THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 15, pp. 11842–11849, April 6, 2012

Seminal Plasma Accelerates Semen-derived Enhancer of Viral Infection (SEVI) Fibril Formation by the Prostatic Acid Phosphatase ($PAP_{248-286}$) Peptide*

Received for publication, October 17, 2011, and in revised form, February 6, 2012 Published, JBC Papers in Press, February 21, 2012, DOI 10.1074/jbc.M111.314336

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Background: SEVI is an amyloid fibril that enhances HIV infectivity. To date, it has been produced from its precursor peptide only under nonphysiologic conditions.
Results: Seminal plasma (SP) accelerates SEVI formation and protects SEVI from proteolytic degradation.
Conclusion: SEVI forms spontaneously from its precursor peptide under physiologic conditions in SP.
Significance: These findings may explain the presence of SEVI in human semen.

Amyloid fibrils contained in semen, known as SEVI, or semen-derived enhancer of viral infection, have been shown to increase the infectivity of HIV dramatically. However, previous work with these fibrils has suggested that extensive time and nonphysiologic levels of agitation are necessary to induce amyloid formation from the precursor peptide (a proteolytic cleavage product of prostatic acid phosphatase, $PAP_{248-286}$). Here, we show that fibril formation by $PAP_{248-286}$ is accelerated dramatically in the presence of seminal plasma (SP) and that agitation is not required for fibrillization in this setting. Analysis of the effects of specific SP components on fibril formation by $PAP_{248-286}$ revealed that this effect is primarily due to the anionic buffer components of SP (notably inorganic phosphate and sodium bicarbonate). Divalent cations present in SP had little effect on the kinetics of fibril formation, but physiologic levels of Zn^{2+} strongly protected SEVI fibrils from degradation by seminal proteases. Taken together, these data suggest that in the *in vivo* environment, $PAP_{248-286}$ is likely to form fibrils efficiently, thus providing an explanation for the presence of SEVI in human semen.

Amyloid fibrils are ordered aggregates that form from a wide variety of soluble proteins and peptides and are associated with a range of human pathologies, including Alzheimer disease, type II diabetes, and Parkinson disease (1). Although a great diversity of peptides have been shown to form amyloid fibrils, these fibrils all share certain properties, including self-assembly by a nucleation-dependent process, a nonbranching fibrillar cross-β structure, with β strands perpendicular to the fibril axis, and the ability to specifically bind to dyes such as Congo Red and thioflavin T (ThT) (2–4).

Amyloid fibrils that enhance HIV-1 infection *in vitro* have been isolated from semen (5). These fibrils, known as semen-derived enhancer of virus infection, or SEVI,[2] self-assemble from the peptide $PAP_{248-286}$, which is a proteolytic cleavage product of prostatic acid phosphatase, a protein found in abundant quantities in semen. SEVI fibrils are highly cationic, with a pI of 10.21 (6), and enhance HIV infection in a charge-dependent manner, likely by decreasing the electrostatic repulsion between the surface of the cell and the HIV virion and by binding to virions and increasing their sedimentation onto the cell surface (6, 7).

SEVI fibrils have been shown to have a well defined nonbranched fibrillar structure, identical to that of other amyloid fibrils; to bind specifically to ThT, indicating the presence of a cross-β structure; and to follow the expected nucleation-dependent elongation mechanism (8). The nucleation-dependent self-assembly of amyloid fibrils consists of an initial lag phase, during which small oligomers are formed by the entropically unfavorable process of association between monomers. Once a nucleus is formed, fibril formation enters the growth phase, in which the fibrils aggregate rapidly until equilibrium is reached (9). The process of nucleation can be affected by a variety of environmental factors, including pH, salt concentration, metal cations, and temperature (10–13). $PAP_{248-286}$ may be especially sensitive to factors that affect electrostatic interactions, as its numerous cationic residues would likely lead to strong charge repulsion between the monomers.

SEVI fibrils have been isolated from semen, yet *in vitro*, their self-assembly requires extensive time and agitation (5). This suggests that semen may contain components that decrease the lag phase of fibril formation by $PAP_{248-286}$. Semen contains many components that could modify fibrillization of this peptide, including the metal cations zinc and magnesium, which

* This work was supported, in whole or in part, by National Institutes of Health Grants R01AI084111 (to S. D.), T32GM007356 (to J. S. O.), and T32DA007232 (to J. S. O.). This work was also supported by a Creative and Novel Ideas in HIV Research award (to B. L. N.), by University of Rochester Developmental Center for AIDS Research Grant P30AI078498, and by a DuPont young professors award (to B. L. N.).
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[2] The abbreviations used are: SEVI, semen-derived enhancer of virus infection; AS, artificial semen; HFIP, hexafluoroisopropyl alcohol; PAP, prostatic acid phosphatase; PSA, prostate-specific antigen; SP, seminal plasma; ThT, thioflavin T.

have been shown to influence fibril formation by amyloid β protein; the anionic buffers phosphate and bicarbonate; as well as high concentrations of sodium, protein, and citrate (10, 14, 15).

The presence of divalent metal cations, and especially of zinc, is also important because seminal proteases can degrade SEVI fibrils under conditions in which Zn^{2+} concentration is low (16). In contrast, physiologic concentrations of zinc in semen are high (low millimolar levels) and act to maintain seminal proteases in an inactive state (17). Therefore, these cations may serve to protect SEVI fibrils from proteolytic degradation.

