Anionic poly(amino acid)s dissolve F-actin and DNA bundles, enhance DNase activity, and reduce the viscosity of cystic fibrosis sputum

Jay X. Tang,1,2 Qi Wen,1 Andrew Bennett,2 Brian Kim,2 Catherine A. Sheils,2,3 Robert Bucki,4 and Paul A. Janmey2,4

1Department of Physics, Brown University, Providence, Rhode Island; 2Hematology Division, Brigham and Women’s Hospital, Boston; 3Pulmonary Division, Children’s Hospital, Boston, Massachusetts; and 4Department of Physiology, Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, Pennsylvania

Submitted 3 February 2005; accepted in final form 11 June 2005

Tang, Jay X., Qi Wen, Andrew Bennett, Brian Kim, Catherine A. Sheils, Robert Bucki, and Paul A. Janmey. Anionic poly(amino acid)s dissolve F-actin and DNA bundles, enhance DNase activity, and reduce the viscosity of cystic fibrosis sputum. Am J Physiol Lung Cell Mol Physiol 289: L599–L605, 2005. First published June 17, 2005; doi:10.1152/ajplung.00061.2005.—Bundles of F-actin and DNA present in the sputum of cystic fibrosis (CF) patients but absent from normal airway fluid contribute to the altered viscoelastic properties of sputum that inhibit clearance of infected airway fluid and exacerbate the pathology of CF. Previous strategies to remove these filamentous aggregates have focused on DNase to enzymatically depolymerize DNA to constituent monomers and gelsolin to sever F-actin to small fragments. The high densities of negative surface charge on DNA and F-actin suggest that the bundles of these filaments, which alone exhibit a strong electrostatic repulsion, may be stabilized by multivalent cations such as histones, antimicrobial peptides, and others positively charged molecules prevalent in airway fluid. This study reports that bundles of DNA or F-actin formed after addition of histone H1 or lysozyme are efficiently dissolved by soluble multivalent anions such as polyaspartic acid or polyglutamic acid. Addition of poly-aspartate or poly-glutamate also disperses DNA and actin-containing bundles in CF sputum and lowers the elastic moduli of these samples to levels comparable to those obtained after treatment with DNase I or gelsolin. Addition of poly-aspartic acid also increased DNase activity when added to samples containing DNA bundles formed with histone H1. When added to CF sputum, poly-aspartic acid significantly reduced the growth of bacteria, suggesting activation of endogenous antibacterial factors. These findings suggest that soluble multivalent anions have potential alone or in combination with other mucolytic agents to selectively dissociate the large bundles of charged biopolymers that form in CF sputum.

multivalent anions; polyelectrolyte; antimicrobial; LL37; cathelicidin

Address for reprint requests and other correspondence: R. Bucki, Dept. of Physiology, Inst. for Medicine and Engineering, Univ. of Pennsylvania, 1010 Vagelos Labs., 3340 Smith Walk, Philadelphia, PA 19104 (e-mail: buckirob@mail.med.upenn.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
electrolyte effects is tested by measurements of the effects of soluble anions such as poly-aspartate (poly-ASP) and poly-glutamate (poly-GLU) on the viscoelastic and morphological features of both purified systems and sputum obtained from CF patients.

MATERIALS AND METHODS

Proteins, DNA, and other reagents. G-actin was prepared from rabbit skeletal muscle as previously described (25) and stored in G-actin buffer composed of 2 mM Tris, 0.2 mM CaCl$_2$, 0.2 mM dithiothreitol, 0.5 mM ATP, pH 8.0, and polymerized to F-actin by addition of 2 mM Mg$^{2+}$, 10 mM Tris, pH 7.4; and either 50 or 150 mM NaCl as specified. LL37 was synthesized with an automatic peptide synthesizer monitored by mass spectrometry and capillary electrophoresis at the Louisiana State University Medical Center Core Laboratories. Calf thymus DNA (Sigma, St. Louis, MO) was dissolved in solution containing 100 mM NaCl and 2 mM HEPES, pH 7.5. Bovine mucin (Sigma) was dissolved at a concentration of 3% for rheological measurements. All chemicals were supplied at analytical grade. Histone H1, turkey egg white lysozyme (3× crystalized), poly-aspartic acid, and poly-glutamic acids of various molecular weights were purchased from Sigma and underwent no further purification.

