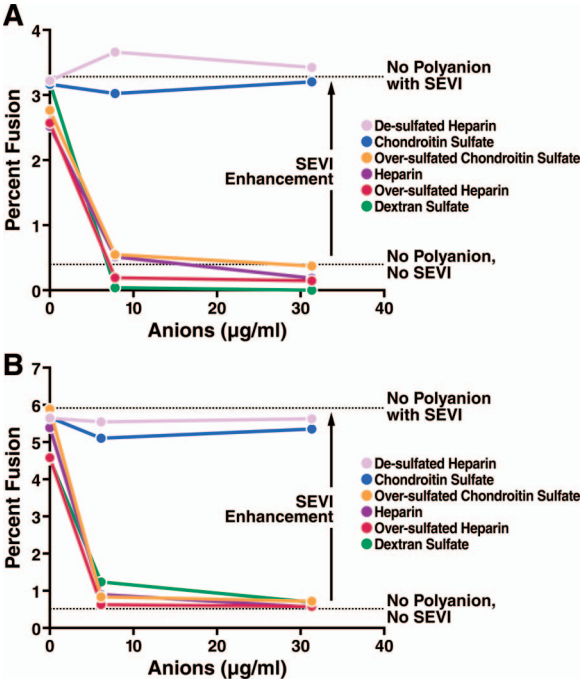

FIG. 4. Anionic polymers inhibit SEVI-mediated enhancement of viral fusion. (A) BlaM-Vpr-containing NL4-3 virions were incubated with SEVI and different concentrations of the indicated polyanionic compounds. The virions were then diluted fivefold to a final concentration of 50 ng/ml and added to $CD14^-$ $CD4^+$ T cells. The level of viral fusion in the absence of SEVI is indicated by the bottom dotted line, whereas the level of viral fusion in the presence of SEVI is indicated by the top dotted line. At concentrations greater than 8 μg/ml, heparin, oversulfated heparin, dextran sulfate, and oversulfated chondroitin sulfate inhibited SEVI activity. Desulfated heparin and chondroitin sulfate had minimal inhibitory effect at all of the concentrations tested. Results are representative of data from five separate experiments. (B) $CD14^-$ $CD4^+$ T cells were incubated with SEVI and different concentrations of the indicated anionic polymers. The cells were then washed and infected with BlaM-Vpr-containing NL4-3 and analyzed by flow cytometry for virion fusion. The level of viral fusion in the absence of SEVI is indicated by the bottom line, whereas the level of viral fusion in the presence of SEVI is indicated by the top dotted line. At concentrations greater than 8 μg/ml, heparin, oversulfated heparin, dextran sulfate, and oversulfated chondroitin sulfate inhibited SEVI activity. Desulfated heparin and chondroitin sulfate had minimal inhibitory effect at all of the concentrations tested. Results are representative of data from five separate experiments.

SEVI action at concentrations of greater than ~8 μg/ml (Fig. 4A).

Some of the polyanionic compounds, such as dextran sulfate, impaired virion fusion in the absence of SEVI (Fig. 5A), likely reflecting direct inhibitory effects on the HIV virions (17). To examine the effect of the anionic polymers on SEVI while minimizing their effect on the virions, the fusion assay was modified. The target cells, rather than the virions, were pretreated with SEVI in the presence or absence of the anionic polymers, washed extensively to remove excess polymers, and infected with HIV. Virion fusion was then assessed by flow cytometry. HIV infection of SEVI-treated cells was enhanced 10-fold relative to that of mock-treated cells (Fig. 4B), consistent with previous observations that pretreatment of cells with SEVI enhances subsequent

