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Naturally Occurring Fragments from Two Distinct Regions of the Prostatic Acid Phosphatase Form Amyloidogenic Enhancers of HIV Infection

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Semen is the major vector for HIV-1 transmission. We previously isolated C-proximal fragments of the prostatic acid phosphatase (PAP) from semen which formed amyloid fibrils that potently enhanced HIV infection. Here, we used the same methodology and identified another amyloidogenic peptide. Surprisingly, this peptide is derived from an N-proximal fragment of PAP (PAP85-120) and forms, similar to the C-proximal fragments, positively charged fibrillar structures that increase virion attachment to cells. Our results provide a first example for amyloid formation by fragments of distinct regions of the same precursor and further emphasize the possible importance of amyloidogenic peptides in HIV transmission.

The majority of HIV-1-infected individuals acquire the virus through sexual intercourse, and semen is the major vehicle for the spread of the AIDS pandemic (3, 17). Accumulating evidence shows that semen boosts HIV-1 infection when analyzed under conditions minimizing its cytotoxic effects (4, 7, 10, 11, 13, 14, 15). One factor that contributes to the HIV-1-enhancing effect of semen is an amyloid termed SEVI (semen-derived enhancer of virus infection) (10). SEVI forms by self-assembly of various C-proximal fragments of prostatic acid phosphatase (PAP), with PAP248-286 being the predominant peptide (10). SEVI captures viral particles and promotes their attachment to target cells to boost viral fusion and infection (10). Notably, the HIV-1-enhancing effect of semen is donor dependent and correlates with the level of SEVI (7). Thus, semen and SEVI may play relevant roles in the sexual spread of the virus, and counteracting this enhancing activity will provide a novel strategy to reduce virus transmission. Indeed, the first inhibitors of SEVI-mediated enhancement of HIV-1 infection have been identified, and most of these compounds also block semen-mediated infectivity enhancement (4, 11, 13, 14, 16).

SEVI was identified by screening a complex seminal fluid (SF)-derived peptide library for compounds that modulate HIV-1 infection (10). We observed that besides fractions containing SEVI in pH pool 7 (10), fractions 27 to 29 of pH pool 4 of this library also promoted HIV-1 infection (Fig. 1A). Peptide sequencing after two rounds of purification demonstrated that these fractions contained an N-proximal fragment of PAP (4,464 Da) corresponding to residues 85 to 120 (PAP85-120) (Fig. 1A and B). Given that the peptide library contains several thousand peptides and small proteins that are present in semen, the independent identification of a second fragment of the same precursor as an HIV enhancer was unexpected.

To confirm the infection-enhancing activity of PAP85-120, we chemically synthesized the peptide, dissolved it in hexafluoroisopropanol (HFIP; 10 mg/ml) to remove preformed aggregates, transferred 100-μl aliquots into 1.5-ml vials, and evaporated the HFIP, resulting in 1-mg peptide aliquots. The lyophilized peptide was then dissolved in 200 μl of H_2O (5 mg/ml) and agitated at 1,200 rpm as described previously (10). Next, we examined the effects of PAP85-120 preparations on CCR5 (R5)-tropic HIV-1 infection of TZM-bl reporter cells as described previously (10). Freshly dissolved PAP85-120 had no effect on infection (Fig. 2A), but the agitated, slightly turbid solution potently enhanced HIV-1 infection (Fig. 2A). This gain of function was associated with a shift in the fluorescence spectrum of the amyloid-specific dye Thioflavin T (10) (Fig. 2B). Formation of amyloid fibrils was further confirmed by atomic force microscopy (AFM) using agitated PAP85-120 and PAP248-286 (SEVI) samples (0.05 μg/ml). As expected from previous findings (10), SEVI formed homogeneous aggregates of typical needle-like amyloid fibrils with lengths of 5 to 10 μm and an average diameter (Ø) of 5.3 ± 0.1 nm (average ± standard deviation). In contrast, AFM of PAP85-120 revealed more heterogeneous aggregates comprising small oligomeric particles (Ø, 3.58 ± 1.22 nm) and worm-like structures (Ø, 2.58 ± 1.22 nm), respectively, as well as typical 1- to 5-μm-long mature fibrils (Ø, 4.01 ± 1.23 nm) (Fig. 2C) (5, 6). Thus, semen contains fragments derived from two distinct regions of PAP (amino acid residues 85 to 120 and 248 to 286) that form amyloid enhancers of HIV-1 infection.

