Semen-Derived Amyloid Fibrils Drastically Enhance HIV Infection

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SUMMARY

Sexual intercourse is the major route of HIV transmission. To identify endogenous factors that affect the efficiency of sexual viral transmission, we screened a complex peptide/protein library derived from human semen. We show that naturally occurring fragments of the abundant semen marker prostatic acidic phosphatase (PAP) form amyloid fibrils. These fibrils, termed Semen-derived Enhancer of Virus Infection (SEVI), capture HIV virions and promote their attachment to target cells, thereby enhancing the infectious virus titer by several orders of magnitude. Physiological concentrations of SEVI amplified HIV infection of T cells, macrophages, ex vivo human tonsillar tissues, and transgenic rats in vivo, as well as *trans*-HIV infection of T cells by dendritic or epithelial cells. Amyloidogenic PAP fragments are abundant in seminal fluid and boost semen-mediated enhancement of HIV infection. Thus, they may play an important role in sexual transmission of HIV and could represent new targets for its prevention.

INTRODUCTION

HIV-1, the causative agent of AIDS, has infected about 60 million people and caused over 20 million deaths. More than 80% of these HIV-1 infections are acquired through sexual intercourse. Despite its dramatic spread in the human population, the efficiency of HIV-1 transmission via the sexual route is surprisingly poor. For instance, the risk of male-to-female intravaginal HIV-1 transmission is estimated at about 1 event per 200–2000 coital acts (Gray et al., 2001). This rate is about 10-fold increased during acute infection when the viral load is particularly high (Pilcher et al., 2004). Moreover, the presence of other sexually transmitted diseases and sexual practices associated with bleeding and lesions of the mucosal barrier can increase this risk to up to 3% per sexual contact (Galvin and Cohen, 2004). Nevertheless, the poor transmissibility of HIV-1 is clearly a major factor restricting the AIDS pandemic.

Globally, most infections result from genital exposure to semen (SE) of HIV-positive men (Royce et al., 1997). Women who acquired HIV-1 through vaginal intercourse constitute almost 60% of new infections in Africa (reviewed in Haase, 2005). The infectivity of HIV-1 in male genital fluid together with the susceptibility of the host, the type of sexual practice, and the viral load are major determinants of sexual transmission (Chakraborty et al., 2001; Gupta et al., 1997; Pilcher et al., 2004). The factors modulating HIV infectiousness in SE are poorly understood (reviewed in Miller and Shattock, 2003).

To identify natural agents that might play a role in sexual transmission of HIV/AIDS, we screened a complex peptide/protein library derived from human seminal fluid (SE-F) for novel inhibitors and enhancers of HIV infection. We found that fragments of prostatic acidic phosphatase (PAP) drastically enhance HIV infection. Functional and structural analyses showed that these peptides form amyloid fibrils that capture HIV particles and strongly enhance the number of productively infected cells by promoting virion-cell attachment and fusion. In agreement with a relevant role in vivo we found that semen and

seminal fluid also drastically enhance HIV infection and provide evidence that fibril-forming PAP fragments contribute to this effect. Our data support that amyloidogenic peptides are abundant in semen and promote sexual transmission of HIV/AIDS.

RESULTS

Purification of an Enhancer of HIV-1 Infection from Semen

Our recent isolation of a novel HIV-1 entry inhibitor (Münch et al., 2007) shows that screening of peptide libraries from human body fluids is a useful approach to discover as-yet-unknown molecules modulating HIV-1 infection. To identify factors that play a role in sexual transmission of HIV-1, we analyzed a complex peptide/protein library derived from pooled human SE. This library encompassed 294 fractions and ought to represent all peptides and small proteins (MW < 50 kDa) present in seminal fluid (SE-F). We found that fraction 29 of pH pool 7 significantly enhanced HIV-1 infection (Figure 1A). This fraction contained only small amounts of peptide/protein (Figure 1A, left inset). Mass spectrometry (MS) after one additional round of purification identified several peptides in the active fractions, ranging from 4028 to 4551 Da (Figure 1A, right inset). Peptide sequencing identified them as fragments of PAP (Table S1). This protein is produced by the prostatic gland, secreted in large amounts (1–2 mg/ml) into SE (Rönnberg et al., 1981), and used as a SE marker (Graves et al., 1985). All peptides mapped to the same region of PAP but differed in length from 34 to 40 residues. The predominant form of 4551 Da (Figure 1A, right inset) corresponds to amino acids 248 to 286 of PAP (EMBL accession number AAB60640).

Next, we verified that chemically synthesized PAP peptides also enhance HIV-1 infection, whereas control peptides, including a sequence scrambled variant of the predominant PAP fragment (PAPscr), had no effect (Figure 1B and Table S1). The PAP peptides promoted HIV-1 infection in the absence of fetal bovine serum (Figure S1) indicating that no serum cofactor is required. If not mentioned otherwise, all subsequent experiments were performed with the synthetic peptide corresponding to the major form detected in the SE-F (PAP248-286). Examination of CEMx174 5.25 M7 (CEMx M7) cells containing the GFP reporter gene under the control of the HIV-1 promoter (Hsu et al., 2003) by fluorescence microscopy (Figure 1C) and flow cytometry (Figure 1D) confirmed that PAP248-286 drastically increases the number of HIV-1-infected cells. In contrast, it did not affect the transcriptional activity of the HIV-1 LTR promoter or Env-mediated cell-cell fusion (Figure S2). Fresh PAP248-286 solutions were inactive in promoting HIV-1 infection. After overnight incubation, however, they enhanced HIV-1 infection more efficiently and were less cytotoxic than the polycation polybrene (Figure 1E), commonly used to boost HIV-1 infection or retroviral gene transfer. In comparison, full-length PAP nei-

ther promoted HIV-1 infection nor inhibited the enhancing activity of PAP248-286 (Figure 1F).

PAP Fragments form Amyloid Fibrils that Promote HIV Infection

We noted that PAP248-286 and other PAP fragments increased HIV-1 infection only when the solutions became turbid either spontaneously during storage or after agitation and found that the precipitate contains the active form (Figure 2A). It has been previously shown that amyloid fibrils associated with Alzheimer's disease enhance HIV-1 infection (Wojtowicz et al., 2002). Thus, we examined whether PAP fragments also form amyloid fibrils. We found that agitation of fresh PAP248-286 solutions induced a strong increase in Thioflavin T binding (Figure 2B), in green birefringence upon staining with Congo red (data not shown), and in β sheet content (Figure S3). Electron microscopy confirmed effective fibril formation (Figure 2C), and X-ray powder diffraction demonstrated reflections at 4.7 and 10.6 Å, which correspond to the regular interstrand spacing and intersheet distances, respectively (Figure 2D). All these properties are typical for amyloid fibrils (reviewed in Nilsson, 2004). Length variations at the N terminus of the PAP fragments did not impair fibril formation or enhancement of HIV-1 infectivity, whereas deletion of the four C-terminal (LIMY) residues reduced both effects (Figures 1B, S4, and S5). However, the PAP247-282 fragment lacking the LIMY region did not exert efficient transdominant-negative effects on fibril formation by other PAP fragments (Figure S4). Notably, PAP fragments were substantially more potent in enhancing HIV-1 infection than other amyloidogenic peptides (Figure S5). Since the amyloid fibrils strongly enhanced the infectivity of HIV-1 we refer to them herein after as Semen-derived Enhancer of Virus Infection (SEVI).