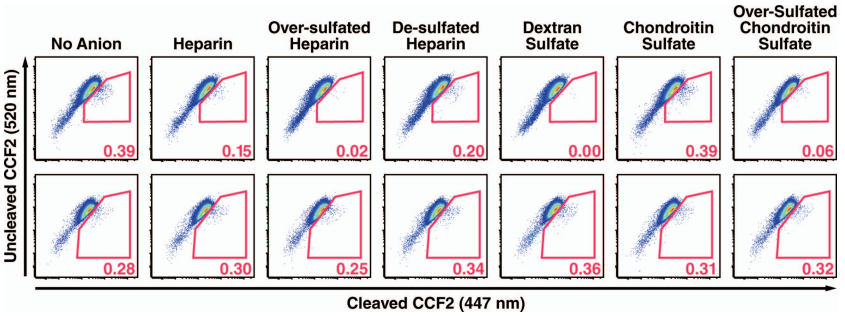

FIG. 5. Anionic polymer treatment of HIV and T cells in the absence of SEVI. (A) BlaM-Vpr-containing NL4-3 virions treated with the indicated polyanionic compounds were added to $CD14^-$ $CD4^+$ T cells. Values at the bottom right of plots reflect the percentages of cells that fused with virions. Treatment of virions with dextran sulfate and oversulfated heparin essentially eliminated viral fusion. (B) $CD14^-$ $CD4^+$ T cells were treated with the indicated anionic polymers, washed to remove excess polymers, and infected with BlaM-Vpr-containing NL4-3 virions. Values at the bottom right of plots reflect the percentages of cells that fused with virions. Importantly, none of the polyanionic compound-treated cells were completely unable to fuse with virions.

viral infection (9). Exposure of cells to heparin, dextran sulfate, oversulfated heparin, and oversulfated chondroitin sulfate inhibited the enhancing activity of SEVI, whereas chondroitin sulfate and desulfated heparin did not (Fig. 4B). Significantly, under these conditions, none of the anionic polymers abolished viral fusion in a SEVI-independent manner (Fig. 5B), presumably because these polymers were removed before the addition of the HIV. Decreasing the concentration of the inhibitory polyanionic compounds to below ~8 μg/ml greatly reduced their ability to block SEVI enhancement (Fig. 4B). The anionic polymers that inhibited SEVI-mediated enhancement of viral fusion also inhibited the ability of SEVI to enhance HIV infectivity in TZM-bl cells regardless of whether the virions or the target cells were pretreated with the anions (Fig. 6).

Next, we investigated whether the active anionic polymers inhibited the attachment of SEVI to the surface of host cells. In order to simultaneously visualize SEVI and the virions, we preincubated FITC-conjugated SEVI with CFP-labeled HIV virions in the presence or absence of anionic polymers and added this mixture to TZM-bl cells. SEVI fibrils efficiently attached to cells in the absence of polyanions (Fig. 7A and B), but this attachment was abrogated in the presence of heparin (Fig. 7C) or dextran sulfate (data not shown). As expected, heparin-treated samples, which lacked detectable surface-bound SEVI, exhibited decreased virion attachment to cells (Fig. 7C). These results suggest that polyanions can inhibit SEVI enhancing activity by inhibiting the attachment of SEVI/HIV complexes to the surface of cells.

Polyanions inhibit semen-mediated enhancement of viral infection. We next examined whether the anionic polymers inhibited the activity of endogenous SEVI in semen. In the absence of these agents, treating target cells with 10% semen enhanced HIV fusion approximately fivefold (Fig. 8). Addition of polyanionic compounds decreased this enhancement to various extents, depending on the specific polymer. Interestingly, desulfated heparin and chondroitin sulfate, the anionic polymers that did not effectively inhibit the enhancement activity of synthetic SEVI (Fig. 4), were also ineffective in decreasing semen-mediated enhancement of viral fusion. Dextran sulfate and oversulfated chondroitin sulfate were the most effective, whereas heparin and oversulfated heparin displayed intermediate effects (Fig. 8). Polyanion-mediated inhibition of semen's viral enhancement activity was similarly observed in an infection assay with TZM-bl cells (Fig. 9).