Next, we compared the HIV-1-enhancing activity of PAP85-120 and SEVI fibrils. We either treated HIV-1 virions with amyloid prior to inoculation of TZM-bl cells (virion treatment) or first incubated the cells with the fibrils and then proceeded with infection (cell treatment). The fibrils boosted HIV-1 infection under both conditions, although virion treatment was more effective (Fig. 2D). We observed some batch-to-batch variation in the efficiency of PAP85-120 to form amyloid aggregates (Fig. 2E), but we
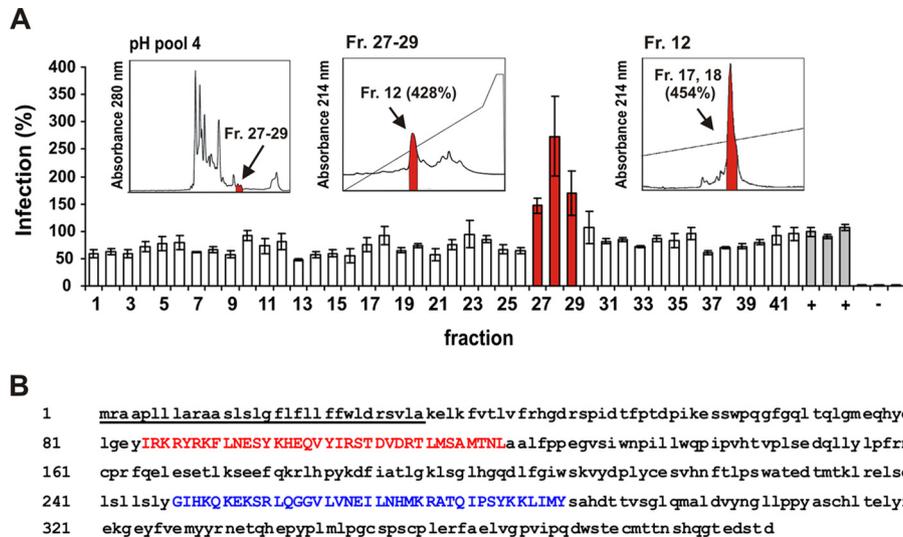
FIG 1 Purification of a second fibril-forming PAP fragment that enhances HIV-1 infection from semen. (A) Fractions 27 to 29 of pH pool 4 (red) promote HIV-1 infection of TZM-bl cells. The inlets show the high-performance liquid chromatography results of pH pool 4 (left) and of the active fractions 12 (middle) and 17/18 (right) obtained after the different purification steps. Fractions 17 and 18 used for peptide sequencing are shown in red. +, no peptide added; −, uninfected cells. (B) Amino acid sequence of PAP, with the signal peptide underlined, residues 85 to 120 in red, and residues 248 to 286 in blue.

consistently found that once PAP85-120 formed fibrils, these fibrils enhanced HIV-1 infection almost as efficiently as SEVI. Notably, PAP85-120 amyloid was not cytotoxic (Fig. 2F). Since semen contains both of these amyloidogenic peptides, we also tested whether SEVI and PAP85-120 cooperate to boost HIV-1 infection, and we found that PAP85-120 amyloid and SEVI exert additive effects on virus infection of TZM-bl cells (Fig. 2G).

In the next set of experiments, we tested whether PAP85-120 amyloid can enhance infection by different HIV-1 variants (see the materials and methods described in reference 10). Infection of CEM-M7 cells in suspension and adherent TZM-bl cells by viruses differing in their coreceptor tropism (Fig. 3A and B), various subtypes of HIV-1 M and group O viruses, and drug-resistant HIV-1 variants (Fig. 3C) were all enhanced in the presence of PAP85-120 fibrils. We also demonstrated that the fibrils boosted HIV-1 infection of primary cells, including peripheral blood mononuclear cells (PBMCs) and monocyte-derived macrophages (Fig. 3D). The effect on X4-tropic HIV infection of macrophages was relatively weak, most likely because they express low levels of CXCR4 and the fibrils do not bypass the requirement for the appropriate coreceptor (10). Additionally, a virion fusion assay (2) revealed that the PAP85-120 fibrils enhanced HIV-1 fusion to CD4$^+$ T cells purified from the female genital mucosa (see the methods described in reference 13) (Fig. 3E).