To assess whether SEVI interacts directly with HIV-1 particles, we preincubated virus stocks with the fibrils for 5 min in a small volume and subsequently added the virus/SEVI mixture to the cell culture, thereby diluting it 50-fold. Diluting the HIV-1/SEVI mixture did not reduce the magnitude of infectivity enhancement (Figure S6A), implying that SEVI efficiently bound to the virions. However, preincubation of the target cells with SEVI also enhanced HIV-1 infection, even after extensive washing (Figure S6B). To clarify whether SEVI promotes a physical interaction of virions with target cells, we incubated TZM-bl cells with HIV-1 particles in the presence or absence of SEVI. Viral binding was assessed by the amount of cell-associated p24 after extensive washing. SEVI significantly enhanced binding of both wild-type HIV-1 particles and virions lacking Env, although the absolute levels of cell-associated p24 were about 30-fold lower in the absence of Env (Figure 2E). To directly visualize the effects of SEVI, we monitored infection of TZM-bl cells microscopically using fluorescently labeled HIV-1 virions. We found that SEVI drastically enhanced virion binding to the cells and the coverslips (Figure 2F). Parallel bright-field phase images demonstrated that SEVI fibrils are loaded with
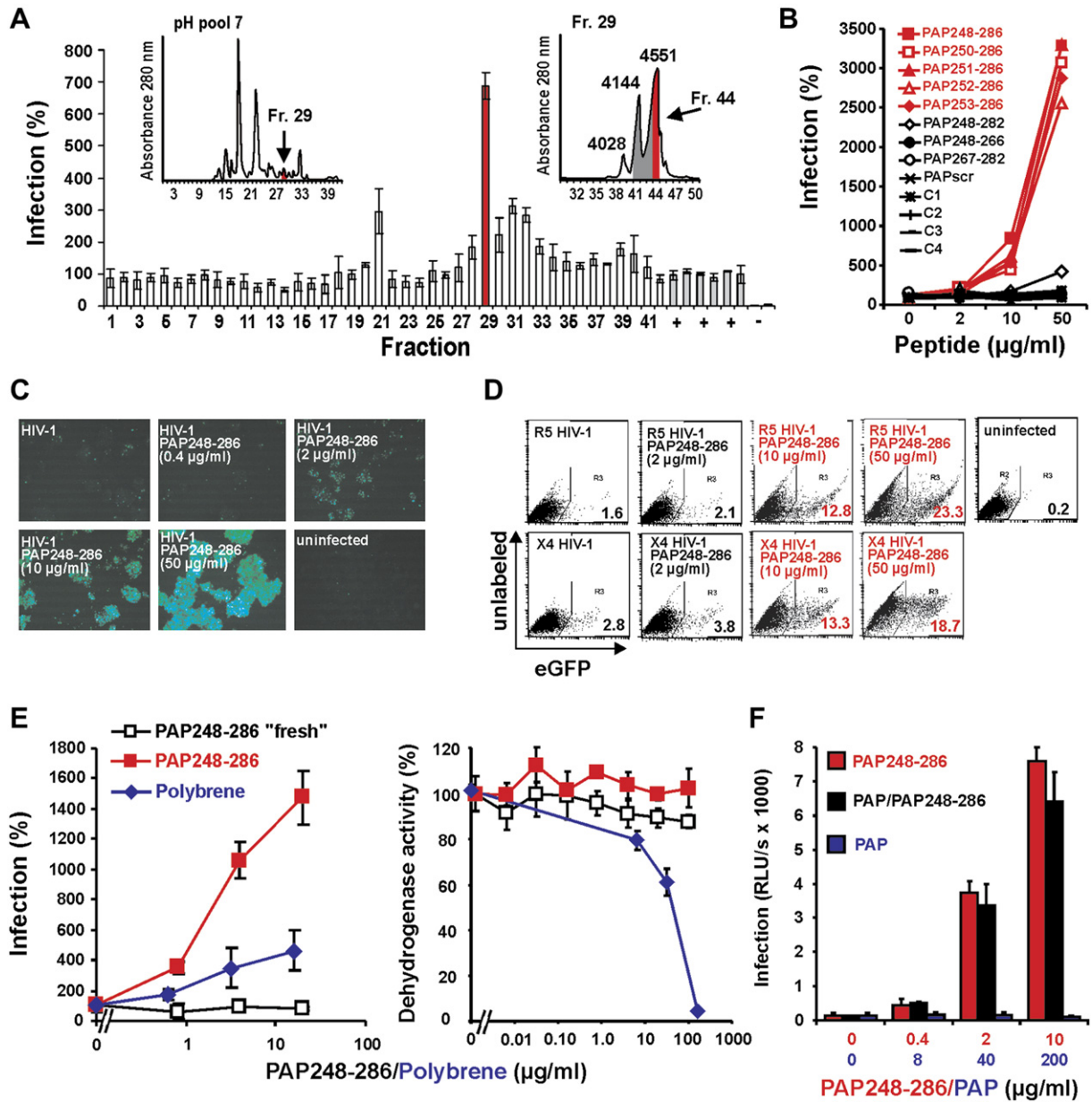
Figure 1. Purification of an Enhancer of HIV-1 Infection from Seminal Fluid

(A) SE-derived fraction 29 of pH pool 7 (red) promotes HIV-1 infection of P4-CCR5 cells. The inlets show the HPLC chromatograms of pH pool 7 (left) and of the active fraction 29 (right). Fraction 44 used for peptide sequencing is shown in red and the molecular masses of the major peaks are indicated. +, no peptide added and −, uninfected cells. Average values (± standard deviation [SD]) of triplicate measurements are shown in panels (A) and (F).

(B) Synthetic PAP fragments enhance HIV-1 infection. Infectivities were determined in TZM-bl cells and are shown relative to those measured in the absence of peptide (100%). Data represent mean values obtained from quintuple infections. Numbers correspond to the amino acid positions in full-length PAP; PAPscr represents a scrambled form of PAP248-286. C1 to C4 are structurally and functionally unrelated control peptides. Active peptides are indicated in red.

(C and D) Analysis of CEMx M7 cells infected with HIV-1 in the presence of PAP248-286 by (C) fluorescence microscopy and (D) flow cytometric analysis. The numbers in (D) indicate the percentages of HIV-1-infected (GFP+) cells.

(E) Active PAP248-286 enhances HIV-1 infection of TZM-bl cells more efficiently and is less cytotoxic than Polybrene. The effects of PAP248-286 that was freshly diluted or incubated overnight and Polybrene on viral infectivity (left) and metabolic activity (right) are shown relative to those measured in the absence of peptide (100%) and were derived from sextuple measurements.

(F) Full-length PAP does not enhance HIV-1 infection. TZM-bl cells were infected in the presence of PAP248-286 and full-length PAP agitated overnight or mixtures thereof. Similar results were obtained with full-length PAP agitated for up to one week.
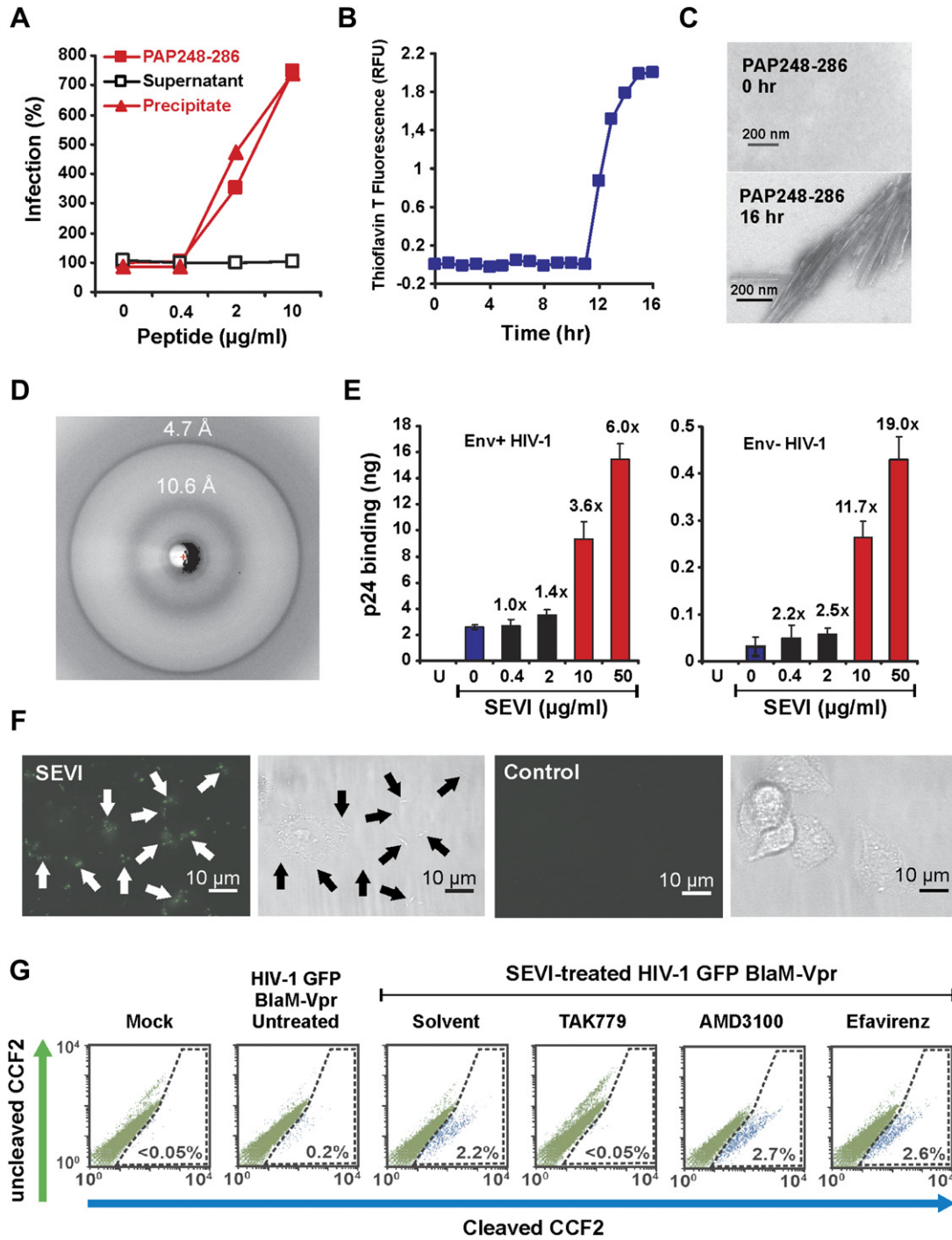
Figure 2. PAP248-286 Forms Fibrils that Enhance Virion Attachment and Fusion