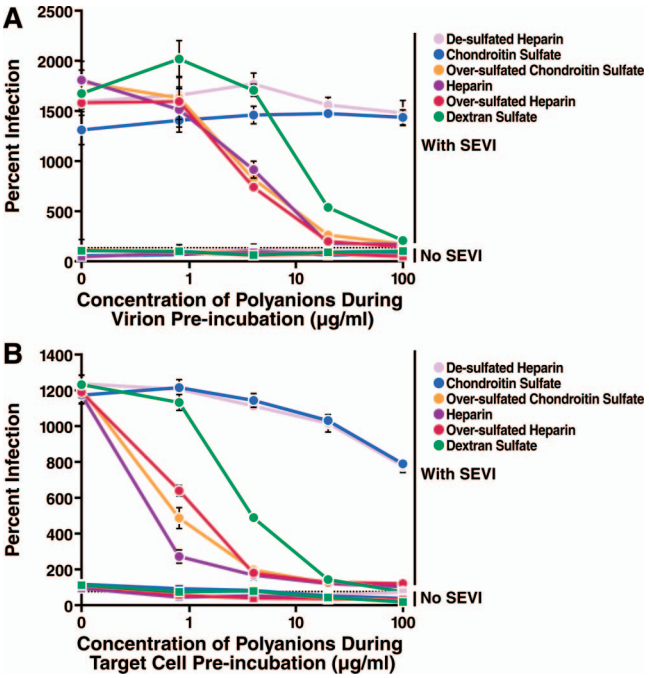

FIG. 6. Polyanionic compounds inhibit SEVI-mediated enhancement of HIV infection. (A) Virions were pretreated with SEVI and the indicated polyanionic compounds and then added to TZM-bl cells. (B) Alternatively, TZM-bl cells pretreated with the indicated anionic polymers were infected with HIV in the presence of SEVI. Cells were assayed for luciferase activity 2 days postinfection. Samples containing SEVI are graphed with circles, while samples lacking SEVI are graphed with squares. The polyanionic compounds that were used for the SEVI-treated samples are labeled. Samples treated with polyanions in the absence of SEVI are not labeled; however, the colors for these samples correspond to those for the samples containing SEVI. The values on the *y* axis were calculated based on the percent infection relative to samples infected in the absence of SEVI and anionic polymers. Shown are data representative of three independent experiments.