At very low multiplicities of infection, HIV-1 infection was frequently only detectable in the presence of amyloid. To further examine the magnitude of HIV-1 infection enhancement, we performed limiting dilution assays. CEM-M7 cells were infected with HIV-1 in the presence or absence of PAP85-120 fibrils (50 μg/ml), and productive infection was monitored by detection of green fluorescent protein (GFP)-expressing cells 1 week later. PAP85-120 fibrils increased the threshold for infection by 3 orders of magnitude, from a 10^4-fold virus dilution to 10^7 (Fig. 3F). Together, these data demonstrate that, similar to SEVI (10), the enhancing effect of PAP85-120 is independent of the viral genotype

and is apparent with both adherent (TZM-bl) and suspension (CEM.M7) cell lines, as well as primary cells.

To further examine the mechanism underlying infectivity enhancement, we stained PAP85-120 fibrils with an amyloid-specific red fluorescent dye (ProteoStat amyloid plaque detection kit) (Fig. 4A). Fibrils stained by the dye were not impaired in their ability to enhance HIV-1 infection (data not shown). After incubation of stained amyloid with yellow fluorescent protein (YFP)-tagged virions for 5 min (10), we observed complex formation that was remarkably rapid and efficient, since no residual virions could be detected (Fig. 4A). Further analyses showed that PAP85-120 amyloid also interacts with TZM-bl cells (Fig. 4B). To directly investigate whether the fibrils increase virion attachment, we inoculated TZM-bl cells with X4- and R5-tropic HIV-1 that was pretreated with PAP85-120 fibrils, removed the inoculum 1 h later, and measured the quantity of cell-associated virus by p24 enzyme-linked immunosorbent assay (ELISA). Our results demonstrated that PAP85-120 efficiently increased virion attachment to the cells in a dose-dependent manner (Fig. 4C).