(A) Effect of PAP248-286 agitated overnight, the clear supernatant, and the redissolved pellet on HIV-1 infection of CEMx M7 cells. Average values obtained from sextuple infections are shown.

(B) PAP248-286 aggregation to active fibrils monitored by Thioflavin T fluorescence. RFU, relative fluorescence units.

(C) Electron micrographs of freshly diluted PAP248-286 and after agitation for 16 hr.

(D) SEVI was subjected to X-ray powder diffraction. The resulting diffraction pattern, exhibiting strong reflections at 4.7 and 10.6 Å, is characteristic of an amyloid cross β sheet structure.

(E) SEVI enhances binding of wild-type (left) and Env-defective (right) HIV-1 particles to TZM-bl cells. Shown are average values (±SD) of sextuple measurements. The numbers above the bars give n-fold enhancement of p24 binding relative to that measured in the absence of SEVI.

sequestered HIV-1 virions, and timelapse microscopy revealed that they are efficiently captured and internalized by cellular protrusions (Movies S1 and S2). Electron microscopy confirmed uptake of PAP248-286 fibrils into the cells (Figure S7). However, despite evidence for the internalization of fibrils into cells, enhancement of HIV-1 infection by SEVI was not affected when TZM-bl cells were pretreated with various inhibitors of phosphatidylinositol 3-kinase and contractile actin microfilaments that all block phagocytosis (Figure S8). Thus, phagocytosis is not critical for the ability of SEVI to promote HIV-1 infection.

To further elucidate how SEVI promotes viral infection we monitored both HIV-1 virion fusion and viral gene expression from the same infection (Cavrois et al., 2002; Goffinet et al., 2007). We found that SEVI enhanced HIV-1 virion fusion and subsequent gene expression about 10-fold (Figures 2G and S9). The fusion inhibitor T20 and the CCR5 antagonist TAK779 blocked CCR5-tropic HIV-1 YU2 virion fusion, whereas the CXCR4 ligand AMD3100 had no inhibitory effect. Predictably, the RT inhibitor Efavirenz blocked viral gene expression but not virion fusion (Figure S9). Altogether, these results suggest that SEVI enhances HIV-1 infection by capturing virions and promoting their physical interaction and fusion with target cells but does not bypass the requirement for the appropriate coreceptor, e.g., by disrupting the integrity of the cell membrane.

SEVI Is a General Enhancer of HIV-1 Infection

To examine whether SEVI-mediated enhancement of HIV-1 infection depends on the viral geno- or phenotype we analyzed its effect on a large panel of HIV-1 variants. We found that SEVI enhanced infection by R5- X4-, and dual-tropic HIV-1 clones at concentrations $\geq$ 0.8 μg/ml (Figure S10). Irrespective of the viral pheno- or genotype, SEVI also increased infection by all primary group M and O HIV-1 strains tested (Figure 3A). The most dramatic effects were observed after infection with low viral doses (Figure 3B), and the magnitude of SEVI-mediated HIV-1 infectivity enhancement was inversely correlated to the virus inoculum or infectious dose (Figures S11 and 3C). Thus, the enhancing activity of SEVI is most pronounced when the levels of infectious virus are low and hence resemble the conditions of sexual HIV-1 transmission. Notably, the inverse correlation between viral infectivity and the magnitude of viral infectivity enhancement by SEVI was stronger for individual HIV-1 molecular clones than for different primary HIV-1 isolates (Figures 3C and S11B). Thus, viral properties, such as CD4 and coreceptor affinities, may also impact SEVI-mediated enhancement of HIV-1 infection.

Next, we analyzed whether the observed effects may be cell type dependent. Our data demonstrate that SEVI enhanced infection of various indicator cell lines with similar efficiency (Figure S12). More importantly, SEVI also efficiently increased HIV-1 infection of peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) by both R5 and X4 HIV-1 (Figures 4A and 4B). The effect of SEVI on X4 HIV-1 infection of macrophages was relatively weak, most likely because MDM express low levels of CXCR4 and the amyloid fibrils do not bypass the requirement for the appropriate coreceptor. In addition to the infection of cells at the site of exposure, binding of viral particles to DC-SIGN expressed on dendritic cells (DCs) and their subsequent dissemination to lymphatic organs may play a role in sexual transmission and systemic spread of HIV (Geijtenbeek et al., 2000). Therefore, we examined whether SEVI affects HIV-1 infection in DC-T cell cocultures. Predictably, binding of HIV-1 to DCs strongly enhanced trans-HIV-1 infection of T cells. Remarkably, SEVI amplified infection of T cells by HIV-1 particles bound to DCs even further by up to 24-fold (Figure 4C). It has been observed that SE-F enhances binding of virions to epithelial cells in ex vivo cervicovaginal tissue (Maher et al., 2005). Following this observation, we examined whether SEVI might also promote trans-HIV-1 infection in the absence of DC-SIGN. The HeLa human epithelial carcinoma cell line (Scherer et al., 1953) remained nonpermissive for HIV-1 infection even in the presence of SEVI (data not shown). However, SEVI increased the ability of HeLa cells to transmit R5- and X4-tropic HIV-1 to T cells by 30- to 70-fold (Figure 4D). Thus, SEVI may promote virus attachment to genital surfaces, penetration of the mucosal barrier, and subsequent dissemination to lymphoid organs by increasing HIV-1 virion binding to epithelial cells and to migrating DCs.

SEVI Increases the Infectious Titer of HIV-1 up to Five Orders of Magnitude

The magnitude of HIV-1 entry enhancement in infectivity assays may underestimate the real potency of SEVI because 0.5% to 5% of the target cells already became infected in its absence. To determine the effect of SEVI on the $TCID_{50}$ (50% tissue culture infectious dose) of virus stocks more accurately, we performed thorough limiting dilution infection assays. Treatment with SEVI enhanced the $TCID_{50}$ of HIV-1 in CEMx M7 cells by four orders of magnitude (Figures 5A and 5B). Amazingly, up to 400,000-fold enhancement was measured in PBMC cultures (Figures 5A and 5B). Notably, these assays detected spreading HIV-1 infection, implying that the detected virions were replication competent. We calculated the number of virions in our virus stocks assuming that there are

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(F) Effect of SEVI on binding of HIV-1 virions to TZM-bl cells. Cells were infected for 30 min with equal doses of infectious fluorescently labeled HIV-1 virions in the absence or presence of SEVI (20 ug/ml). The arrows indicate the positions of fibrils.

