Negatively Charged Peptide Nanofibrils from Immunoglobulin Light Chain Sequester Viral Particles but Lack Cell-Binding and Viral Transduction-Enhancing Properties

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ABSTRACT: Positively charged naturally occurring or engineered peptide nanofibrils (PNF) are effective enhancers of lentiviral and retroviral transduction, an often rate-limiting step in gene transfer and gene therapy approaches. These polycationic PNF are thought to bridge the electrostatic repulsions between negatively charged membranes of virions and cells, thereby enhancing virion attachment to and infection of target cells. Here, we analyzed PNF, which are formed by the peptide AL1, that represents a fragment of an immunoglobulin light chain that causes systemic AL amyloidosis. We found that negatively charged AL1 PNF interact with viral particles to a comparable extent as positively charged PNF. However, AL1 PNF lacked cell-binding activity, and consequently, did not enhance retroviral infection. These findings show that virion capture and cell binding of PNF are mediated by different mechanisms, offering avenues for the design of advanced PNF with selective functions.

1. INTRODUCTION

Self-assembling peptide nanofibrils (PNF) are versatile tools for a growing number of applications in biomedicine. Among the most advanced PNF are those that facilitate retroviral gene transfer and gene therapy approaches. These polycationic PNF are thought to bridge the electrostatic repulsions between negatively charged membranes of virions and cells, thereby enhancing virion attachment to and infection of target cells. Here, we analyzed PNF, which are formed by the peptide AL1, that represents a fragment of an immunoglobulin light chain that causes systemic AL amyloidosis. We found that negatively charged AL1 PNF interact with viral particles to a comparable extent as positively charged PNF. However, AL1 PNF lacked cell-binding activity, and consequently, did not enhance retroviral infection. These findings show that virion capture and cell binding of PNF are mediated by different mechanisms, offering avenues for the design of advanced PNF with selective functions.
2. RESULTS AND DISCUSSION

2.1. AL1 Peptide Forms Negatively Charged PNF Lacking Infection-Enhancing Activity. AL1 PNF were analyzed by transmission electron microscopy (TEM) confirming the formation of twisted fibrils with diameters varying roughly between 8.0 and 22.0 nm (Figure 1A), due to fibril polymorphism. \(^{18}\) Amyloid formation was further corroborated by changes in the fluorescence intensities of the amyloid-specific dye Thioflavin T (Figure 1B). To further characterize and compare the fibrils to the seminal amyloid PAP248-286, we performed \(\zeta\)-potential measurements of both native peptides and fibrils. AL1 PNF have an isoelectric point of 5.52 and featured a net negative surface charge \((-12\,mV)\), whereas PAP248-286 fibrils were positively charged \((16\,mV)\) in KCl solution, confirming previous data.\(^{3,10,12,26}\) Native AL peptide features small negative \((-3\,mV)\) and PAP284-286 minor positive \((0.3\,mV)\) surface charges (Figure 1C). Due to the rapid assembly of EF-C peptide into fibrils, \(^{3}\) \(\zeta\)-potentials of freshly dissolved peptide could not be determined; however, the assembled EF-C PNF were positively charged \((+12\,mV)\), as shown before.\(^3\)