Preparation of sputum samples. Sputum samples were collected by spontaneous expectoration as previously reported (20). Undiluted samples were used for rheological measurements and each sample was diluted 1:10 in PBS (138 mM NaCl, 27 mM KCl, 10 mM Na$_2$HPO$_4$, pH 7.4), vortexed, split into equal aliquots, and then used for microscopy or the bacterial assay. Frozen 1-ml aliquots were thawed at room temperature under conditions where previous studies determined that the rheological properties were similar to those of the fresh sample before freezing (20, 26). They were used for rheological measurements and microscopic imaging.

Light scattering measurements. Formation of lateral aggregates of F-actin or DNA was detected by measuring the light scattering intensity at a fixed angle of 90° with a Perkin-Elmer LS-5B luminescence spectrometer (25). For the light-scattering data shown in Figs. 1 and 2, rectangular cuvettes of 10 mm path length and 5 mm width were used, containing initially 600 μl of either 0.1 mg/ml F-actin or 0.3 mg/ml DNA solution. Scattering intensity was measured when the solution reached steady state following addition of a stock solution of concentrated histone H1 or lysozyme. Light-scattering data were plotted in arbitrary units, with the scattering intensities consistent within the individual graphs only.

Optical microscopy. Aggregates were observed under a Nikon TE-300 microscope using both fluorescence and phase-contrast modes. The images were taken with a Sanyo charge-coupled device TE-300 microscope using both fluorescence and phase-contrast within the individual graphs only.

Light-scattering intensities were plotted in arbitrary units, with the scattering intensities consistent within the individual graphs only.

Rheological measurements. In most studies a torsion pendulum was used to measure the elastic modulus of sputum samples. The pendulum response to a sudden deflection to an initial strain <10%, resulting in a resonance frequency of ~1 Hz, was analyzed by fitting to a sinusoidal function with exponential decay (8). A commercial RFS II rheometer (Rheometric Scientific, Piscataway, NJ) was also used to determine the frequency-dependent G' and G" values for selected sputum samples (e.g., sample 5) and provided an internal control for the accuracy of G" values derived from the torsion pendulum. In these measurements, the strain was 5%, and the frequency varied from 0.6 to 100 rad/s. The magnitude of the G' and G" moduli and their frequency dependence (data not shown) were very similar to those of sputum samples previously studied by this method (17). Each value of elastic modulus shown in Fig. 6 is the average of three to six measurements taken on separate 500-mg sputum aliquots, and the error bars show the calculated SD. Three methods of preparing the samples were examined: 1) addition of additives, such as buffer and polyelectrolyte solutions, followed by 1 h of slow rotation of the mixture sealed in Eppendorf tubes; 2) addition of additives, 10 s of vortexing, 1/2 h of incubation at rest in Eppendorf tubes; and 3) addition of additives, placement of samples in a humid container, 10 cycles of gentle swirling with a pipette tip, followed by 2 h of incubation at rest. Frozen samples were thawed at room temperature for 30–45 min before the treatment described. Once the sample was applied between the rheometer plates, the rheological measurements took <30 min to complete.

DNase assay. The enzymatic activity of DNase I from bovine pancreas (Sigma) was evaluated by adding 60 U/ml into solutions of 0.01 mg/ml DNA in 100 mM NaCl, 5 mM HEPES, pH 7, and 3 μM poly-Asp-244. The activity is obtained from kinetic measurements by monitoring optical density of the samples with light-scattering effect subtraction [optical density at 260 nm (OD$_{260}$)–OD$_{320}$]. Because the OD$_{260}$ increases as DNA is digested by DNase, the initial rate of change of OD$_{260}$ is proportional to the DNase activity.