In this study, we examined the effects of seminal plasma (SP), and the specific components of seminal fluid, on fibril formation and enhancement of HIV infection by $PAP_{248-286}$. We found that SP dramatically accelerated fibril formation by $PAP_{248-286}$ but still yielded fibrils that were morphologically and functionally identical to fibrils formed in PBS. Additionally, physiologic concentrations of zinc protected SEVI from degradation by seminal proteases. Taken together, these results provide an explanation for the presence of SEVI fibrils in semen.

MATERIALS AND METHODS

Peptide Synthesis and Purification—$PAP_{248-286}$ and $PAP_{248-286}$Ala were synthesized on a CEM Liberty microwave-equipped peptide synthesizer utilizing standard *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) solid phase chemistry with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/ hydroxybenzotriazole (HOBt) activation. NovaPEG Wang resin was purchased from Novabiochem (Gibbstown, NJ), and all amino acids used were purchased from AAPPTec (Louisville, KY). Peptides were cleaved from the resin by suspension in TFA/ triisopropylsilane/H_2O (95:2.5:2.5, v/v/v) for 2 h and isolated by precipitation in cold diethyl ether. Peptides were purified by reverse phase HPLC on a Shimadzu LC-AD HPLC with a reverse phase C18 column (19 × 250 mm; Waters, Milford, MA). A linear gradient of acetonitrile and water (0.1% TFA) was used as a mobile phase at 55 °C while eluent was monitored by UV absorption at 215 and 254 nm. The purity and relative hydrophobicity of the peptides were determined by analytical HPLC analysis using a reverse phase C18 column (BEH300 10 μM, 4.6 × 250 mm; Waters). Products were verified using MALDI-TOF mass spectroscopy. HPLC fractions containing the correct peptide by mass were lyophilized.

Peptide Disaggregation—Lyophilized peptide was dissolved in TFA/HFIP (1:1, v/v, 1 ml), and the solution was evaporated under a stream of dry N_2. The resulting film was dissolved in HFIP (1 ml) and again evaporated under a stream of N_2. This material was dissolved in HFIP (500 μl), and concentration was determined by correlation to a standard concentration curve by analytical HPLC. The concentration curve was calibrated by amino acid analysis performed by AIBiotech (Richmond, VA). Once the concentration was determined, the HFIP solution was frozen and lyophilized for 12 h. The disaggregated peptide was used immediately.

Processing of Human SP—Semen samples were obtained from the Infectious Diseases Division (Rochester, NY) and Fairfax Cryobank (Fairfax, VA) and stored at −80 °C. Samples were collected only from normal healthy subjects. Semen samples were thawed, centrifuged for 30 min at 10,000 × *g*, and SP was collected and used immediately.

Semen Simulant Solution—A described previously, semen simulant (14) was synthesized. The formulation of the semen simulant is as follows: pH 7.7; citrate (mg/100 ml), 523 (18 mM); chloride (mg/100 ml), 142 (40 mM); calcium (mg/100 ml), 27.6 (7 mM); magnesium (mg/100 ml), 11.0 (4.5 mM); potassium (mg/100 ml), 109 (28 mM); sodium (mg/100 ml), 484 (220 mM); zinc (mg/100 ml), 16.5 (2 mM); fructose (mg/100 ml), 272 (15 mM); glucose (mg/100 ml), 102 (6 mM); protein (g/100 ml), 5.04; lactic acid (mg/100 ml), 62 (7 mM); urea (mg/100 ml), 45 (7.5 mM); all in a 123 mM sodium phosphate base.

Cell Lines and Viruses—CEM M7 cells (a gift from N. Landau, New York University, New York, NY) were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (50 units/ml), and streptomycin (50 μg/ml). HIV-1_{IIIB} was obtained from Zeptometrix (Buffalo, NY).

Kinetics of Fibril Formation—Peptide self-assembly kinetics were characterized by a ThT fluorescence assay. Freshly disaggregated peptide was dissolved at 10 mg/ml and agitated at 1,400 rpm and 37 °C. For unagitated conditions, peptide was placed at 37 °C without agitation. At various time points, peptide was diluted to 0.1 mg/ml in 25 μM ThT in 96-well black bottom microtiter plates, and fluorescence was measured using a Beckman Coulter DTX880 fluorometer with excitation at 465 nm and emission at 535 nm (20 nm bandwidth). Where a lag time is reported, fluorescence data were fit to the empirical sigmoid Equation 1, where y_0 and y_{max} are initial and maximum fluorescence intensities, respectively; k is the apparent rate constant, and $t_{½}$ is the time at which the fluorescence intensity reaches one-half the maximum value (18). Lag time was calculated using Equation 2 (18).

$$f(t) = y_0 + \frac{y_{max}}{1 + e^{-(t - t_{1/2})k}} \quad \text{(Eq. 1)}$$

$$t_{lag} = t_{1/2} - \frac{2}{k} \quad \text{(Eq. 2)}$$

Electron Microscopy—Mature fibrils from a 100-μl suspension were collected by centrifugation (14,000 × *g*, 1 h, 4 °C). The supernatant was removed, and the fibrils were washed in 100 μl of deionized water. The fibrils were again harvested by centrifugation, and the resulting supernatant was removed. The fibrils were resuspended in 100 μl of water, and a 10-μl aliquot was applied to 200 mesh, carbon-coated copper grids and allowed to adsorb for 5 min. Excess fluid was removed by capillary action, and residual salts and buffer were washed by application of water (10 μl) for 10 s followed by removal by capillary action. The grids were then stained via application of 10 μl of 5% uranyl acetate for 3 min, and the excess staining solution was removed via capillary action. The stained grids were allowed to air dry for at least 10 min prior to imaging. Electron microscopy images were obtained using a Hitachi 7650 transmission electron microscope in high contrast mode with an accelerating voltage of 80 kV.