Before studying the impact of AL1 PNF on infection, we verified that they did not impair cellular metabolic activity at concentrations of up to 200 \(\mu g/mL\) (Figure 1D). We then determined the infection-enhancing activity of AL1 PNF, and as controls, PAP248-286 fibrils (SEVI) and EF-C PNF. As shown in Figure 1E, EF-C PNF and SEVI fibrils enhanced viral infection in a concentration-dependent manner, with up to 39-fold increased infection rates for EF-C PNF, and 21-fold increased infectivity in the presence of SEVI, at the highest tested concentration. On the contrary, AL1 PNF only marginally increased viral infection (2-fold) at 40 and 200 \(\mu g/mL\). Thus, AL1 fibrils lack potent infection-enhancing activity, presumably due to the negative surface charge, which may prevent the interaction with viral and cellular membranes.
2.2. AL1 PNF Bind Viral Particles. To prove that AL1 PNF featuring a negative z-potential indeed lack virion binding activity, we performed scanning electron microscopy and cryo-transmission electron microscopy of fibrils that were exposed to noninfectious env-deleted HIV-1 or MLV particles. Unexpectedly, our analysis showed that virions were sequestered by the AL1 PNF network (Figure 2 A,B). Individual fibrils aligned to the viral envelope bilayer (Figure 2A,B) without causing obvious alterations in the viral membrane (Figure 2B). Confocal microscopy confirmed this finding and revealed complex formation of virions and fibrils (Figure 2C), very similar to those previously obtained for SEVI,8 Semenogelin,27 and EF-C PNF.3 Virion binding by AL1 PNF, EF-C PNF, and SEVI fibrils was further quantified by flow cytometry. For this, a constant concentration of fibrils (100 μg/mL) was incubated with increasing concentrations of the fluorescently labeled MLV particles, and complex formation was analyzed via flow cytometry. A viral-dose-dependent increase in fluorescence was observed for all fibril species (Figure 2D,E). To exclude that the increase in fluorescence is due to unspecific effects, different concentrations of MLV-Gag virions lacking the YFP expression cassette were tested for fibril binding in parallel, using a constant AL1 fibril concentration of 0.1 mg/mL (Figure S1A). Furthermore, flow cytometry analysis of MLV-YFP particles only (in the absence of fibrils) did not result in virus-specific fluorescence signals excluding that free virions interfered with analysis or cause false-positive results (Figures 2D, S1B). Interestingly, the fluorescence signals for AL1 fibrils were significantly higher than for the respective EF-C or SEVI-fibril/virion complexes (Figure 2D). However, we found AL1 PNF to exhibit autofluorescence, giving rise to higher signals. To exclude unspecific signals from fibrils with increasing virion concentrations, we compared fluorescence signals of AL1 PNF and fluorescent retroviral particles with AL1 PNF incubated with the same concentrations of nonfluorescent virions. For AL1 fibrils and nonfluorescent virions, no fluorescence increase of fibrils with increasing virion concentrations was observed, proving the specificity of signals (Figure S1A).

2.3. Low Cell-Binding Activity of AL1 PNF Compared to EF-C PNF and SEVI. AL1 PNF bind virions but do not enhance viral infectivity. Thus, we next analyzed if AL1 PNF are capable of binding to the cell membrane, as previously reported for EF-C PNF3 and SEVI.8 To test this, the cells were incubated with the three Proteostat-labeled fibril species for 1 h and then analyzed by confocal microscopy before or after thorough washing. As shown in Figure 3A, the three labeled fibril species were readily detectable as red-fluorescent patches on the cell surface. To analyze whether the fluorescence is caused by sedimentation of the fibril complexes or a specific interaction with the cell surface, we next washed the cells to remove unbound fibrils. Washing hardly affected EF-C PNF fluorescence, indicating a strong interaction of these fibrils with the cell surface (Figure 3A). Washing removed only some of the SEVI fibrils while AL1 PNF fluorescence disappeared (Figure 3A). Flow cytometry results confirmed these findings: after extensive washing, almost all EF-C PNF and ~61% of SEVI remained cell-associated. In contrast, more than 80% of the AL1 PNF were removed by washing. We next analyzed whether changes in the incubation time may affect AL1 PNF binding. However, even after 2 h of incubation, AL1 PNF could be easily removed from the cell surface, whereas SEVI and EF-C fibrils remained bound (Figure 3C). Thus, AL1 PNF do not efficiently attach to the cell surface, which may explain the lack of viral infection-enhancing activity.