It has previously been shown that the cationic properties of SEVI underlie its ability to enhance HIV-1 infection and that polyanionic compounds abrogate this activity (13). Like PAP248-286, the PAP85-120 sequence contains a relatively high number of positively charged residues near its N terminus (net charge, +4; isoelectric point, 9.99). To directly determine the surface charge of SEVI and PAP85-120 amyloid, we performed zeta potential measurements (8) by using a zeta nanosizer (Malvern Instruments, United Kingdom). This analysis showed that in contrast to the respective monomeric peptides, both amyloid samples displayed positive zeta potentials, providing direct evidence for a positively charged surface (Fig. 4D). Consequently, addition of the polyanion heparin abrogated the ability of PAP85-120 amyloid to bind cells (Fig. 4B) and to promote HIV-1 infection (Fig. 4E). To examine whether polyanions directly interact with PAP85-120 amyloid, we incubated the fibrils with phosphate-buffered saline
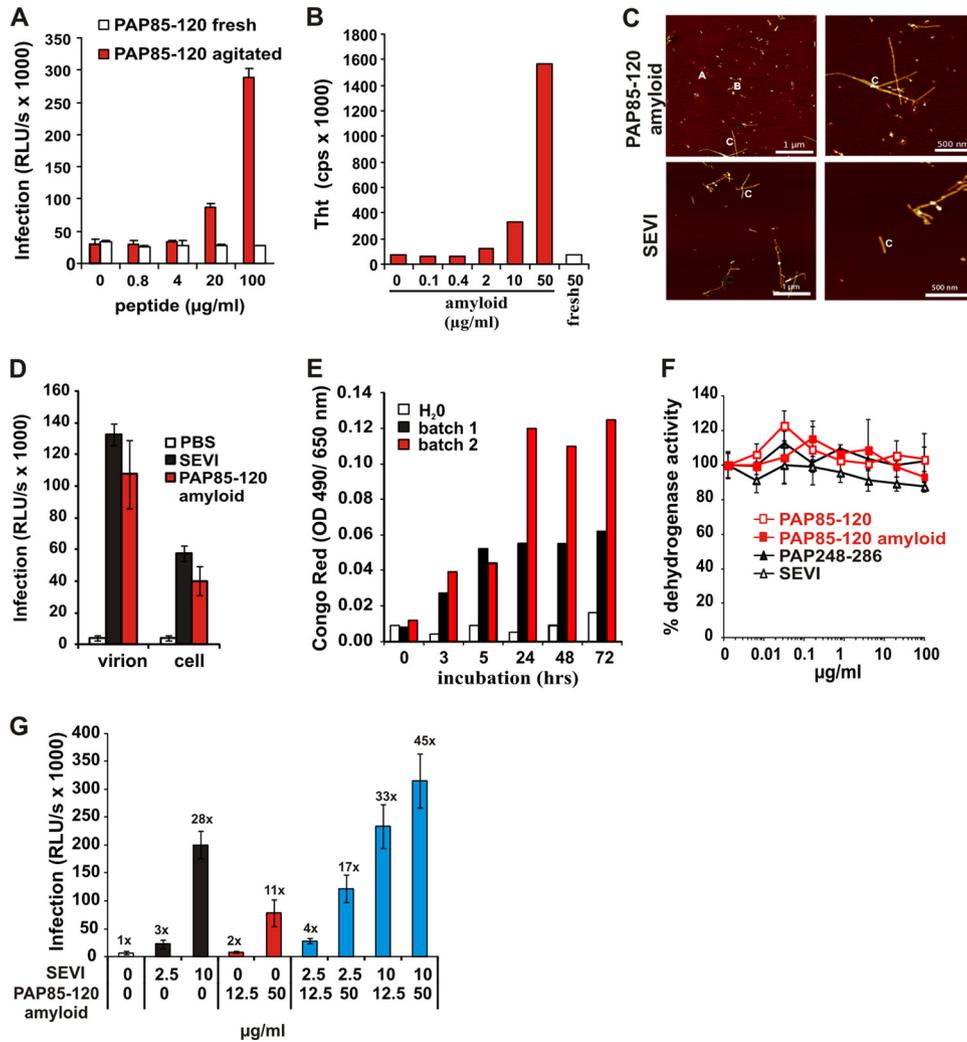
FIG 2 PAP85-120 forms amyloid and enhances HIV infection. (A) Only agitated PAP85-120 boosts HIV-1 infection of TZM-bl cells. All infection experiments in Fig. 2 to 4 were performed in triplicate, and values shown are average values ± standard deviations. (B) Agitated PAP85-120 forms amyloid as assessed by fluorescence in the presence of Thioflavin T (ThT) (10). (C) AFM images of PAP85-120 and SEVI amyloid (0.05 μg/ml) deposited on mica; images were obtained using a Nanoscope IIIa AFM (Digital Instruments) using silicon cantilevers (Omicron; spring constant, 2 N/m). A, oligomeric particles; B, protofibrils; C, mature fibrils (5, 6). (D) PAP85-120 amyloid promotes HIV-1 infection of TZM-bl cells following virion or target cell pretreatment. The final cell culture concentration of PAP85-120 amyloid was 10 μg/ml. (E) Fibril formation kinetics of two batches of PAP85-120 (5 mg/ml, 200 μl; agitation at 1,200 rpm). At the indicated time points, aliquots were taken and amyloid formation was quantified in a Congo red-based amyloid staining assay (10). (F) PAP-derived amyloid does not diminish cell viability. PBMCs were incubated for 3 days with the indicated PAP preparations, and cell viability was measured in an MTT (3-[4,5-diemthyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay (10). (G) Additive HIV enhancing effects of fibrillar PAP fragments. TZM-bl cells were infected with HIV-1 incubated with the indicated PAP fibrils alone or in combination.

(PBS) or heparin, centrifuged the samples (10 min; 14,000 rpm), and determined the zeta potentials of the precipitated material. As expected, untreated PAP85-120 amyloid displayed a positive charge, whereas heparin treatment resulted in a strong negative zeta potential of the precipitated fibrils (Fig. 4F). These results are evidence for a direct interaction between the positively charged amyloid and the highly sulfated glycosaminoglycan and suggest that polyanions interfere with the ability of PAP85-120 amyloid to neutralize the negative charge repulsions between virions and target cells.