Bacterial assay. A single colony of Pseudomonas aeruginosa (PAO1) was selected from a plate and grown to mid-log phase (OD$_{600}$ ~0.3) in 2 ml of tryptic soy broth medium (Becton-Dickinson, Cockeysville, MD). One milliliter of the bacterial suspension was centrifuged at 4,000 rpm for 5 min at room temperature, and the bacterial pellet was resuspended in PBS. Serial dilutions of the antibacterial peptide LL37 were mixed with the diluted bacterial suspension, with or without poly-aspartic acid, in 0.3-ml aliquots. Similarly, tubes containing 90 μl of CF sputum samples (1:10 dilution) were treated with LL37 peptide or poly-aspartic acid. All tubes were incubated at 37°C for 1 h and transferred to ice. Duplicate 10-μl aliquots of 10-fold dilutions (undiluted, 1:10, 1:100, 1:1,000) of these mixtures were plated on sectors of agar plates, and plates were incubated overnight at 37°C. The number of colonies in the duplicate samples at each dilution was counted the following morning, and the colony forming units of the individual mixture were determined from the dilution factor.

RESULTS

Formation and dissolution of bundles containing F-actin and DNA. As predicted from consideration of polyelectrolyte electrospectrostatics, independent of specific chemical structure, two structurally distinct cationic proteins expected to be present in CF airway fluid have potent ability to bundle both DNA and F-actin in solutions of physiologically relevant salt concentrations. Figure 1A shows the effect of histone H1 on bundling of DNA and F-actin. Because DNA found in CF sputum results from rupture of neutrophils and exposure of heterochromatin to the extracellular fluid, histones are presumably also released where they could interact with both F-actin and DNA. Lysozyme, a cationic protein normally secreted into the airway...
fluid as a bactericidal agent, is also capable of bundling F-actin and DNA (Fig. 1B). To test that the bonds stabilizing the histone-containing bundles are primarily electrostatic, Fig. 2 shows that the bundles of DNA or F-actin formed by histones are dissociated when the polyvalent anions poly-ASP or poly-GLU are added. Both species of anionic polypeptides (coions for DNA) can dissolve DNA bundles, but less poly-ASP is required for the same outcome.

Microscopic visualization of filament bundles. Previous studies have shown separately that polyvalent cationic agents such as polylysine form bundles of DNA or F-actin alone (24). We also find that when these polymers are mixed, histone H1 forms bundles containing both types of filaments. Figure 3 shows by optical microscopy the formation of heterologous bundles of DNA and F-actin induced by histone, followed by disappearance of large aggregates upon addition of poly-ASP. Similar results were also obtained using poly-GLU of higher concentrations (not shown), consistent with the light-scattering results. These in vitro results demonstrate the concept that polyvalent cations can bundle F-actin, DNA, and mixtures of the two and that the bundles so formed are dissolved when polyvalent anions (either poly-ASP or poly-GLU) are added.

Extension of these results to the more complex environment of whole CF sputum is shown in Fig. 4. Here the large aggregates of both actin and DNA common to CF sputum are dissociated by poly-ASP, as we predict from the results of Fig. 3, assuming that what holds the bundles together in sputum are polyvalent cations. The effect of poly-ASP under these conditions is limited to F-actin and DNA, as the endogenous mucin
network, as visualized by fluorescent lectin retains much of its structure.

The possibility that the actin and DNA bundles in CF sputum may be stabilized by histones is confirmed by the immunoblotting results shown in Fig. 5. Analysis of four different sputum samples showed a prominent band at \( \sim 31 \) kDa that reacted specifically with an antibody to histone H1.

**Fig. 4.** Effects of poly-ASP (right) on the sputum of a cystic fibrosis (CF) patient. A: dissolution of DNA and F-actin bundles was visualized by rhodamine phalloidin labeling for F-actin (top row) and YOYO-1 labeling for DNA (middle row), respectively. The change in overall morphology is shown by phase-contrast microscopy (bottom row). B: presence of mucin in CF sputum visualized using Texas red-conjugated lectin (top row). The morphology of corresponding samples is shown by phase-contrast microscopy (bottom row). Data shown are representative of 3 experiments.