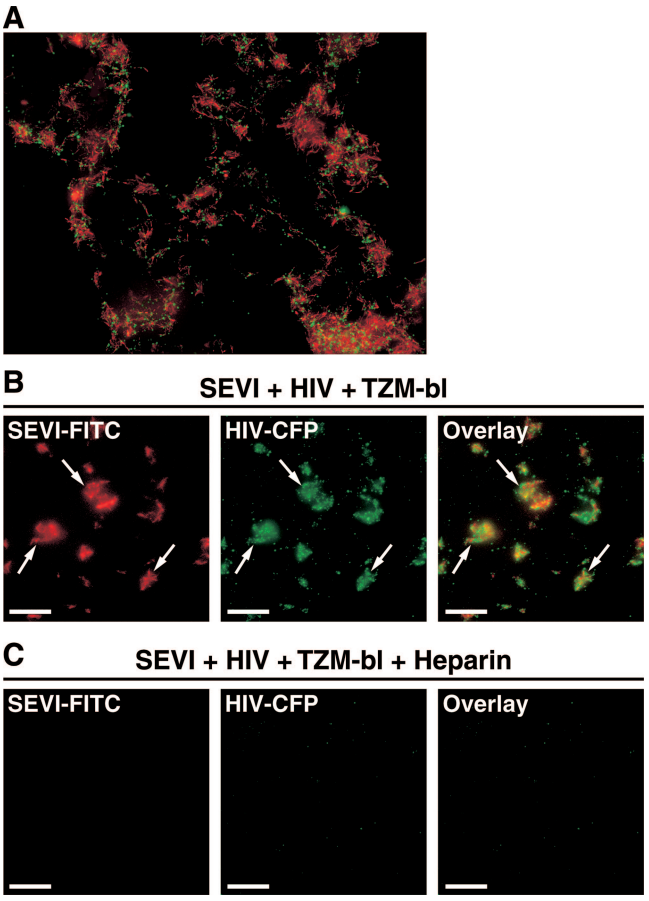

FIG. 7. Anionic polymers inhibit binding of SEVI to host cells. HEK293 supernatants containing CFP-labeled HIV virions were preincubated with FITC-conjugated SEVI in the absence or presence of heparin and then added to TZM-bl cells. Samples were then fixed and visualized by fluorescence microscopy. SEVI-FITC is shown in red, and HIV-CFP is shown in green. (A) High-resolution merged image demonstrating the binding of HIV virions to SEVI. (B) Binding of HIV virions to SEVI in the absence of heparin. Arrows indicate examples of SEVI/HIV complexes. (C) Heparin abolishes binding of SEVI and HIV virions to cells. Bars = 10 μm.

DISCUSSION

This study reveals that the cationic property of SEVI is crucial for its ability to efficiently enhance HIV infection. This enhancement results from the direct binding of SEVI to both HIV virions and target cells, thereby facilitating subsequent virion fusion. The mutant PAP248-286(Ala) peptide, which lacks lysines and arginines, forms fibrils that do not markedly enhance HIV infectivity. Furthermore, anionic polymers inhibit the binding of SEVI to HIV virions and host cells and also diminish both SEVI- and semen-mediated enhancement of HIV infectivity. The inhibition of SEVI activity by the polyanionic compounds was manifested both at the level of virion fusion and upon HIV infection of a reporter cell line. Of note, the magnitude of SEVI enhancement in the virion fusion assay was smaller than that in the reporter cell line, likely because the fusion assay requires exposure to substantially higher quantities of infectious HIV-1 particles, which translates to a lower enhancing effect mediated by SEVI (9).

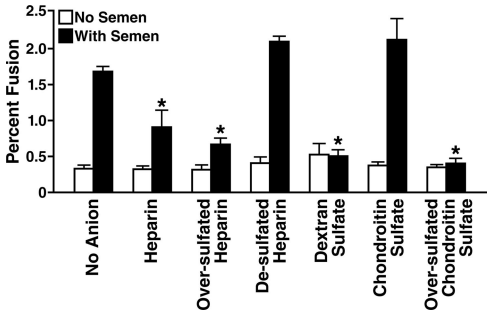

FIG. 8. Anionic polymers inhibit semen-mediated enhancement of viral fusion. $CD14^-$ $CD4^+$ T cells were incubated with medium or 10% semen in the presence of polyanionic compounds. The cells were then washed and infected with BlaM-Vpr-containing NL4-3, and the level of virion fusion was determined by flow cytometry. Values are the mean ± standard deviation from one of four experiments that yielded similar results. *, $P < 0.01$ versus with semen in the absence of anion (one-tailed t test).

Other cationic species have been shown to enhance viral infection. The best studied of these is Polybrene, a synthetic cationic polymer that enhances the infectivity of a variety of retroviruses, including HIV. Polybrene appears to enhance viral infectivity by neutralizing the charge repulsion between the virions and the cell surface (5). SEVI activity also enhances both R5 and X4 HIV-1 infections (Fig. 1C), and the magnitude of the effect is dependent on the infectivity of the virus stock rather than viral coreceptor tropism (9). Recent studies suggest that its effects also extend to a variety of other viruses (J.M. and F.K., unpublished data). Interestingly, SEVI appears to have a greater ability to enhance infection than Polybrene and is significantly less toxic (9). Therefore, SEVI may be useful in enhancing retrovirus-mediated gene transfer.