To the best of our knowledge, PAP is the first human protein described so far that contains two distinct regions capable of amyloid formation. Given that our library contains essentially all peptides and small proteins that are present in semen, it was sur-

prising that the second HIV-1-enhancing fraction also contained a PAP fragment. In total, we isolated ~1.13 mg of PAP85-120 from fractions 27 to 29 of pH pool 4 (Fig. 1A) that were derived from 29 ml of pooled semen. This roughly corresponds to a concentration of PAP85-120 of ~39 μg per ml of semen, which is close to the concentration of PAP248-286 (35 μg/ml) (10). Interestingly, additional peptides derived from the abundant semen proteins semenogelin 1 and 2 are also amyloidogenic and boost HIV infection (15). Notably, semen is the only body fluid in which multiple amyloidogenic peptides are detected. In contrast, we did not find amyloid-containing fractions in peptide/protein libraries generated from human plasma, serum, or hemofiltrate. Thus, amyloid formation appears to be semen specific. It is noteworthy that these amyloidogenic peptides most likely form small amyloid ag-
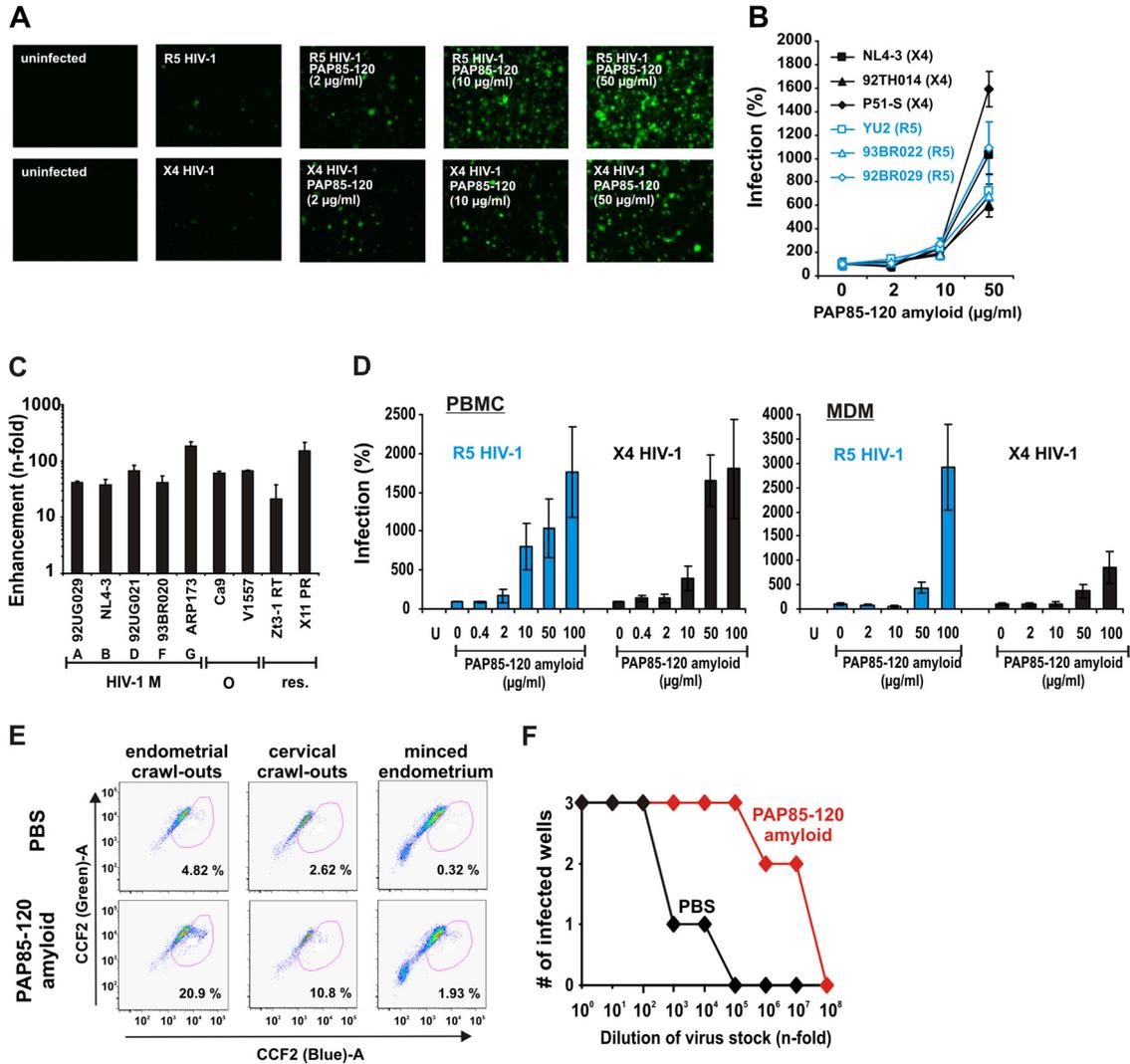
FIG 3 Fibrillar PAP85-120 enhances R5- and X4-tropic HIV-1 infection of cell lines and primary target cells (10, 12). (A) UV-microscopy images taken 3 days postinfection of CEM-M7 with PAP85-120 amyloid-treated X4- and R5-tropic virus (12). (B and C) TZM-bl cells were infected with various X4- and R5-tropic HIV-1 NL4-3 V3 recombinants (12) treated with various concentrations of PAP85-120 fibrils (B) or primary HIV-1 isolates and drug-resistant virus (10) treated with 50 μg/ml of PAP85-120 fibrils (C). (D) PAP85-120 amyloid enhances infection of interleukin-2/phytohemagglutinin-stimulated PBMCs (left) and monocyte-derived macrophages (MDM) (right) by luciferase-encoding HIV-1 (10). Concentrations shown are those of PAP85-120 amyloid during virion preincubation. (E) The fibrils (31.35 μg/ml) boost fusion of HIV-1 to CD4+ T cells purified from endometrial biopsies (13). These cells were isolated by allowing T cells to "crawl out" of endometrial biopsies (13) or by positive selection from minced tissue ("minced endometrium"). Percent values give fusion rates in the absence and presence of amyloid fibrils. (F) Limiting dilution analysis of HIV-1. CEM-M7 cells were infected in triplicates with 10-fold dilutions of R5-tropic HIV-1 virus stock in the absence or presence of PAP85-120 fibrils (50 μg/ml). Shown are the numbers of infected cultures 7 days postinfection as determined by UV-microscopy.