To corroborate this finding, we quantified the amount of virus that was delivered to the cellular membrane by the three types of fibrils using a p24 antigen ELISA that quantifies the...
viral capsid protein. As expected, SEVI and EF-C PNF resulted in a dose-dependent increase of cell-bound viral capsid antigen (Figure 3D). Cell-associated p24 was increased up to 23-fold upon virus incubation with EF-C PNF and up to 14-fold with SEVI. However, no increase in virus binding was observed with AL1 PNF (Figure 3D). Collectively, these findings demonstrate that AL1 PNF effectively sequester viral particles but lack cell binding and consequently viral infection-enhancing activity.

3. CONCLUSIONS

To identify functional amyloids of the human body that may play roles in innate immunity or be developed as nanotechnological tools, we here analyzed amyloid-like fibrils formed by a specific peptide derived from the antibody light chain, the AL1 peptide.17 In contrast to viral infection enhancing fibrils such as SEVI or EF-C that have a positive surface charge, AL1 PNF feature a net negative surface charge. AL1 PNF lacked viral infection-enhancing activity, which was expected because the anionic fibrils might not bridge or neutralize the negative charge repulsions that exist between viral and cellular membranes. In fact, no binding to the cellular membrane was observed for AL1 PNF, in contrast to cationic EF-C and SEVI fibrils. It came to a surprise, however, that AL1 PNF featured no deficit in virion binding, and interacted with viral particles to a similar extent as positively charged EF-C and SEVI fibrils. Thus, fibrils with a net negative charge are also capable of sequestering viral particles and the net positive surface charge of fibrils is not the only driving force for binding to virions. However, it needs to be considered that AL1 PNF may feature positively charged surface patches, which may mediate electrostatic interactions with negatively charged virions. In fact, a recently established model of AL1 PNF suggests the presence of positive charges at the fibril surface (Figure S2). Thus, even though the fibril has a

Figure 3. AL1 PNF lack cell-binding activity. (A) Representative confocal microscopy images of TZM-bl HeLa cells incubated with Proteostat-labeled (red) EF-C, SEVI, and AL1 PNF (each 5 μg/mL). After 1 h, the cells were imaged, washed three times in PBS, and imaged again. Nuclei were stained with Hoechst (blue). (B) Quantitative evaluation of Proteostat-labeled fibril binding using ImageJ from two individual experiments with three to four images each using ImageJ. Fluorescence intensity was normalized to intensities obtained before washing. (C) Flow cytometry evaluation of Proteostat-labeled fibril binding to TZM-bl HeLa cells. TZM-bl HeLa cells were incubated with the Proteostat-labeled fibrils, washed, trypsinized, and analyzed by flow cytometry. Shown are mean (±SD) of two independent experiments. Asterisks indicate background signal only. (D) TZM-bl HeLa cells were inoculated with HIV-1 that was exposed to indicated concentration of the fibrils. After 3 h, the cells were washed three times, lysed, and analyzed for viral capsid antigen by p24 enzyme-linked immunosorbent (ELISA). Shown are mean (±SD) of two independent experiments.
net negative net charge, cationic regions on the fibril surface may be sufficient for virion capture, but not for cell binding. The selective interaction of AL1 PNF with membranes of virions but not cells suggests that differences in the composition of the viral and cellular lipid bilayer may account for this observation. The retroviral envelope is derived from the cell membrane, which resembles a typical lipid bilayer and is negatively charged due to the anionic phospholipid phosphatidylserine. However, viral and cellular membranes are not identical. First, embedded into the viral membrane are viral glycoproteins that mediate attachment and infection of target cells and may serve as binding partners of AL1 PNF. However, as shown by TEM, Cryo-EM, confocal microscopy, and flow cytometry, AL1 PNF fibrils captured lentiviral or retroviral particles lacking viral glycoproteins, which were used in these experiments for safety reasons as they are not infectious. Second, retroviral particles are budding through membrane microdomains, which are rich in cholesteryl, sphingolipids, as well as GPI-linked and fatty acylated proteins. Consequently, the viral membrane is enriched in these fatty acids. Notably, a recent article showed that HIV-1 particles are also heavily enriched in phosphoinositides, in particular phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5-trisphosphate (PIP₃). PIP₃ is the most negatively charged plasma membrane lipid and might render viral particles much more polyanionic than the cytoplasmic membrane. Consequently, the highly negatively charged viral membrane may allow binding into cavities between the positive patches in AL1 PNF, whereas AL1 PNF are not electrostatically attracted by cells with lower charge density, explaining the lack of viral-enhancing activity.