Infectivity Assays—For infection of CEM M7 cells, X4 tropic HIV-1_{IIIB} (21 ng/ml p24 antigen) was pretreated for 10 min at

room temperature with 10 μg/ml SEVI. Treated virions were then added to 5×10^4 CEM M7 cells/well in 96-well flat-bottomed tissue culture plates. Infection was assayed after 48 h by quantifying luciferase expression in cell lysates using the Promega luciferase assay and a Beckman Coulter DTX880 plate reader.

RESULTS

SP Accelerates Fibril Formation by $PAP_{248-286}$—SP contains a diversity of molecules that could potentially impact fibril formation, including a unique buffer salt profile; high concentrations of zinc, calcium, and magnesium; as well as high concentrations of protein. Therefore, we were interested in examining whether SP had an effect on fibril formation by $PAP_{248-286}$. To test this, we resuspended $PAP_{248-286}$ directly into SP and allowed it to form fibrils under standard conditions (37 °C incubation, with rapid agitation (1,400 rpm)); we then monitored fibril formation over time using ThT fluorescence. SP dramatically accelerated fibril formation by $PAP_{248-286}$, reducing the lag time from 13 h, with a S.D. of 1.5 h (when $PAP_{248-286}$ was suspended in PBS) to an average of 0.9 h, with a S.D. of 0.17 h (Fig. 1*A*) among three different donors. In the three donors, the lag time ranged from 0.74 to 1.13 h.

Having observed this pronounced effect of SP on the formation of SEVI fibrils, we wanted examine to more closely which components of SP might be responsible for this effect. To do this, we took advantage of a well characterized semen simulant that mimics the pH, buffering capacity, osmolarity, ionic strength, and metal concentrations found in human semen (14). The use of this semen simulant (referred to hereafter as "artificial semen" (AS)) allowed us to dissect the requirements for SP-enhanced fibril formation while eliminating the possibility that preexisting SEVI fibrils or oligomers might contribute to the accelerated kinetics of fibril formation (as could, conceivably, be the case with native SP samples).

We first wanted to confirm that AS replicated the effects of SP on SEVI fibril formation. Therefore, we performed a time course analysis of fibril formation by $PAP_{248-286}$ resuspended in AS. As seen in Fig. 2*B*, the kinetics of fibril formation in AS closely mimicked those observed in SP and differed dramatically from the kinetics of fibril formation in PBS. In AS, the lag phase for SEVI fibril formation was 0.3 h, with a S.D. of 0.07 h (Fig. 2*B* and Table 1).

In light of the profound effect of SP and AS on the kinetics of SEVI fibril formation under standard (agitated) conditions, we were interested to know whether $PAP_{248-286}$ might be able to form fibrils without agitation in these solutions. To address this question, we suspended $PAP_{248-286}$ in AS or PBS and incubated the material at 37 °C without agitation; we then examined fibril formation over time by measuring ThT fluorescence. As shown in Fig. 1*C*, $PAP_{248-286}$ was able to form fibrils effectively without agitation in AS, but not in PBS.

Fibrils Formed in SP and AS Are Functionally and Morphologically Identical to Those Formed in PBS—We examined SEVI fibrils formed in AS by transmission electron microscopy to see whether they exhibited a structure similar to previously characterized SEVI fibrils formed in PBS. Fig. 3*A* shows that the ThT-reactive fibrils formed in PBS and AS exhibit broadly similar structures (unbranched long fibrils that coalesce into a mesh-like lattice). This suggests that AS changes the kinetics of fibril formation but does not grossly alter the structure of the fibrils. Due to the complex composition of SP, it was difficult to obtain images of the fibrils in SP; therefore, we chose to assess these fibrils from a functional, rather than structural standpoint.