(G) SEVI enhances virus fusion. Shown are representative FACS dot plots for the detection of CCF2 substrate cleavage in TZM-bl cells that were either mock-infected or infected with untreated or SEVI-treated HIV-1R7/3YU-2 Env GFP BlaM-Vpr. The CCR5 antagonist TAK779, the CXCR4 antagonist AMD3100, and the NNRTI Efavirenz were added to the cells 1 hr prior to virus challenge.
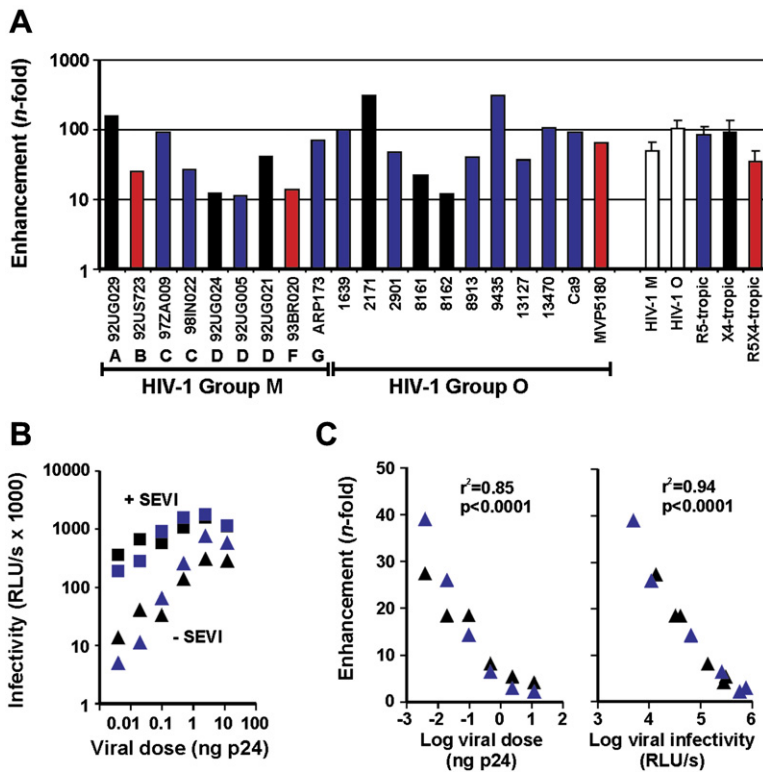
Figure 3. SEVI Enhances HIV-1 Infection Independently of the Viral Geno- and Phenotype

(A) Relative effect of SEVI (5 μg/ml) on infection of CEMx M7 cells by primary R5- (blue), X4- (black), and dual-tropic (red) HIV-1 M and O isolates. (A) and (C) show the average enhancement (n = 2) of the infectivity levels relative to those measured in the absence of SEVI.
(B) SEVI enhances HIV-1 infection most efficiently after low-dose infection. TZM-bl cells were infected with the indicated doses of X4 (black) or R5 (blue) HIV-1 in the presence (squares) or absence (triangles) of SEVI (10 μg/ml). Each symbol represents the average β-galactosidase activity (n = 3) measured 3 days after virus exposure. RLU/s: relative light units per second.
(C) Correlation between the magnitude of SEVI-mediated enhancement of HIV-1 infection and the p24 content (left) or the infectivity (right) of the viral stocks used for infection. Shown is the average (n = 3) enhancement of HIV-1 infection by SEVI (10 μg/ml) measured after inoculation of TZM-bl cells with virus stocks containing the indicated quantities of p24 antigen content (left) or as a function of the β-galactosidase reporter gene activities measured after infection with the untreated virus stocks (right).

5000 p24 CA proteins per viral particle (Briggs et al., 2004) and determined the genomic HIV-1 RNA copy numbers by quantitative real-time PCR analysis. The results obtained by both methods showed that just one to three virions are usually sufficient for productive HIV-1 infection of PBMC in the presence of SEVI.

To assess a potential role of SEVI in oral HIV-1 transmission, we inoculated unstimulated ex vivo human tonsillary tissue (HLT) with a dose of HIV-1 (0.1 pg p24) that is usually subinfectious. Efficient replication of HIV-1 was observed, however, in the presence of SEVI (Figure 5C). Control experiments showed that AZT blocked p24 production by HLT infected ex vivo with X4 and R5 HIV-1 clones both in the absence or presence of amyloid fibrils (Figure 5D). Thus, SEVI enhances HIV-1 replication in ex vivo infected HLT and not the desorption of inoculated virions. These results demonstrate that SEVI lowers the viral threshold required for productive HIV-1 infection of human tonsillar tissues ex vivo.

PAP Fragments Boost Semen-Mediated Enhancement of HIV Infection

We isolated about 10 mg SEVI/PAP fragments from 283 ml pooled SE-F, which corresponds to a yield of about 35 μg/ml. PAP, the precursor of SEVI, is secreted into SE at quantities of 1 to 2 mg/ml (Rönnberg et al., 1981). Thus, this concentration of SEVI/PAP fragments can be achieved by degradation of about 20% of its precursor. Importantly, SEVI already enhanced HIV-1 infection at concentrations $\geq$ 2 μg/ml (Figures 1E, 4, and 5B). To

directly test whether SE contains PAP fragments in sufficient quantity for HIV-1 infectivity enhancement, we analyzed partially purified peptide samples from individual SE donors. Proteins > 50 kDa were removed from the SE-F by ultracentrifugation and the resulting peptide/protein mixture was separated by a single RP chromatography step. Infection of TZM-bl cells with HIV-1 in the presence of fractions predicted to contain amyloidogenic PAP fragments revealed that they efficiently enhance HIV-1 infection after short-term storage without agitation (Figure S13).

Next, we assessed whether SEVI is active under conditions resembling the deposition of HIV-infected SE in the genital tract. As expected from published data (Kiessling, 2005), SE and SE-F were highly cytotoxic (Figure S14). To minimize these toxic effects we mixed virus stocks with equal volumes of fresh SE spiked with SEVI and added 25 μl of these mixtures to 975 μl CEMx M7 cell cultures. The results showed that SEVI enhances the infectiousness of HIV-1 in SE and SE-F in a dose-dependent manner (Figures S15). To assess whether active PAP aggregates may form in vivo, we spiked SE-F with unassembled PAP248-286. Unexpectedly, the PAP248-286 fragments boosted the ability of SE-F to enhance HIV-1 infection even at the first time point analyzed (Figure S16A), indicating that fibrils or other active aggregates already formed during freezing and thawing of the SE-F samples. At an acidic pH of 4.2, which resembles the vaginal environment, this enhancing effect became apparent after 30 min and was more pronounced after 2 hr (Figure S16A). Our finding
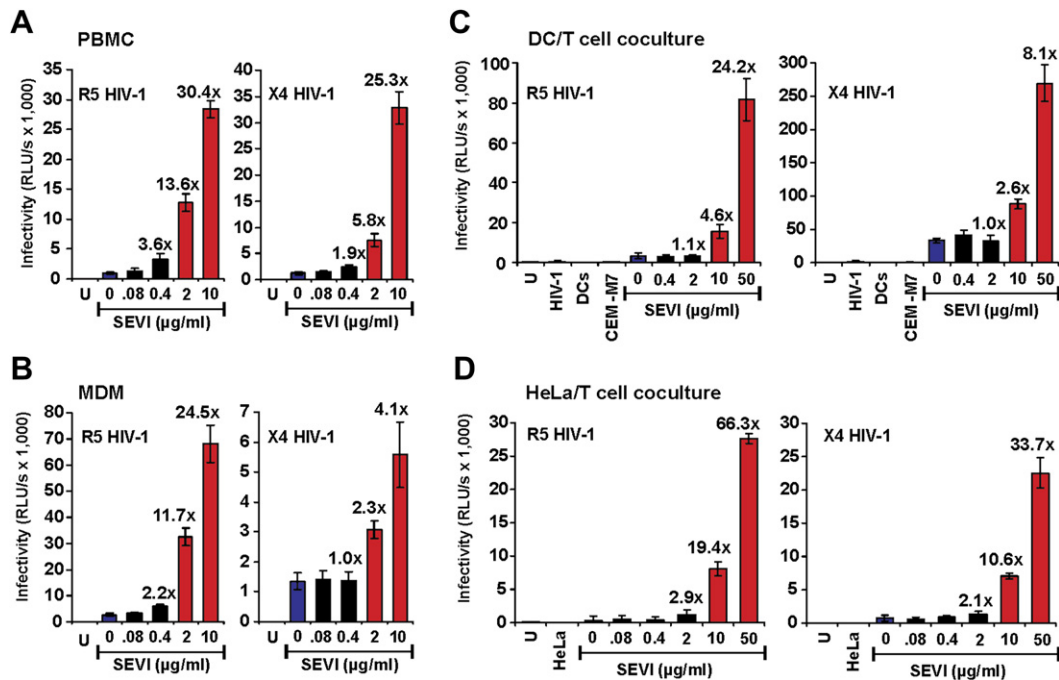
Figure 4. The Effect of SEVI on HIV-1 Infection Is Cell Type Independent
(A and B) Effect of SEVI on R5- or X4-tropic HIV-1 infection of PBMCs (A) and macrophages (B).
(C and D) SEVI enhances in *trans*-infection of T cells by viral particles bound to DCs (C) or HeLa cells (D). The numbers indicate n-fold infectivity enhancement observed in the presence of the indicated concentrations of SEVI compared to those measured in the absence of SEVI. U, uninfected cells; HIV-1, wash control; DCs, HeLa and CEMx M7, indicated cell type only. All data shown in this figure give average values ± SD obtained from triplicate or quintuple infections.