Another cationic species reported to enhance HIV infectivity is a multimeric form of the chemokine RANTES (1). At low

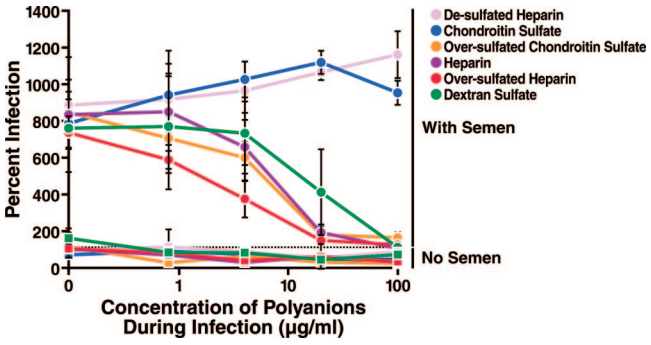

FIG. 9. Polyanionic compounds inhibit semen-mediated enhancement of HIV infection. Virions were treated with 10% diluted semen or mock treated for 10 min and then added to TZM-bl cells in the presence of the indicated anionic polymer. After 2 h of incubation at 37°C, the supernatant was removed and the cells were further cultivated in fresh medium. Cells were assayed for β-galactosidase activity 2 days postinfection. Samples containing semen are graphed with circles, while samples lacking semen are graphed with squares. Labeling of the graph is similar to the labeling used in Fig. 6. The values on the y axis were calculated based on the percent infection relative to samples infected in the absence of semen and polyanionic compounds. Similar results were obtained in two independent experiments.

concentrations, multimeric RANTES inhibits HIV infection by binding directly to CCR5 and inhibiting the binding of CCR5-tropic HIV virions to their coreceptors. However, at high concentrations, this form of RANTES aggregates and enhances viral infectivity. The enhancement is mediated by the binding of the highly positively charged RANTES multimers to proteoglycans on the surface of target cells (15). Whether SEVI binds directly to cell surface proteoglycans remains to be determined. Of note, unlike RANTES multimerization, which only occurs at high, nonphysiologic concentrations, the estimated concentration of PAP248-286 in semen is ~35 μg/ml (9) and thus is well within the range that enhances viral infectivity (Fig. 1C). Therefore, unlike RANTES, SEVI may play a role during natural transmission of HIV. It will be interesting to assess whether SEVI levels differ among males and whether HIV infection increases SEVI production. In addition, since HIV fusion to target cells shows many similarities to sperm-to-egg fusion (6), it will be interesting to determine whether SEVI has a physiological role in enhancing fertilization.

Only recently has the influence of the different components of semen on HIV infectivity been analyzed in a systematic manner. Semen appears to harbor factors such as SEVI that enhance HIV infection (9). It may also contain factors that inhibit viral infection, including a recently described factor that impairs *trans*-infection of HIV by binding directly to DC-SIGN on dendritic cells (12). The overall influence of semen on HIV infectivity may be dependent on the target cell type, as well as the experimental conditions during semen treatment. In the present study, we show that diluted pooled semen enhances both HIV fusion with $CD4^+$ PBLs and infection of a reporter cell line. Significantly, the same anionic polymers that inhibited the ability of SEVI to enhance infection also inhibited the viral enhancement activity mediated by semen.

We predict that these anionic polymers act both on SEVI and on other positively charged SEVI-like factors. Polyanionic compounds have long been investigated as prophylactic microbicides against HIV. They are thought to interfere with HIV infection by binding a basic region of the V3 loop of gp120 and inhibiting the attachment of virions to either CD4 or proteoglycans (3, 11, 17). Unfortunately, anionic polymers such as cellulose sulfate have failed in clinical trials as they enhanced rather than inhibited HIV transmission (16). The failure of these polymers likely stemmed from their induction of an inflammatory environment in the female genital tract, a condition that favors HIV infection. An effective microbicide will need to maximally inhibit HIV infectivity without inducing tissue inflammation or other conditions favoring HIV transmission. Given our data that at least some anionic polymers can block the activity of SEVI, we suggest that combination microbicides incorporating compounds that have virucidal properties and those that block the activity of viral enhancement factors present in semen should be tested. Such an approach, attacking both the virus and host proinfectivity factors, could lead to a highly effective topical approach for preventing HIV transmission.

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