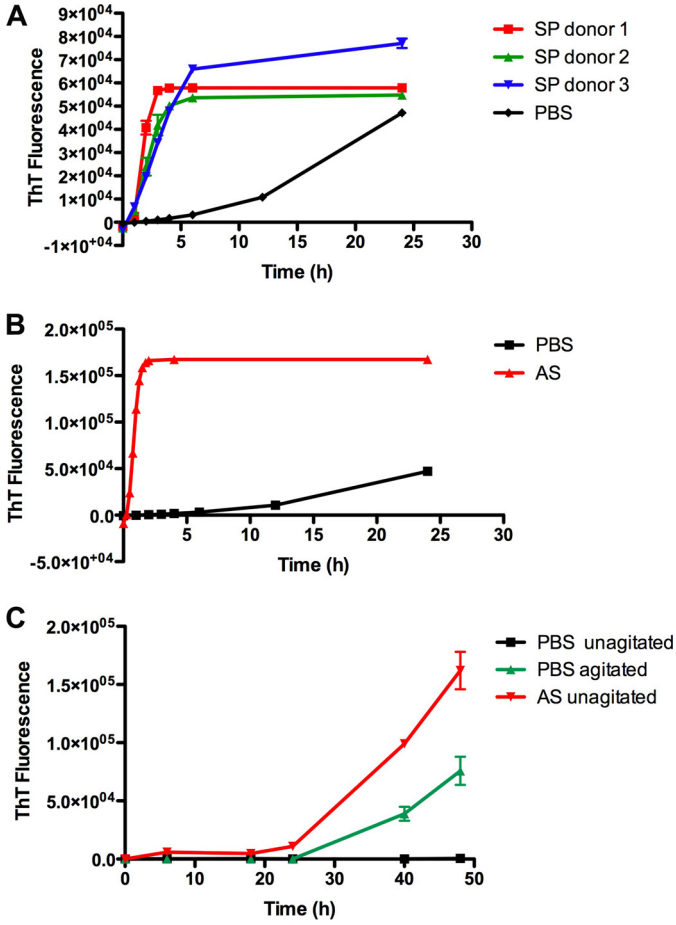

FIGURE 1. **SP greatly accelerates fibril formation by $PAP_{248-286}$.** *A*, SP accelerates fibril formation by $PAP_{248-286}$. $PAP_{248-286}$ peptide was resuspended in PBS and SP from three different donors at 10 mg/ml and agitated at 37 °C and 1,400 rpm. ThT fluorescence (indicative of fibril formation) was then measured at timed intervals; the results are presented. *B*, AS accelerates fibril formation by $PAP_{248-286}$. This experiment was performed as described in *A*, except that the $PAP_{248-286}$ peptide was resuspended in AS. *C*, $PAP_{248-286}$ forms fibrils without agitation in AS. $PAP_{248-286}$ peptide was resuspended in PBS or AS at 10 mg/ml and incubated at 37 °C without agitation. ThT fluorescence (indicative of fibril formation) was then measured at timed intervals; the results are presented. Results shown in all *panels* are average values ± S.D. (*error bars*) of triplicate measurements from one of three independent experiments, which yielded equivalent results.

We next wanted to ensure that SEVI fibrils formed in SP and AS retained the infection-enhancing properties of SEVI fibrils formed in PBS. To do this, we compared the ability of these fibrils to enhance infection of the CEM M7 reporter cell line with $HIV\text{-}1_{IIIB}$. As seen in Fig. 3*B*, SEVI fibrils formed in both SP and AS enhanced HIV-1 infection to an extent equivalent to SEVI fibrils formed in PBS. Unagitated fibrils also efficiently enhanced HIV infection. The magnitude of HIV-1 infection enhancement was statistically equivalent ($p > 0.05$) for fibrils formed in SP or AS, under both agitated and unagitated conditions compared with fibrils formed under standard conditions

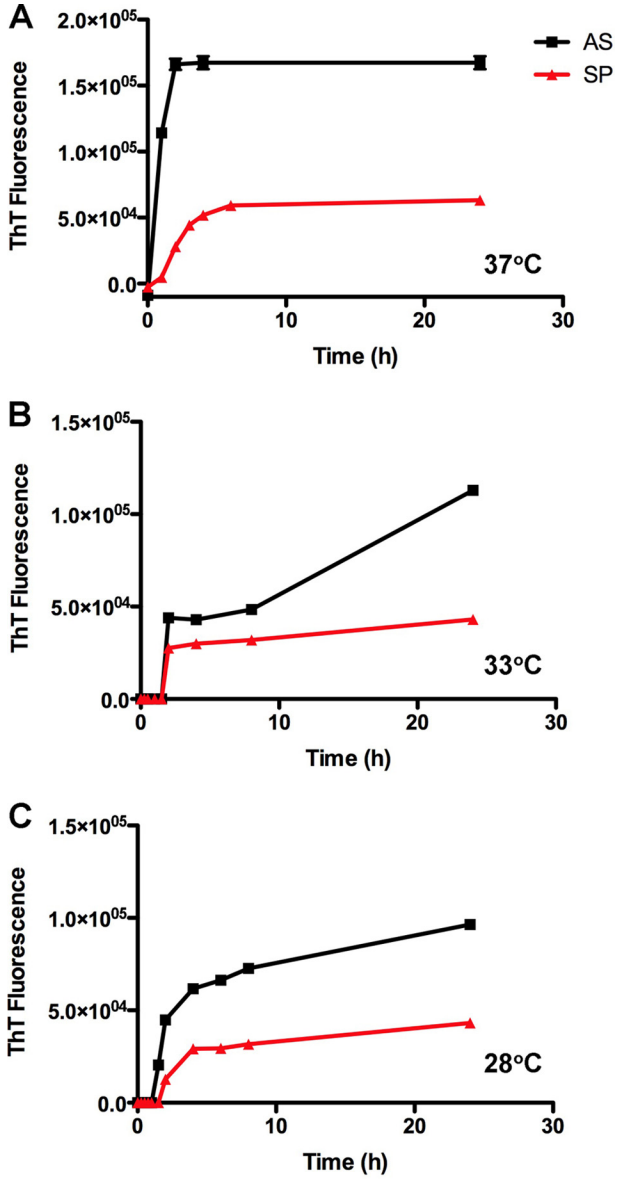

FIGURE 2. **SP and AS accelerate fibril formation by $PAP_{248-286}$ with similar kinetics, regardless of temperature.** $PAP_{248-286}$ peptide was resuspended in AS or SP at 10 mg/ml and incubated at the indicated temperatures, with continuous agitation at 1,400 rpm. ThT fluorescence (indicative of fibril formation) was then measured at timed intervals; the results are presented. Results shown are average values ± S.D. of triplicate measurements. Note that the results shown in *A* (37 °C) correspond to the same data shown in Fig. 1*B* (AS) and Fig. 1*A* (SP; the SP data values shown in Fig. 2*A* represent the mean values for the three different donors shown in Fig. 1*A*).