Amyloid fibrils have long been associated with diseases but it became increasingly evident that fibrils also exert “functional” roles. One example of functional amyloid in humans are fibrils formed by fragments of PAP or Semenogelin, which are naturally present in semen and are responsible for removal of apoptotic sperm and bacterial pathogens in the female reproductive tract. Other examples are amyloidogenic amyloid-β/peptide variants, which are involved in Alzheimer’s disease but have recently been shown to induce microbial agglutination and to exert antimicrobial and antiviral activity. Even though AL1 PNF sequestered viral particles, the fibrils did not abrogate viral infectivity, even at elevated concentrations. Whether AL1 PNF serve any functional role, e.g., antibacterial activity, needs to be addressed in follow-up studies. It would also be of interest to analyze more negatively charged natural amyloids or synthetic PNF for their ability to sequester virions to further dissect the underlying mechanism(s) and to clarify whether virus binding is a general feature of negative fibrils, or specific only for AL1 PNF. Our results show that binding of fibrils to virions and cells underlies shared but also separable mechanisms and are seemingly more complex than previously anticipated. However, the identification of fibrils that selectively bind viral particles without enhancing viral infection may open novel avenues for research. For example, AL1 PNF equipped with membrane destroying agents such as molecular tweezers may act as direct antiviral nanomaterials that specifically target the viral envelope and not the cell. Alternatively, AL1 PNF carrying specific cell targeting moieties may allow selective delivery of viral vectors or drugs to specific cell types or tissues.

4. EXPERIMENTAL SECTION (MATERIALS AND METHODS)

4.1. Reagents and Fibrils. Synthetic peptides PAP248-286 and EF-C were purchased from Celtek peptides (Franklin, Tennessee) or Synpeptide Co., Ltd. (Shanghai, China). For fibril formation, peptides were reconstituted and assembled as previously described. AL1 peptide was chemically synthesized at IZKF Leipzig, Core Unit Peptid-Technologien with the sequence IGNNVVTWYQQL. To prepare fibrils, a 5 mg/mL concentration of lyophilized AL1 peptide was dissolved in 50 mM Tris–HCl buffer pH 8.0 and incubated at room temperature for at least 3 days. All experiments were performed by diluting preformed fibrils with PBS (pH 7.4).

4.2. Electron Microscopy. Specimens were prepared by placing 5 μL of AL1 PNF suspension onto a formvar and carbon-coated 200 mesh copper grid (Plano) followed by a 1 min incubation period at room temperature. The grid was washed three times with water, stained with 2% (w/v) uranyl acetate solution, and dried. Finally, the dried grid was examined under a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan) at 120 kV. For cryo-transmission electron microscopy (cryo-TEM) and scanning electron microscopy, AL1 PNF and, for safety reasons, noninfectious env-deleted HIV-1 particles or H₂O were incubated for 10 min at room temperature prior to adhesion of 3.5 μL to a freshly glow-discharged holey carbon grid. The grids for SEM were blotted, fixed in 1% glutaraldehyde, and washed with 5% ethanol in H₂O. Both, the grids for cryo-TEM and SEM, were then vitrified in liquid ethane by a Vitrobot FP S350/60 (FEI, Eindhoven, Netherlands). The cryo-TEM grids were analyzed in a JEM-2100F (JEOL) at 200 kV. For SEM, samples were freeze-dried and platinum (2 nm)/carbon-coated (0.5 nm) by electron beam evaporation in a Bal 300 (BAL-TEC AG, Balzers, Principality of Liechtenstein) as described previously (Walther et al.). Grids were analyzed with a Hitachi S-5200 field emission scanning electron microscope (Tokyo, Japan) detecting the secondary electron signal at 10 kV.