that amyloidogenic PAP fragments rapidly acquire the ability to increase HIV-1 infection suggests that smaller aggregates that typically precede mature fibril formation may also enhance HIV-1 infection. In agreement with this possibility, we found that fresh PAP248-286 solutions already moderately enhanced HIV-1 infection after 1 hr of agitation and that this effect saturated after 7 hr (Figure S16B). In comparison, Congo Red or Thioflavin T binding increased with slower kinetics and did not saturate within the 24 hr investigation period. Different assay sensitivities and the possibility that mature fibril formation may occur during the 2 hr infection period prevent definitive conclusions. Nevertheless, these data support that PAP248-286 aggregates forming prior to mature fibrils also promote HIV infection.

Semen Increases HIV-1 Infection
To analyze whether semen directly affects the infectiousness of HIV-1, we mixed virus stocks with SE, SE-F, or the semen pellet (SE-P) containing the spermatocytes and large protein aggregates. Subsequently, these mixtures were added to TZM-bl cell cultures thereby diluting them 15-fold. After 3 hr the virus/semen-containing medium was replaced by fresh medium. Under these conditions, mixtures containing up to 10% SE, SE-F, or the SE-P during virion incubation displayed only weak cytotoxic effects particularly at high cell density (Figure S17A). Treatment of

virus stocks with SE, SE-F, and SE-P drastically enhanced HIV-1 infection (Figures 6A and S17B) and virus-induced cytopathic effects (Figure 6B). Similarly to SEVI-mediated infectivity enhancement (Figure 3B), the most dramatic effects of SE were observed after inoculation with low viral doses (Figure 6C). SE, SE-F, and the SE-P also increased transmission of R5 HIV-1 variants from HeLa cells to TZM-bl cells and infection of CEMx M7 cells (Figures 6D and 6E). Acidification enhanced Thioflavin T binding by SE and SE-F but did not markedly alter their ability to promote HIV-1 infection (Figure S18). Thus, acidification of semen and seminal fluids most likely results in the precipitation of proteins/peptides that increase nonspecific Thioflavin T binding, rather than in the formation of amyloid fibrils. More importantly, the results demonstrate that an acidic pH, resembling the conditions in the vaginal tract, does not inhibit enhancement of HIV-1 infection by SE and SE-F.

To further analyze the enhancing factor(s), we agitated fresh, clear SE-F overnight. The solution became slightly turbid and newly aggregated peptides and proteins were preciptitated by centrifugation. The supernatant was discarded and the pellet dissolved in the same volume of PBS. Treatment of virus stocks with an equal volume of this solution potently enhanced HIV-1 infection (7.2 ± 1.7-fold, n = 3) showing that the precipitate contains a significant proportion of the enhancing activity. Further
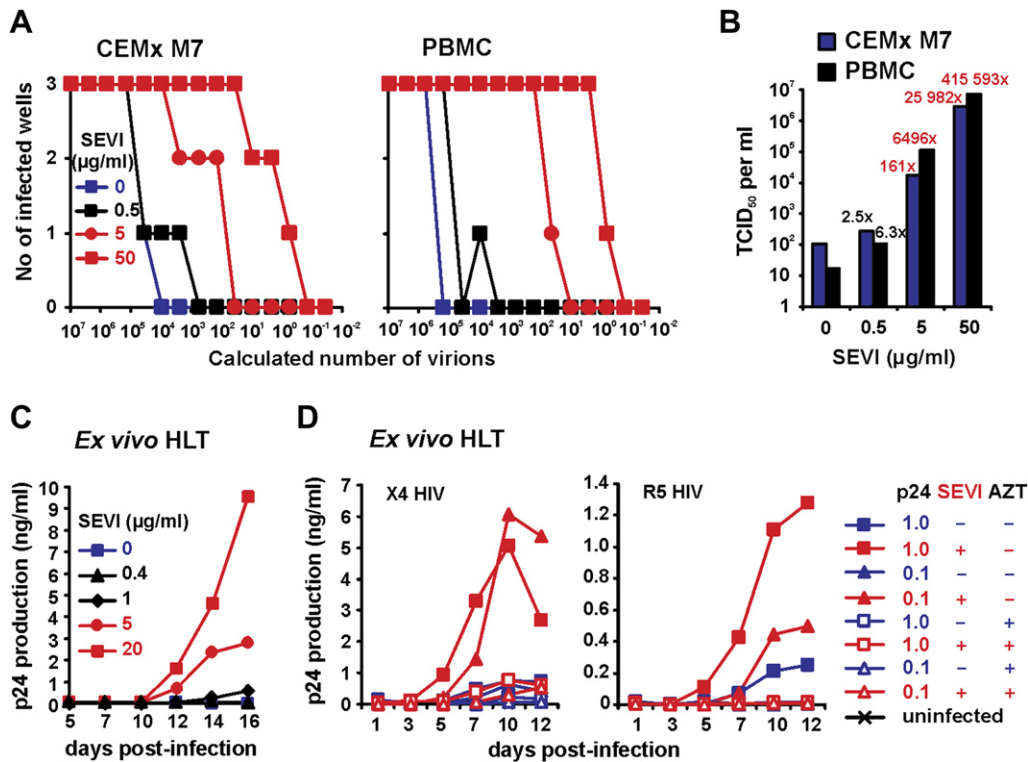
Figure 5. SEVI Lowers the Threshold Required for Productive HIV-1 Infection

(A) Limiting dilution analysis of HIV-1. CEMx M7 cells (left) or PBMCs (right) were infected in triplicate with 4-fold dilutions of an HIV-1 NL4-3 virus stock in the presence of the indicated concentrations of SEVI. Indicated is the number of cultures that became productively infected. Similar results were obtained using different X4- and R5-tropic virus stocks.

(B) Effect of SEVI on the $TCID_{50}$ of HIV-1. $TCID_{50}$ values were calculated based on the infectivity data shown in (A). The numbers above the bars indicate n-fold enhancement compared to the titer measured in the absence of SEVI.

(C) Effect of SEVI on HIV-1 replication in ex vivo-infected human lymphoid tissue. Tonsillary tissue was infected ex vivo with a very low viral dose (0.1 pg p24) of X4-tropic HIV-1 that has been preincubated with different concentrations of SEVI. Similar results were obtained in two independent experiments using tissues derived from different donors.