TABLE 1

Kinetics of fibril formation by $PAP_{248-286}$ in PBS, seminal plasma, artificial semen, sodium phosphate buffer alone, and bicarbonate buffer alone

Peptide was resuspended at 10 mg/ml in the indicated buffers and agitated at 37 °C and 1,400 rpm. Fibril formation over time was assessed by ThT fluorescence and used to calculate $t_{½}$ (h), k (h^{-1}), and lag time (h). $t_{½}$ is the time at which fluorescence intensity reaches half the maximum value, k is the apparent rate constant, and lag time was calculated from $t_{½}$ and k using the equation, $t_{lag} = t_{½} - (2/k)$. Results shown are average values ± S.D. of triplicate measurements from one of four independent experiments that yielded equivalent results. For SP, the values shown are the average of triplicate measurements from all three donors.

Condition	$t_{½}$	k	Lag time
	h	h^{-1}	*h*
PBS	27.7 ± 0.37	0.14 ± 0.01	13 ± 1.5
SP	2.14 ± .43	1.9 ± 1.5	0.39 ± 0.97
AS	0.8 ± 0.019	4.46 ± 0.88	0.34 ± .065
123 mM Sodium phosphate	1.4 ± 0.07	3.8 ± 0.21	0.89 ± 0.02
25 mM Bicarbonate buffer + 40 mM sodium phosphate	2.7 ± 0.03	3.5 ± 0.01	1.99 ± 0.107

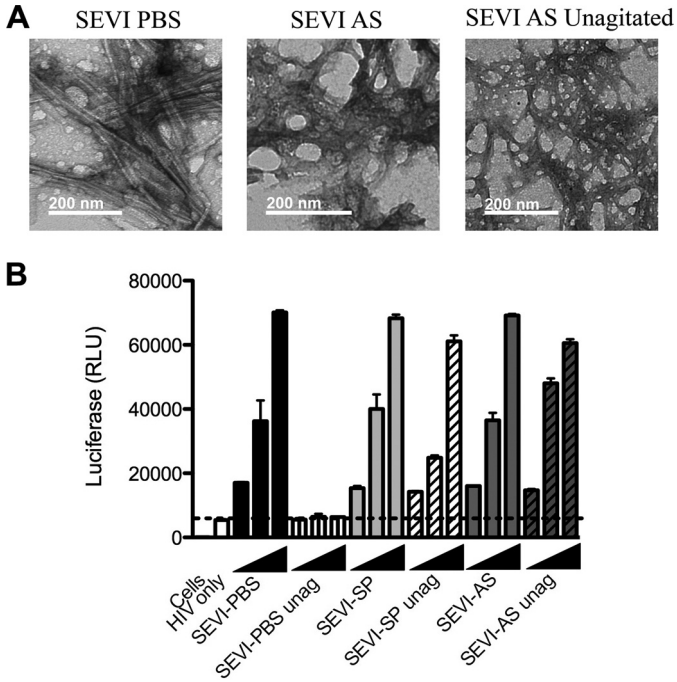

FIGURE 3. **Fibrils formed in SP and AS are morphologically and functionally equivalent to those formed in PBS.** *A*, SEVI fibrils were formed in PBS and AS under agitated and unagitated conditions, and then analyzed by transmission electron microscopy. Representative images are shown. *Scale bars*, 200 nm. It can be appreciated that the fibrils formed in PBS and AS exhibit broadly similar structures (unbranched long fibrils that coalesce into a mesh-like lattice). *B*, fibrils formed in seminal plasma (SEVI-SP) and artificial semen (SEVI-AS), under both agitated and unagitated conditions, enhance infection comparably with SEVI fibrils formed under agitation in PBS (SEVI-PBS). HIV-1_{IIIB} virions were mixed with increasing concentrations of SEVI fibrils formed in PBS, SP, or AS (with or without agitation) and then incubated for 10 min at 37 °C (peptide concentrations used for this experiment were 5, 15, or 30 μg/ml, as represented by the *triangles* below the *x* axis). Samples were then added to CEM M7 cells, and virus infection was assayed 48 h later by measuring Tat-driven luciferase expression in cell lysates. Results shown are average values ± S.D. (*error bars*) of triplicate measurements from one of three independent experiments that yielded equivalent results. The *dashed line* indicates the luciferase level of cells infected with HIV alone, in the absence of SEVI fibrils.

(agitated PBS) by ANOVA with Tukey's post test. It is important to note that the SP samples did not enhance infection of HIV on their own (data not shown), presumably because our high speed centrifugation preparation method removed all preexisting fibrils from the sample (as reported previously by Münch *et al.*) (5).

Anionic Buffer Components Are Responsible for Accelerated Fibril Formation by $PAP_{248-286}$—Having observed that $PAP_{248-286}$ forms fibrils rapidly and without agitation in both SP and AS, we were interested in examining which components of these solutions were responsible for their effects on fibril formation. Therefore, we formulated several variants of AS, in which one or more components were removed. Removal of the metal cations zinc and magnesium, the protein, and the calcium ions all had minimal effects on the kinetics of fibril formation (data not shown). In fact, a solution containing only the anionic buffer component of AS (0.123 M sodium phosphate) had nearly