**Fig. 5.** A: protein separation of CF sputum (4 different samples) by SDS-PAGE electrophoresis on 12% polyacrylamide gel. B: presence of histone H-1 (arrow) in CF sputum detected by immunoblotting analysis with a monoclonal anti-histone H-1 antibody.

**Fig. 6.** Changes in the shear elastic modulus in 8 CF sputum samples after addition of buffer alone (solid bars) 25 \( \mu \)M poly-ASP (striped bars) or 250 \( \mu \)M poly-ASP (open bars). Data were normalized to shear storage \( (G') \) for untreated sputum = 1. Samples 4, 5, and 8 came from the same patient on different days, whereas each of the others came from different patients. The samples were slowly rotated for 1 h after addition of buffer and peptide solutions, which contributed 5% by weight.

**Ability of polyvalent anions to relieve the abnormal viscoelasticity of CF sputum.** If polycation-mediated binding between F-actin and DNA polymers in CF sputum contributes to the viscoelasticity of the sputum, then dissolution of these bundles by soluble polyvalent anions would be expected to lower the elastic moduli of sputum in concert with the disappearance of bundles visible by microscopy. Figure 6 shows that the poly-ASP treatment that dissociates the observable bundles in CF sputum also reduces the elastic modulus of CF sputum, as measured by a low strain rheometer used in previous studies of the effects of gelsolin on sputum rheology (20, 26). The extent of decrease in \( G' \) is similar to that observed after treatment with gelsolin or DNase (26). Figure 6 compares the effects of two different concentrations of poly-ASP on eight sputum samples obtained from six different patients. Six of the eight samples showed a significant, concentration-dependent lowering of the shear modulus to an extent that is in the same range as previously reported for effects of DNase or gelsolin.
Two samples showed no significant response, and this finding is also consistent with previous reports that not all sputum samples contain F-actin and that samples with low elastic moduli are unlikely to be altered by actin-depolymerizing agents (20). When the elastic moduli of each sample are normalized to the value of the untreated sputum, 25 μM poly-ASP decreased G’ by 26% (P < 0.001 vs. control value by the Student’s t-test) and 250 μM poly-ASP reduced G’ by 39% (P < 0.001 vs. control value).

The decrease in elasticity of CF sputum was not due to an effect of poly-ASP on the mucin glycoproteins directly. Figure 7A shows the complex shear modulus (G* = G’ + G”) of bovine submaxillary gland mucin measured over a range of frequencies. Triplicate measurements of mucin with or without addition of 250 μM poly-ASP show little variability within each sample and no significant effect of poly-ASP. In contrast, Fig. 7B shows a significant effect of poly-ASP on a representative CF sputum sample measured at the same strain (5%) over the same range of frequencies.

DNase I activity. To test whether poly-ASP-mediated dissolution of bundled DNA affects its degradation by DNase, we evaluated DNase enzymatic activity after adding pancreatic DNase to the samples containing DNA bundles formed with histone H1 and different concentration of poly-aspartic acid.

DNase activity was very low in samples that contained DNA bundled with histone H1 but increased by over an order of magnitude after addition of poly-ASP (Fig. 8). The increase in DNase activity reached a maximum with 3 μM poly-ASP under these conditions and decreased slightly at higher poly-ASP concentrations. When added to DNA without histone H1, poly-ASP at concentrations up to 25 μM had no significant effect on DNase activity. The concentration range over which poly-ASP increased DNase activity is very similar to the range where the same molecular weight of poly-ASP dissolved DNA/histone H1 bundles as measured by light-scattering changes shown in Fig. 2. These data suggest that polyvalent anions might have a positive impact on currently used CF treatments using recombinant human DNase when DNA is assembled in bundles.