(D) Influence of AZT on HIV-1 replication in ex vivo HLT. Tissues were infected with untreated (blue symbols) or SEVI-treated (50 μg/ml; red symbols) X4 (left panel) or R5 (right panel) HIV-1 virus stocks containing 1.0 or 0.1 ng of p24 antigen, respectively, and subsequently cultured in the presence (open symbols) or absence (filled symbols) of AZT.

analysis of the compounds present in this small precipitate by RP-HPLC showed that fractions eluting at retention times similar to synthetic PAP fragments contained primarily a peptide of 4553 Da, corresponding to PAP248-286 (Figure 6F). These results clearly support that endogenous PAP248-286 fragments form precipitable aggregates in SE-F that contribute to the enhancing effect of semen on HIV-1 infection.

SEVI Enhances HIV-1 Infection In Vivo

It has been established that rats transgenically expressing human CD4 and CCR5 on T cells and macrophages are susceptible to HIV-1 infection (Keppler et al., 2002; Goffinet et al., 2007). To explore the ability of SEVI to enhance HIV-1 infection in vivo, hCD4/hCCR5-transgenic rats were challenged with HIV-1 YU2 by tail vein injection. Four days after inoculation between 3 and 12 copies of HIV-1 cDNA per ng of total DNA were detected in splenocyte extracts derived from four rats infected with untreated virus stocks

(Figure 7). In comparison, the relative viral cDNA copy numbers were about 5-fold higher ranging from 21 to 49 of cDNA per ng of total DNA in five rats that received the same dose of SEVI-treated HIV-1. Thus, SEVI significantly enhanced the infectivity of R5 HIV-1 in vivo. Notably, this result demonstrates that SEVI does not only enhance target cell infection at the site of viral exposure but also markedly increases the levels of HIV-1 infection in a lymphatic organ by viral particles transported via the blood stream.

DISCUSSION

SEVI—a Key Factor in Sexual HIV-1 Transmission?

The limited ability of HIV-1 to cross the mucosal barrier and to infect sufficient numbers of cells in the genital tract to establish a sustained infection constitutes a major barrier for sexual transmission (reviewed in Haase, 2005). In this study, we show that fragments of PAP, a highly abundant SE marker, form amyloid fibrils (SEVI) that drastically
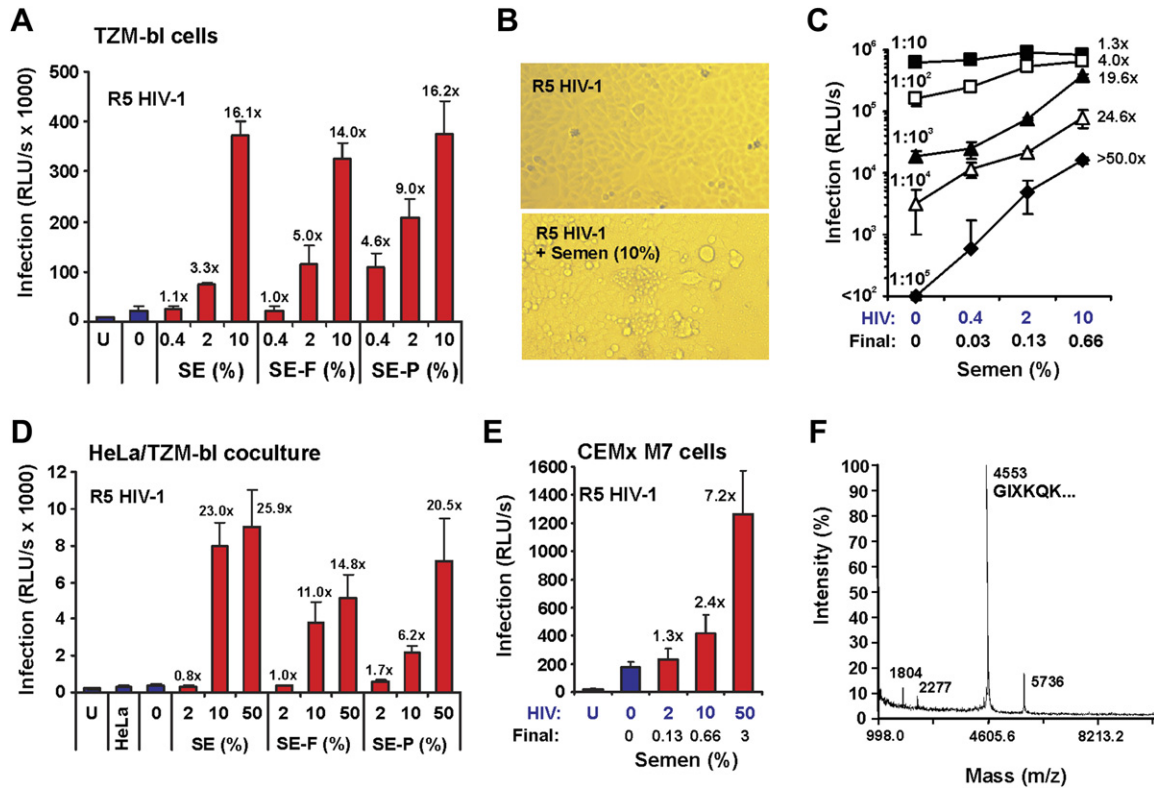
Figure 6. Semen and Seminal Fluid Enhance HIV-1 Infection

(A) Effect of SE, SE-F, and SE-P on R5-tropic HIV-1 infection of TZM-bl cells. The numbers indicate n-fold infectivity enhancement observed after treatment of HIV-1 with the indicated concentrations of SE, SE-F, and SE-P compared to those measured after infection with the PBS-treated virus stock. U, uninfected cells. Average values ± SD (n = 3) are shown in (A) and (C)–(E).

(B) Microscopic examination of TZM-bl cells infected with equal doses of HIV-1 that was either untreated or treated with semen.

(C) Semen enhances HIV-1 infection most efficiently after low-dose infection. TZM-bl cells were infected with 10-fold dilutions of R5-topic HIV-1 treated with the indicated concentrations of semen. The numbers indicate n-fold enhancement compared to the infectivity measured using PBS-treated virus stocks. The X-axes in (C) and (E) indicate the percentage of semen during virus incubation (blue) and the final concentration in the cell culture (black).

(D and E) Semen enhances in *trans*-infection of TZM-bl by virus-exposed HeLa cells and infection of CEMx M7 cells. TZM-bl cells were exposed to the virus/semen mixture for 3 hr and CEMx M7 cells for 1 hr, respectively. Numbers indicate n-fold infectivity enhancement observed in the presence of the indicated concentrations of SE, SE-F, and SE-P. U, uninfected cells; HeLa, HIV-1-exposed HeLa cells only.

(F) MALDI-MS analysis of aggregated protein/peptides pelleted from seminal fluid. Molecular masses and the N-terminal sequence of the major peak are indicated.

enhance the infectiousness of HIV-1 by promoting virion-cell attachment and fusion (Figure 2). SEVI amplified HIV-1 infection of CD4+ T lymphocytes and macrophages (most likely the cell types first targeted by HIV-1 in vivo; reviewed in Pope and Haase, 2003; Haase, 2005) and increased *trans*-HIV-1 infection of CD4+ T cells by an epithelial cell line and by primary DCs (Figure 4). An intact mucosal epithelium provides a strong physical barrier to HIV-1 infection (Miller and Shattock, 2003). However, epithelium integrity is often compromised after sexual intercourse as well as in the presence of ulcerative sexually transmitted diseases. Moreover, semen itself might cause local inflammation or epithelial breaks and induce DC projections to the luminal surface (Sharkey et al., 2007). Thus, SE components—such as amyloidogenic PAP fragments—might frequently be able to access CD4+ T cells, macro-

phages, and DCs in the subepithelium to enhance HIV-1 attachment, infection, and dissemination. Our data suggest that amyloidogenic peptides in SE may help HIV to pass the early "bottleneck" in infection by assisting the virus to attach to genital surfaces, to establish a self-propagating infection at the point of entry, and to cross the mucosal barrier with migrating DCs.