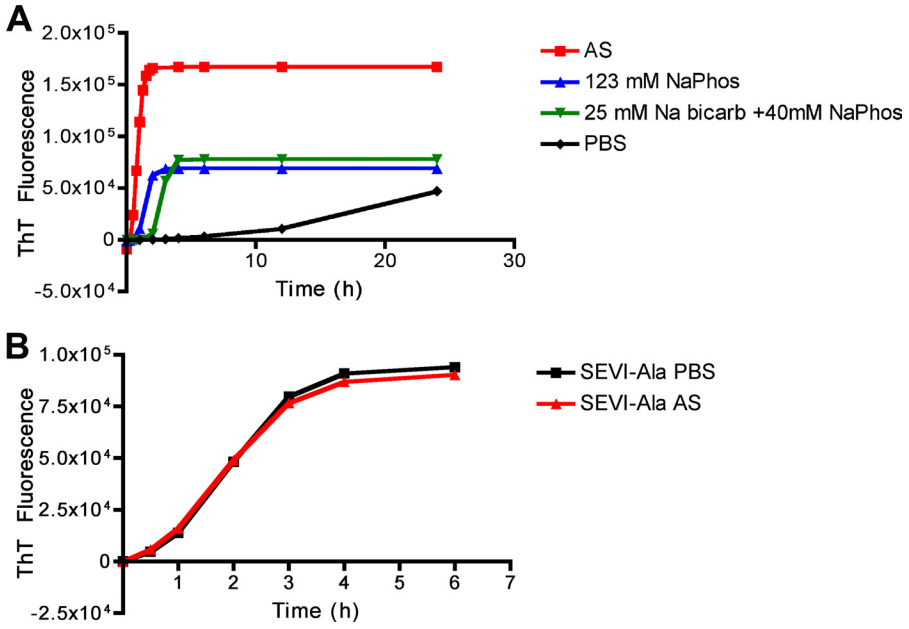

FIGURE 4. **Anionic buffer components are responsible for effect of AS on fibril formation by $PAP_{248-286}$.** *A*, sodium phosphate buffer or sodium bicarbonate buffer alone accelerates fibril formation by $PAP_{248-286}$, to an extent equivalent to complete AS. $PAP_{248-286}$ peptide was resuspended at 10 mg/ml in PBS, AS, 0.123 M sodium phosphate (123 mM *NaPhos*), or 25 mM sodium bicarbonate + 40 mM sodium phosphate (25 mM *Na bicarb* + 40 mM *NaPhos*) and agitated at 37 °C and 1,400 rpm. ThT fluorescence (indicative of fibril formation) was then measured at timed intervals; the results are presented. *B*, AS has no effect on the kinetics of fibril formation by a noncationic variant of $PAP_{248-286}$, $PAP_{248-286}$Ala. $PAP_{248-286}$Ala peptide, which contains Ala substitutions in place of the Arg and Lys residues in wild-type $PAP_{248-286}$, was resuspended in PBS or AS at 10 mg/ml and agitated at 37 °C and 1,400 rpm. ThT fluorescence (indicative of fibril formation) was then measured at timed intervals; the results are presented. Results shown in both *panels* are average values ± S.D. of triplicate measurements from one of three independent experiments that yielded equivalent results.

the same effects on fibril formation as complete AS, with a lag phase of 0.89 h (Fig. 4*A*, Table 1).

SP has a unique buffering capacity because of its role in neutralizing the pH of cervicovaginal fluid during coitus, which is essential for fertilization (19). However, previous reports on the buffer components of SP have described somewhat conflicting findings (14, 15, 20) Therefore, we formulated an additional buffer solution containing an alternative concentration of sodium bicarbonate and sodium phosphate that has been reported in other literature on the composition of seminal fluid (15). This solution contained 25 mM sodium bicarbonate, with 40 mM inorganic phosphate (15). All solutions were adjusted to pH 7.7. Fig. 4*A* shows that this solution accelerated fibril formation to an extent nearly equivalent to the original AS buffer. The kinetics of fibril formation by $PAP_{248-286}$ in these various solutions, including lag time, $t_{1/2}$, and k, are summarized in Table 1.

$PAP_{248-286}$ is a highly cationic peptide, containing six lysine and two arginine residues. Therefore, it is possible that these anionic buffers accelerate fibril formation by reducing charge-charge repulsion between peptide subunits. If so, we would not expect AS to enhance fibril formation by an otherwise similar, but noncationic, peptide. To test this prediction, we examined the effects of AS on fibril formation by $PAP_{248-286}$Ala, a variant of the SEVI-forming peptide in which all of the lysine and arginine residues have been mutated to alanine. This peptide has been shown to form fibrils that have no effect on HIV infection (6). As expected, this variant peptide formed fibrils more rapidly than wild-type $PAP_{248-286}$ in PBS (likely because of its lower overall charge) (compare Fig. 4, *A* and *B*). However, the kinetics of fibril formation by $PAP_{248-286}$Ala were not affected by AS (Fig. 4*B*). This suggests that the effects of AS on SEVI fibril formation are due to its ability to reduce the electrostatic repulsion between cationic $PAP_{248-286}$ peptide subunits.

AS Protects SEVI from Degradation by Seminal Proteases—As noted above, the metal cations present in SP do not contribute significantly to accelerating fibril formation by $PAP_{248-286}$. However, we hypothesized that these metals, especially zinc, might play an important role in protecting the fibrils from degradation by seminal proteases. Semen contains a high concentration of zinc, which acts in part to maintain seminal proteases such as prostate-specific antigen (PSA) in an inactive state until after ejaculation (17). Previous reports have suggested that seminal proteases, especially PSA, can degrade SEVI (16). However, in these studies, 1% SP was diluted into PBS, removing the proteases from the physiologic zinc concentration which acts to restrict their activity. We were interested to see whether the addition of seminal proteases had the same effect in the presence of zinc and other components of semen.