Bacterial load of CF sputa. Poly-ASP decreases bacterial outgrowth from CF sputum but has no effect on growth of
isolated P. aeruginosa cultures. Figure 9 shows that addition of 10 and 50 μM poly-ASP decreased the number of bacterial colonies grown from samples of purulent CF sputum but had no effect on growth of isolated P. aeruginosa. The extent of bacterial growth inhibition by 50 μM poly-ASP was as great as that achieved by 10 μM of the antimicrobial peptide LL37, a concentration that completely blocked the growth of isolated P. aeruginosa. These results show that addition of poly-ASP to CF sputum can lessen bacterial growth by a mechanism other than direct bacterial killing by poly-ASP and consistent with its ability to liberate endogenous antibacterial activities within CF sputum. Higher concentrations of poly-ASP could not be used in the bacterial outgrowth assays because control experiments (not shown) verified that high concentrations (100–500 μM) directly inactivated purified antimicrobial peptides like LL37, presumably by the same electrostatic mechanism by which the more highly charged polymers like F-actin or DNA do so at lower concentrations (27).

**DISCUSSION**

Accumulation of long filamentous polymers into bundles is associated with a number of pathological states, and in CF, bundles of DNA and actin have been implicated in the altered sputum viscoelasticity that may contribute to the disease (19, 20, 26). Even apart from the effects of F-actin and DNA on sputum viscosity, these highly charged polymers may have other deleterious consequences in CF. Some therapeutic treatments have been based on identifying agents that can fluidize the sputum, and a recent focus has been on proteins that selectively depolymerize F-actin or DNA, such as DNase (1, 5, 19). In addition to the covalent bonds or specific protein-protein contact sites targeted by these depolymerizing agents, there are also more general electrostatic forces that stabilize anionic polymers and that can inhibit the agents meant to depolymerize them. It is increasingly recognized that the electrostatic features of polyelectrolytes with high surface charge can lead to strong attractive forces that are significant even in solutions of high monovalent salt concentrations.

The importance of polyelectrolyte effects governing the sequestration of mobile ions around DNA has long been a subject of theoretical and experimental work and is an essential aspect of the forces regulating the dense packing of long DNA polymers in the viral head or chromatin (3, 28). Polyelectrolyte effects are also central to the design of strategies using cationic lipids and polymers to improve transfection efficiency of specific DNA in therapeutic applications (4). More recently, these same concepts have been shown to govern the bundling of other anionic biopolymers with high surface charge, such as F-actin, into the bundled arrays characteristic of some intracellular structures (25) and into stable complexes of F-actin and cationic lipid bilayers (30).

The results reported here suggest that polyelectrolyte effects also govern the formation of bundles containing actin and DNA in the complex matrix of the airway fluid and that soluble polyanions have a potential to fluidize these bundles, thereby lowering sputum viscosity and enhancing access of depolymerizing agents to their filamentous targets. The efficacy of poly-ASP and poly-GLU strongly suggests that polycationic ligands are responsible for the stabilization of the bundles. The list of these polycations in CF sputum is not fully known, and several such molecules may act in concert to facilitate bundling. A likely component is histone H1 that is released with DNA from the nuclei of ruptured cells and which potently bundles actin and DNA in vitro, as shown in Fig. 1. Numerous other polycations may also contribute to bundle stabilization. These include interleukin 8, recently shown to be sequestered by F-actin and DNA in CF (18), and a number of antimicrobial proteins and peptides including lysozyme, as shown in Fig. 1B, defensins, and cathelicidins. The loss of antimicrobial activity in CF, despite the secretion of antimicrobial peptides into the airway fluid (21), may therefore be due to the sequestration of cationic antimicrobial peptides in polyanionic filament bundles. Therefore, fluidization of these bundles may lead to liberation of some antibiotic activity, and this effect along with lowered viscosity may have beneficial effects in vivo (27). The prevalence of anionic polyelectrolytes in many insoluble pathological deposits including amyloid and biofilms, suggests that treating these materials with appropriate soluble multivalent anions may also aid in their fluidization.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the help of David Lipson and Marianne Ferrin in facilitating this study and patients of the Adult Cystic Fibrosis Center of the University of Pennsylvania for providing sputum samples.

**GRANTS**

This work has been supported by National Heart, Lung, and Blood Institute Grant HL-67286 and by the Cystic Fibrosis Foundation.

**REFERENCES**