Although the magnitudes of the effects measured in infectivity assays were quite remarkable, they underestimated the real potency of SEVI. More quantitative limiting dilution-infection assays demonstrated that SEVI enhanced the $TCID_{50}$ of HIV-1 by three to five orders of magnitude (Figures 5A and 5B). In the absence of SEVI the ratio of detectable infectious units to virus particles was on the order of 1:1,000 to 1:100,000, which is in agreement with published data (Dimitrov et al., 1993; Rusert et al., 2004).
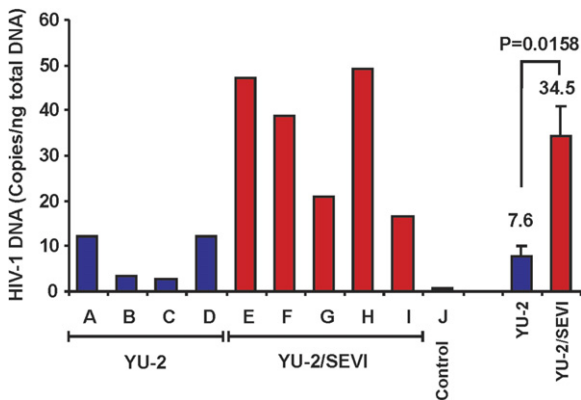
Figure 7. SEVI Enhances HIV-1 Infection In Vivo
hCD4/hCCR5-transgenic rats were challenged intravenously with untreated HIV-1$_{YU-2}$ (animals A–D) or with virus stocks treated with SEVI (animals E–I). As control, a hCD4-single transgenic rat (J) was infected with untreated virus. On 4 days post-challenge, all animals were sacrificed and the spleens were removed for determination of the HIV-1 cDNA load relative to the amount of cellular DNA. The right panel depicts arithmetic mean values ± SEM for the two groups of animals.

In contrast, only one to three virions were usually sufficient for productive infection in the presence of SEVI. Recent data suggest that the apparent high frequency of "noninfectious" HIV-1 particles is largely due to the low frequency of successful virus-cell interactions (Thomas et al., 2007). In agreement with this possibility, our data demonstrate that a few HIV-1 particles are sufficient for spreading infection in the presence of an effective attachment factor.

HIV-1 is detected in SE and cell-free SE-F of most infected men (Tachet et al., 1999), even under HAART (Zhang et al., 1998). However, the quantity of HIV-1 transmitted during sexual intercourse is usually subinfectious (Gray et al., 2001). On average, about 11,000 RNA copies/ml have been detected in SE of HIV-1-infected men (Gupta et al., 1997). Thus, an ejaculate of 4 ml SE would deposit ~22,000 virions in the genital tract, which corresponds to approximately 5 pg of p24 antigen. Interestingly, productive HIV-1 infection of PBMC cultures and of ex vivo tonsillar tissues after exposure to such low viral doses was only observed in the presence of SEVI (examples shown in Figure 5).

Semen-Mediated Enhancement of HIV-1 Infection
Given that semen is the main vector for HIV-1 transmission worldwide, surprisingly little is known about its effects on viral infectivity. One of the most striking findings of our study is that semen potently enhances HIV-1 infection (Figures 6, S17, and S18). Perhaps this effect was missed in previous studies because analysis of semen is complicated due to its high cytotoxicity and frequent bacterial contaminations. We circumvented these problems, at least in part, by adding relatively small volumes of virus/semen mixtures to monolayer cell cultures for limited time periods. To some extent this approach resembles

the deposition of the HIV-1-infected ejaculate in the genital tract and dilution by vaginal fluids. Notably, the ability of SE and of amyloidogenic PAP fragments to promote HIV-1 infection was not reduced by an acidic pH (Figures S16 and S18B), which represents a nonspecific vaginal defense mechanism against various pathogens including HIV-1 (Tevi-Benissan et al., 1997). Thus, the observed effects of semen on HIV-1 infection are most likely relevant for sexual viral transmission.

Our data support that amyloidogenic PAP fragments account for a significant fraction of the enhancing activity in semen. First, the yield of amyloidogenic PAP fragments from pooled SE (~35 μg/ml) considerably exceeds the concentration required to enhance HIV-1 infection (≥2 μg/ml). Second, freshly diluted PAP fragments were highly prone to fibril formation and rapidly achieved the ability to enhance HIV-1 infection in the context of SE and SE-F (Figure S16). The rapid kinetics of HIV-1 infectivity enhancement by freshly diluted PAP248-286 suggest that preexisting aggregates or fibrils in SE-F accelerate amyloid formation. Third, PAP248-286 was detected in the precipitate of SE-F that contains a large fraction of the enhancing activity (Figure 6F). Fourth, amyloidogenic PAP fragments were isolated from a complex semen-derived library that ought to represent all peptides and proteins < 50 kDa. Finally, our preliminary data show that a second semen-derived peptide fraction enhancing HIV-1 infection contains a different PAP fragment that also forms amyloid fibrils. It is noteworthy that seminal vesicle amyloid is a well-known form of localized amyloidosis (Maroun et al., 2003) and that PAP can readily be detected in vaginal washings and hence has been used as a SE marker, e.g., in the case of alleged sexual assaults (Graves et al., 1985). After sexual intercourse, elevated levels of PAP can usually be detected in the vagina for about 24 hr, but not after 48 hr (Collins and Bennett, 2001). Thus, it is easy to envision that the concentrations of SEVI may increase after sexual intercourse because intact PAP is degraded in the vaginal, rectal, or oral environment. Since the concentration of SEVI during virion incubation determines the magnitude of infectivity enhancement (Figures S6A and S15), its subsequent dilution by vaginal fluids would not diminish its potency. Accordingly, SEVI fibrils that form prior to or after sexual intercourse may all increase virion attachment and infectivity. Amyloid fibrils are known to be highly stable and could potentially facilitate HIV-1 infection in the genital tract for relatively long time periods.

Further studies are required to clarify whether amyloidogenic PAP fragments also serve a purpose in normal SE physiology. Moreover, it will be interesting to further investigate how efficiently other possible aggregates, such as low-molecular-weight oligomers, pores, spheres, and protofilaments that typically precede fibril formation (Walsh et al., 1999), promote HIV-1 infection. However, the relative contribution of such intermediates to HIV-1 infectivity enhancement will be difficult to assess because even if they are initially separated from monomers and

mature fibrils, the equilibrium between these species may rapidly be re-established. Notably, our results clearly support a role of immature PAP aggregates in HIV-1 infection. For example, we did not observe large fibrils in SE, although amyloidogenic PAP fragments clearly formed aggregates boosting the potency of semen-mediated enhancement of HIV-1 infection (Figure S16A), and SE showed birefringence under polarized light upon staining with Congo red (data not shown). Moreover, fresh solutions of PAP fragments acquired the ability to enhance HIV-1 infection very rapidly in comparison to other indicators of amyloid formation, such as increased Congo red and Thioflavin T binding (Figure S16B). It has been suggested that short fibrils are particularly effective in promoting the infectiousness of HIV-1 (Wojtowicz et al., 2002). Thus, smaller aggregates that form in semen could be even more potent in assisting HIV-1 infection than the large SEVI fibrils obtained in vitro.