4.3. Thioflavin T Fluorescence. Fibril samples (5 μL of 5, 2.5, 1.25, 0.6 mg/mL) were stained with 145 μL of 50 μM ThT (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in black 96-well plates with clear bottom (Corning). Fluorescence spectra were recorded on a Tecan Infinite M1000 PRO plate reader (Zürich, Switzerland). The excitation wavelength was chosen as 440 nm, and emission was detected at 460–600 nm.

4.4. ζ-Potential. Surface charge of fibrils/peptides was assessed by mixing 50 μL of a 1 mg/mL solution of fibrils with 950 μL of 1 mM KCl solution and measuring in a DTS1061 capillary cell (Malvern, Herrenberg, Germany) using the Zeta Nanosizer and the DLS Nano software (Malvern, Herrenberg, Germany). Per sample, three measurements were performed. For neutralization experiments, 0.5 mg/mL AL fibrils were incubated with the indicated substances (50 or 500 μg/mL heparin/polybrene), centrifuged for 10 min at 14 000 rpm, and resuspended with 50 μL of 1 mM KCl. PBS was used as a particle-free control.

4.5. Cell Culture and Virus Generation. Adherent TZM-bl reporter cells (NIH Aids Reagent: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc.) containing a lacZ reporter gene under the control of an HIV long terminal repeat (LTR) promoter were cultured in DMEM supplemented with 120 μg/mL penicillin, 120 μg/mL streptomycin, 350 μg/mL glucose, and 10% inactivated fetal calf serum.
Bad Wildbad, Germany). Virus stocks of infectious CCR5-tropic HIV-1 NL4-3 92TH014 were generated by transient transfection of 293T cells with respective proviral plasmids as described (Münch et al.). After transfection and overnight incubation, the transfection mixture was replaced with 2 mL of cell culture medium with 2% inactivated FCS. After 40 h, the culture supernatant was collected and centrifuged for 3 min at 3300 g to remove cell debris. Virus stocks were analyzed by p24 antigen ELISA and stored at −80 °C. Virus-like particles of murine leukemia virus tagged with YFP (MLV-Gag-YFP VLPs) were produced by transfection of HEK293T cells with pcDNA3 MLV Gag-YFP using TransIT-LT-1 (Mirus). Two days post-transfection, supernatants were harvested, clarified by centrifugation, aliquoted, and frozen at −80 °C until use.

4.6. Cell Viability Assay. The effect of different fibril concentrations on the metabolic activity of TZM-bl cells was assessed using the ATP-dependent CellTiter-Glo assay (Promega, Madison, Wisconsin (WI)). After 3 day incubation with the fibrils, the supernatant was discarded and 50 μL of PBS and 50 μL of CellTiter-Glo Reagent were added to the cells. After incubation and gentle shaking for 10 min at room temperature, luminescence in the cell-free supernatant was determined via the Orion microplate luminometer (Berthold, Bad Wildbad, Germany).

4.7. HIV Enhancement Assay. To assess the HIV-1 enhancing effect of the different amyloid fibrils, 104 TZM-bl cells in 180 μL of cell culture medium were seeded in 96-well flat-bottom plates 1 day before infection. Concentration series of the different fibrils (0–200 μg/mL) were prepared and then mixed with CCR5-tropic HIV-1 NL4-3 92TH014 (1 ng/mL p24 antigen). After 10 min at room temperature, 20 μL of these mixtures were added to the TZM-bl cells and infection rates were determined 3 days post-infection by detecting β-galactosidase activity in cellular lysates using the Tropix Gal- Screen kit (Applied Biosystems, Life Technologies, Frederick, MD) and an Orion microplate luminometer (Berthold, Bad Wildbad, Germany). All values represent reporter gene activities (relative light units per second; RLU/s) derived from triplicate infections minus background activities derived from uninfected cells.