To address this question, we examined fibril formation in the presence of 1% SP. Fig. 5*A* shows that addition of 1% SP to a suspension of $PAP_{248-286}$ peptide in PBS resulted in a failure to form SEVI fibrils after 48 h. We presumptively attribute this to their proteolytic degradation, as reported by Martellini *et al.* (16). In contrast, addition of 1% SP to a suspension of $PAP_{248-286}$ peptide in AS had no effect on fibril formation, and high levels of fibrils were formed after 48 h (Fig. 5*A*).

To test whether the protective effect of AS could be attributed to zinc ions, 1% SP was added to fully formed fibrils that were suspended either in complete AS or AS without zinc. Fig. 5*B* shows that at both 8 and 24 h, the level of fibrils remaining in AS without zinc was greatly reduced (as reflected by a decline

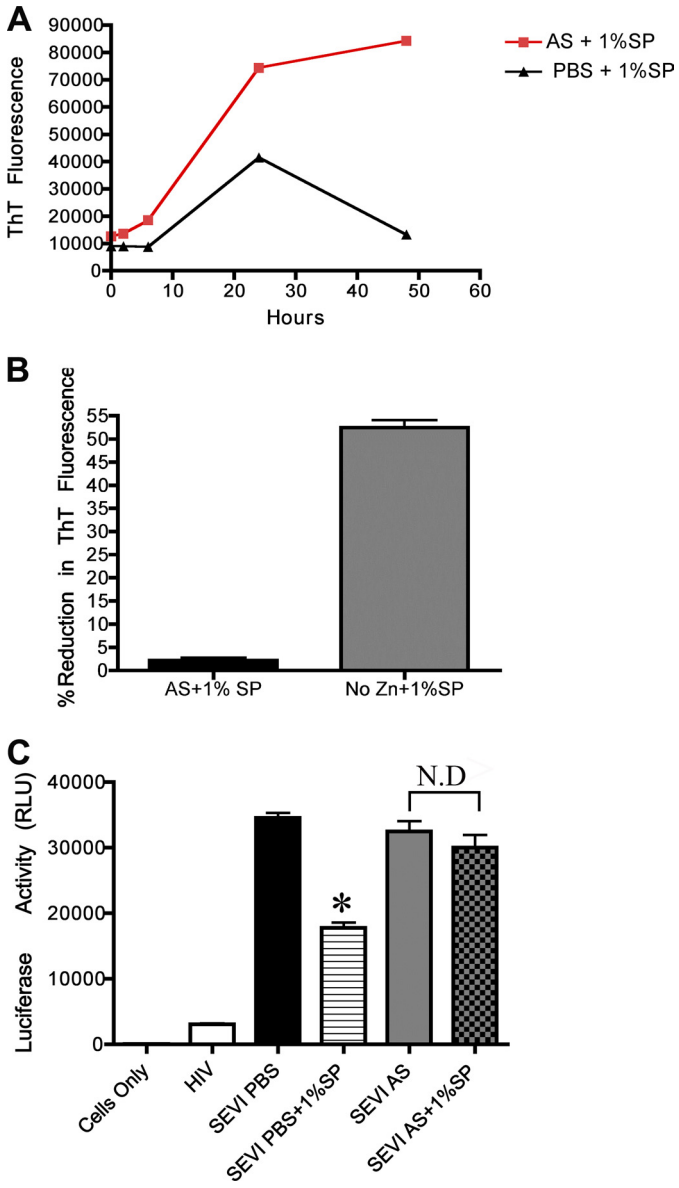

FIGURE 5. **AS protects SEVI from degradation by seminal proteases.** *A*, addition of 1% SP to $PAP_{248-286}$ reduces fibril formation in PBS, but not in AS. $PAP_{248-286}$ was resuspended at 10 mg/ml in PBS or AS containing 1% SP and agitated at 37 °C and 1,400 rpm. ThT fluorescence (indicative of the presence of fibrils) was then measured at timed intervals; the results are presented. *B*, physiologic concentrations of zinc protect preformed SEVI fibrils from degradation by SP. Preformed SEVI fibrils were resuspended at 10 mg/ml in complete AS or AS without Zn^{2+}. One percent SP was added, and samples were agitated at 37 °C and 1,400 rpm; ThT fluorescence was measured at 0, 8, and 24 h. Data are presented as the percentage of the initial ThT fluorescence at base line (0 h). *C*, SEVI formed in the presence of SP is fully functional. CEM M7 cells were infected with $HIV\text{-}1_{IIIB}$ in the presence or absence of 10 μg/ml SEVI fibrils formed in PBS or AS, with and without the addition of 1% SP. Infection was assayed at 48 h by measuring Tat-driven luciferase expression in lysates of the CEM M7 indicator cells. *, $p < 0.001$ compared with SEVI-PBS (ANOVA with Tukey's post test). *N.D.*, no significant difference compared with SEVI-PBS. Results shown in all *panels* are average values ± S.D. (*error bars*) of triplicate measurements from one of three independent experiments that yielded equivalent results.

in the ThT fluorescence). However, fibrils that were suspended in complete AS (containing physiologic levels of zinc) were completely protected from degradation by 1% SP (as reflected by stable ThT fluorescence over time). Finally, we tested whether SEVI fibrils formed in AS containing 1% SP retained their ability to enhance HIV infection. Fig. 5*C* shows that SEVI fibrils formed in AS +1% SP enhanced HIV-1 infection in CEM M7 cells to an extent equivalent to SEVI fibrils formed in PBS (in the absence of SP). In contrast, fibrils formed in PBS +1% SP showed a 50% percent reduction in enhancement of HIV infection (Fig. 5*C*). This suggests that the high zinc concentration found in semen is important for preventing proteolytic degradation of SEVI fibrils.