gregates in semen, such as protofibrils, rather than the large fibrils obtained *in vitro* in homogeneous solutions. The structural elucidation of the various forms of these amyloid aggregates is currently being investigated, and it will be interesting to further examine their possible physiological roles, e.g., in promoting fertilization.

Using PAP248-286/SEVI-specific antibodies, we previously demonstrated that the ability of semen to boost infection correlates with the levels of SEVI (7). Unfortunately, after multiple attempts at raising antibodies against PAP85-120 amyloid in mice and rabbits, we were unable to successfully generate antisera or monoclonal antibodies that were specific for PAP85-120 amyloid and did not cross-react with the full-length protein and mono-

meric forms. Thus, the relative contributions of the different amyloidogenic peptides in semen-mediated enhancement of virus infection remain to be determined. It is noteworthy, however, that all compounds blocking SEVI-mediated infectivity enhancement, such as polyanions (13), the proteoglycan antagonist Surfen (14), and the Thioflavin T derivative BTA-EG6 (11), also antagonize the enhancing effect of semen. Thus, either SEVI is the major component in semen responsible for infection enhancement, or the above-mentioned agents also block the activity of other amyloids. Indeed, we found that the polyanion heparin abrogated infectivity enhancement by PAP85-120, PAP248-286 (13), and semenogelin-derived fibrils (15). Since the distinct amyloidogenic peptides most likely cooperate to boost HIV-1 infection, strategies to ab-
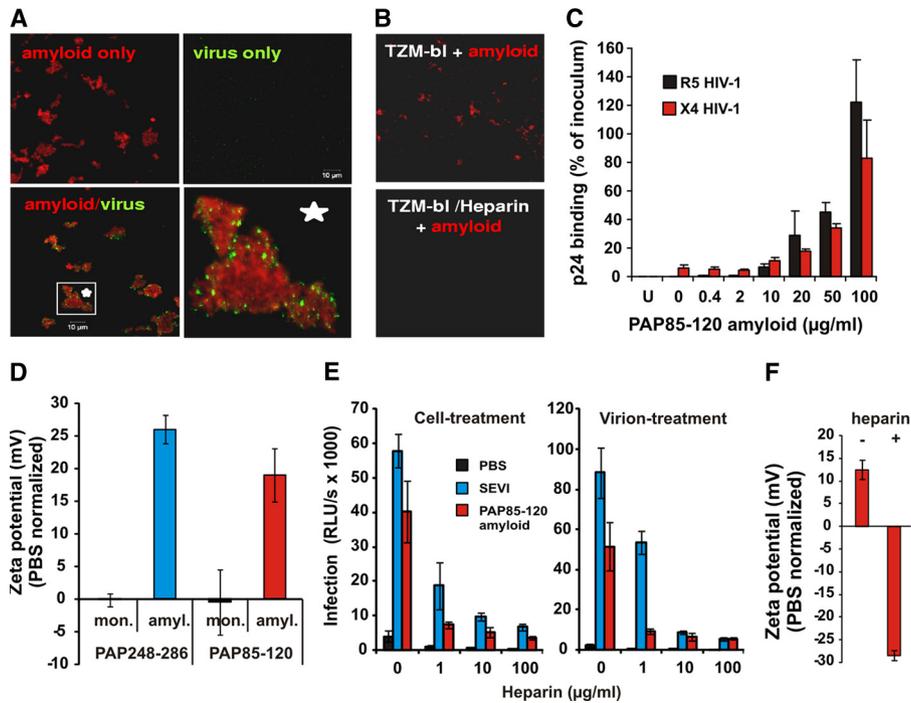
FIG 4 The cationic properties of PAP85-120 fibrils are essential for their ability to enhance HIV infection. (A) PAP85-120 amyloid captures virus. Confocal images of PAP85-120 amyloid stained with a fluorescent dye, YFP-tagged HIV-1, and a mixture of amyloid and virus. The panel with a star indicates a magnified image of the amyloid/virus complexes. (B) PAP85-120 amyloid binds TZM-bl cells in a manner that can be blocked by polyanions. Stained amyloid (50 μg/ml) was added to cells in the presence or absence of heparin (100 μg/ml), and cells were washed 30 min later, and imaged by fluorescence microscopy. (C) Fibrils increase virion attachment. X4- and R5-tropic HIV-1 virions were treated with PAP85-120 fibrils and added to TZM-bl cells. One hour later, cells were washed, and cell-associated p24 was quantified