4.8. Confocal Microscopy. Fibrils (0.5 mg/mL) were stained with Proteostat dye (Enzo Life Sciences, Farmingdale, NY), according to the manufacturer’s instructions (Shen D. et al.). Then, MLV-Gag-YFP particles were added in a ratio of 1:2, resulting in a final fibril concentration of 0.17 mg/mL. After incubating the mixture for 5 min, confocal microscopy was carried out on a Zeiss LSM-710 (Oberkochen, Germany). For analysis of fibril–cell interactions, 20 000 TZM-bl cells were seeded the day before into 1 micrometer eight-well IBIDI slides. They were exposed to 5 μg/mL Proteostat-labeled fibrils of the different species (AL1 PNF, SEVI, or EF-C PNF) and Hoechst. After incubation for 1 h at 37 °C, the samples were analyzed before removing the supernatants. Then, the supernatants were discarded, the cells were washed three times with 200 μL of PBS before 200 μL of DMEM without phenol red were added, and confocal microscopy was carried out again.

4.9. Flow Cytometry. Binding of fluorescent virions to different fibrils was quantified by incubating 250 μL of fibrils (final concentration, 0.1 mg/mL) with 250 μL of an MLV-Gag-YFP particle suspension with increasing virus titer for 10 min before flow cytometry (BD Canto II, Heidelberg, Germany) was carried out. To exclude that the increase in fluorescence is due to unspecified effects, different concentrations of MLV-Gag virions lacking the YFP expression cassette were tested for fibril binding in parallel, using a constant AL fibril concentration of 0.1 mg/mL (Figure S1A). Furthermore, flow cytometry analysis of virions only did not result in virus-specific fluorescence signals excluding that free virions interfered with analysis or cause false-positive results (Figure S1B). For determining cell binding of the fibril species over time, Proteostat-stained SEVI, EF-C PNF, and AL1 PNF fibrils were added to a final concentration of 5 μg/mL to 200 000 TZM-bl cells seeded the day before into 12-well plates. After certain incubation times, supernatants of the cells were discarded and the cells were washed three times with PBS, trypsinated, transferred to FACS tubes, centrifuged, and washed with PBS again. Then, the cells were fixed with 500 μL of 2% PFA and analyzed for bound fibrils via the red fluorescence channel using flow cytometry. For normalization, the complete membrane fluorescence intensity of fibrils was divided by complete fluorescence of 4′,6-diamidino-2-phenylindole (DAPI) to get fibrils/cell.

4.10. p24 ELISA. To quantify the interaction of virions with target cells, 5000 TZM-bl cells were seeded 1 day before into 96-well plates in a volume of 50 μL. The next day, 40 μL of different fibril concentrations was incubated with 40 μL of HIV-1 dilution (40 ng/mL p24 antigen) for 10 min and 20 μL of these mixtures was added to the cells in triplicate. After 3 h incubation at 37 °C, unbound virus was removed and the cells were washed three times with Dulbecco’s modified Eagle’s medium (DMEM) before they were lysed with 1% Triton X-100 for 1 h at 37 °C. Cell-associated HIV-1 capsid antigen was detected using an in-house p24 ELISA.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00068.

Flow cytometry analysis of virion binding to fibrils (Figure S1) and structural model of the main fibril morphology formed by AL1 peptide (Figure S2) (PDF)

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**Author Contributions**

D.S. and A.R. performed all viral experiments and ThT measurements. C.R. did SEM and cryo-EM analyses. R.G. measured $\zeta$-potentials. S.R. supported D.S. in performing experiments. K.A. and M.F. performed TEM, helped C.R. in cryo-EM, and generated the AL1 PNF model. J.M. conceptualized and supervised the work and wrote the manuscript with D.S. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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