DISCUSSION

Previous studies have noted that semen may contain substances that both enhance (5–7, 22, 23) and inhibit HIV-1 infection (24–26). However, recent results reported by Roan *et al.* indicate that the overall effect of semen is to enhance HIV-1 infection, at least *in vitro* (23). Consistent with this, studies conducted using nonhuman primates have shown that human SP can enhance the efficiency of vaginal transmission of SIV_{mac251} in animals exposed to low dose inocula (27, 28). Unfortunately, these studies used small group sizes and failed to achieve statistical significance (27, 28), pointing to the need for additional studies to address this issue, using larger group sizes.

The infection-enhancing effects of semen have been attributed principally to cationic amyloid fibrils, including SEVI fibrils (5–7, 22, 23). Our studies show that the fibril-forming tendencies of $PAP_{248-286}$ are greatly enhanced under physiologic conditions. Previous studies have reported the need for rapid agitation and an extended lag phase of 8–12 h, to induce formation of SEVI fibrils (5, 8). It is hard to reconcile these requirements (especially agitation at 1,400 rpm) with normal human physiology. This creates an important conundrum: how can SEVI fibrils be present *in vivo* (5, 29), if nonphysiologic levels of agitation are required for them to form? Our work provides a resolution of this issue and suggests that the presence of SEVI fibrils in human semen can be attributed to the fact that the components of SP greatly accelerate the kinetics of nucleation of SEVI fibrils.

The $PAP_{248-286}$ peptide is highly cationic. For other fibrils, strong electrostatic repulsion has been shown to induce molecular frustration and disrupt the hydrogen-bonded, cross-β network, resulting in fibril dissociation (30). Therefore, the electrostatic repulsion between $PAP_{248-286}$ peptide subunits may lead to continual dissociation of fibril precursors and is likely to slow formation of SEVI fibrils dramatically. This idea is reinforced by the fact that an uncharged variant of SEVI, SEVI-Ala, undergoes fibril formation much more rapidly than wild-type SEVI. The components of SP, especially the anionic inorganic phosphate and bicarbonate, may serve to reduce the electrostatic repulsion between $PAP_{248-286}$ peptides, thereby greatly accelerating nucleation of SEVI fibrils.

Additionally, seminal components are essential to keeping the action of prostatic proteases in check. Zinc ions, which are present in millimolar concentration in seminal fluid (14), are especially important in this regard. Zn^{2+} strongly inhibits the protease activity of PSA (17), which has been shown to be capable of cleaving SEVI fibrils (16). Because PSA is a major proteolytic component of SP (17), this likely explains why SEVI fibrils are degraded efficiently by 1% SP if they are diluted in PBS (which contains no Zn^{2+}) but not if they are diluted in AS

(which contains physiologic levels of Zn^{2+}). This strongly suggests that SEVI likely persists *in vivo* because of the inhibitory effects of Zn^{2+} on seminal proteases. It is possible that reducing Zn^{2+} concentrations in semen might accelerate the proteolytic degradation of SEVI fibrils. However, this may not be a wise approach to the prevention of HIV transmission because there are multiple reports that physiologic levels of zinc can inhibit infection and/or replication by herpes simplex virus type 2, HIV, and other viruses (31–36). In addition, recent work has demonstrated that zinc-containing gels can protect against vaginal infection by herpes simplex virus type 2 infection and simian-human immunodeficiency virus (37, 38).

The anatomical location in which SEVI fibrils are formed is still unclear. The precursor protein, prostatic acid phosphatase, is found in highest concentrations in the prostatic fluid, and it is conceivable that the $PAP_{248-286}$ peptide could be liberated at this site and that fibril might be initiated here. It is therefore important to note that the composition of prostatic fluid has many important similarities with whole semen. Specifically, prostatic fluid contains high levels of Zn^{2+} (9 mM, compared with 2 mM in semen) (39), as well as spermine and spermidine, which also act as inhibitors of PSA. In addition, prostatic fluid also has a high concentration of phosphate (derived from spermine phosphate) (20, 21), which can be expected to accelerate fibril formation greatly (Fig. 3 and Table 1). Intriguingly, the measured phosphate concentrations in prostatic fluid have also been reported to vary considerably, suggesting the possibility that individual variations in the ionic composition of semen and prostatic fluid may contribute to donor-to-donor variability in the concentration of SEVI fibrils (5, 29).

Taken together, these results suggest that the $PAP_{248-286}$ peptide can form fibrils under physiologic conditions and that these fibrils can persist despite the presence of seminal proteases. This work therefore provides a biochemical explanation for the *in vivo* formation of SEVI fibrils and explains why measurable concentrations of SEVI fibrils have consistently been detected in human semen samples (5, 29). Finally, these studies underscore the importance of follow-up experiments to explore the biological function(s) of SEVI and other amyloid fibrils that may be present in human semen (22, 23, 29).

Acknowledgments—We also gratefully acknowledge Cathy Bunce and Mike Keefer for assistance with obtaining semen samples, as well as Karen Bentley and the URMC EM Core facility for assistance with electron microscopy.

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