by ELISA. Shown are the percentages of the total quantity of p24 present in the viral inoculum. (D) Zeta potential measurements of monomeric and fibrillar PAP fragments. (E) Polyanions abrogate infectivity enhancement. Heparin was added to cells that were subsequently infected with SEVI or PAP85-120 amyloid (50 μg/ml) treated virus (cell-treatment). Alternatively, cells were infected with virus pretreated with amyloid in the presence of the indicated amounts of heparin (virion-treatment). (F) PAP85-120 amyloid binds heparin. Fibrils (100 μg/ml) were mixed with PBS or heparin (100 μg/ml) and centrifuged for 10 min at 14,000 rpm. The pellets were resuspended in PBS, and zeta potentials were measured.

rogate the enhancing activity of semen should focus on compounds that target amyloids in general rather than developing specific inhibitors of a certain type of amyloid.

It is becoming evident that semen enhances HIV-1 infection (4, 7, 10, 11, 13, 14, 15). Two studies that found that semen has HIV-inhibitory effects (1, 9) were performed with concentrations of semen just at the threshold that kills cells. We repeated these experiments and found a direct correlation between semen-induced cytotoxicity and its "antiviral" activity (7, 18), suggesting that cell death rather than a specific antiviral effect accounted for the observed findings (1, 9).

In summary, we have shown here that semen harbors multiple naturally occurring amyloidogenic fragments of highly abundant precursors that are likely generated during semen liquefaction. These peptides form positively charged fibrillar structures that bind HIV-1 particles and promote their binding to target cells to boost subsequent virion fusion and infection. The vaginal environment and changes in the pH might affect the formation and activity of amyloid. However, we previously reported that cervical lavage fluid or the acidic pH in the vagina did not affect SEVI formation or reduce SEVI-mediated enhancement of HIV infection (7, 10), suggesting that these parameters also do not affect PAP85-120 activity, since both amyloids promote infection by a similar mechanism. Further studies on the role of amyloid aggregates in semen for sexual transmission of HIV-1 in appropriate animal models seem highly warranted, and compounds interfering with the HIV-enhancing activity may represent useful additives to candidate microbicides.

ACKNOWLEDGMENTS

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