Possible Role of Amyloid Fibrils in Viral Infections

Recent studies show that amyloid fibrils are much more common than previously recognized. To date, about 30 human diseases are known to be associated with amyloid deposits (reviewed in Termussi et al., 2003; Westermark, 2005). Furthermore, it has become clear that amyloid fibrils are also formed by proteins unrelated to disease (Fowler et al., 2006) and produced by bacteria and fungi (reviewed in Gebbink et al., 2005; Kelly and Balch, 2003). It has been previously shown that β-amyloid fibrils associated with Alzheimer's disease, such as Aß1-40, also enhance viral infection (Wojtowicz et al., 2002). We did not observe an enhancing effect of Aß1-40 (Figure S5A). This apparent discrepancy could be due to the fact that amyloid fibers composed of the same protein can show different conformations with distinct phenotypes (reviewed in Chien et al., 2004). Our results confirmed, however, that other amyloidogenic peptides, i.e., PPI-2480 and α-synuclein, also enhance HIV-1 infection, albeit with much lower efficiency than PAP fragments (Figure S5A). Thus, the ability to enhance HIV-1 infection seems to be a common feature of amyloid fibrils. Moreover, the capability to promote the interaction between virions and the cell surface is independent of the viral Env glycoprotein and hence not restricted to retroviruses. Thus, further studies on the role of amyloids in the transmission and pathogenesis of enveloped viruses are highly warranted. For example, it will be of interest to clarify whether some fungal and bacterial infections may enhance the risk of HIV transmission (and other sexually transmitted viral infections) because they produce amyloid fibrils that facilitate virus adsorption and infection.

Perspectives

Our identification of an amyloid HIV-1 attachment/infectivity factor in SE offers exciting new avenues for further investigation. The high potency of SEVI in promoting viral infection together with its relatively low cytotoxicity suggests that it may not only play a relevant role in sexual HIV transmission but could also help to improve vaccine approaches and gene delivery by lentiviral vectors. For example, pretreatment with SEVI may amplify the potency of single-cycle immunodeficiency viruses (Evans et al., 2005) in mediating virus-specific immune responses and hence mucosal immunity. Finally, agents blocking the generation or enhancing activity of SEVI and potential related virus attachment factors may offer new prospects for preventive strategies.

EXPERIMENTAL PROCEDURES

Identification of SEVI

The peptide library was generated and screened for factors affecting HIV-1 infection essentially as described previously (Münch et al., 2007). See the Supplemental Experimental Procedures for further details.

Peptide Synthesis and Fibril Formation

Peptides were produced by standard Fmoc solid-phase peptide synthesis, purified by preparative RP HPLC, and analyzed by HPLC and MS. Lyophilized synthetic peptides were resuspended in serum-free DMEM or PBS or in SE-F at concentrations of 5 to 10 mg/ml. Fibril formation was induced by overnight agitation at 37°C at 1400 rpm using an Eppendorf Thermomixer and verified by Congo red staining or electron microscopy.

HIV-1 Variants, Virus Stocks, and Infectivity

HIV-1 clones and primary isolates were obtained through the NIH ARRRP. Primary HIV-1 O isolates were kindly provided by Matthias Dittmar (Dittmar et al., 1999). Virus stocks were generated as described previously (Münch et al., 2007). The $TCID_{50}$ was determined as described by the ACTG Laboratory Technologist Committee (http://aactg.s-3.com/pub/download/labmanual/43-ALM-TCID50-Determination.pdf). See the Supplemental Experimental Procedures for further details.

Electron Microscopy

PAP250-286 was dissolved in PBS at 2 mg/ml and incubated at 37°C on a rotating mixer to induce fibril formation. A 0.02 mg/ml suspension of SEVI was adsorbed for 60 s onto 200-mesh carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA, USA). Grids were washed with distilled water and subsequently stained with 1% aqueous uranyl acetate (Electron Microscopy Sciences) for 60 s. Fibrils were visualized with a Philips (New York, USA) CM100 transmission electron microscope.

Thioflavin T Binding Assay

SEVI aggregation was performed as described in the electron microscopy section. Aliquots were withdrawn from the aggregation reaction every hour and the increase in thioflavin T fluorescence (excitation: 440 nm, emission 485 nm) was monitored using a Cary Eclipse fluorimeter.

X-Ray Diffraction Analysis

SEVI was aggregated as described in the electron microscopy section. Aggregates were lyophilized, producing a fine powder. Powder diffraction of about 1.0 mg SEVI in quartz capillaries was recorded using a 6 KW Bruker Direct Drive Rotating anode X-ray generator with a Xenocs focusing mirror (50 kV × 100 mA, 0.3 × 3 mm focus, 0.5 mm slits, Copper [Cu] Target) and a Mar 345 mm IP scanner. The distance from sample to scanner was 250 mm and CuK radiation (1.5418 Å) was utilized.

HIV-1 Infection In trans

1×10^4 HeLa or DCs, isolated as described previously (Rücker et al., 2004), were sown out in a volume of 50 μl RPMI or DMEM, respectively. Thereafter, 10 μl SEVI dilutions and 0.5 ng p24 antigen of HIV-1 NL4-3 or HIV-1 005-pf-103 in a volume of 40 μl were added. After 2 hr of incubation the cells were washed twice in RPMI and resuspended in

150 μl of CEMx M7 cell cultures. Luciferase assay was performed 2 days post-cocultivation.

Infection of Ex Vivo Tonsillary Tissues

Human tonsillar tissues removed during routine tonsillectomies were dissected, maintained, and infected within 5 hr of excision, as described previously (Glushakova et al., 1995; Rücker et al., 2004), except that aliquots of the virus stocks were preincubated with SEVI. Briefly, the tonsils were dissected into 2 to 3 mm³ blocks and infected by inoculating each block with viral stock suspensions derived from transfected 293T cells. Infection doses were normalized based on p24 content. Productive HIV-1 infection was evaluated by measuring the amount of p24 core antigen released into the medium as described (Rücker et al., 2004). To assess the effect of AZT each block was infected with wild-type HIV-1 NL4-3 or an R5-tropic 92UG037 V3 recombinant (Münch et al., 2007), respectively, that were either preincubated for 5 min with various concentration of SEVI or with PBS only. After overnight incubation, the virus inoculum was removed by extensive washing and the tissues were cultured in fresh medium with or without AZT (10 μM).

p24 Binding

First, 5×10^3 TZM-bl cells were sown in 96-well dishes in a volume of 50 μl. Next, 10 μl of DMEM containing different concentrations of SEVI and 40 μl of 10-fold dilutions of the viral stocks were added to the cells. After 3 hr incubation at 37°C unbound virus was removed by washing with DMEM. Thereafter, the cells were lysed in DMEM containing 1% Triton X-100. Cell-associated HIV-1 core antigen was detected using the p24 antigen ELISA obtained by the NIH ARRRP.

Imaging Fluorescently Labeled HIV-1

Fluorescently labeled HIV-1 was generated and infection of TZM-bl cells was monitored essentially as described (Sherer et al., 2003). See the Supplemental Experimental Procedures for further details.

Detection of PAP248-286 in Seminal Fluid

Freshly collected semen was centrifuged (5 min, 14000 g) to remove spermatocytes and the SE-F was agitated (1300 rpm) overnight at 37°C in the presence of gentamycin (50 μg/ml). Aggregated peptide and protein was precipitated by centrifugation for 15 min at 14000 g. The supernatant was discarded and the pellet was dissolved in 6 M Guanidin HCl, diluted 1:5 with chromatography buffer A (0.1% TFA in water), and subjected to an analytical RP HPLC. The peptides/protein mixture was separated by a linear gradient of buffer B (80% acetonitril in A) and the resulting fractions were analyzed by MALDI-MS and Edman sequencing.

Effect of Semen on HIV-1 Infection

Semen and seminal fluid were obtained and analyzed for their effects on HIV-1 infection and transmission as described in the Supplemental Experimental Procedures.

Transgenic Rat Model

The hCD4/hCCR5-transgenic rat model and the generation of replication-competent HIV-1$_{YU-2}$ stocks have been reported previously (Keppler et al., 2002). For details, see the Supplemental Experimental Procedures.

HIV-1 Virion-Fusion and Gene Expression

This sensitive flow cytometry-based HIV-1 virion-fusion assay was conducted in principle as described (Cavrois et al., 2002; Goffinet et al., 2007). For details, see the Supplemental Experimental Procedures.

Data Analysis

The PRISM package version 4.0 (Abacus Concepts, Berkeley, CA) was used for all statistical calculations. If not specified otherwise, data present mean values ± SD obtained from at least triplicate measurements. Nonparametric statistical analyses were performed by using the Mann-Whitney u test.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, eighteen figures, one table, and two movies and can be found with this article online at http://www.cell.com/cgi/content/full/131/6/1059/DC1/.

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