Engineering actin-resistant human DNase I for treatment of cystic fibrosis

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Communicated by James A. Spudich, Stanford University School of Medicine, Stanford, CA, April 10, 1996 (received for review February 7, 1996)

ABSTRACT Human deoxyribonuclease I (DNase I), an enzyme recently approved for treatment of cystic fibrosis (CF), has been engineered to create two classes of mutants: actin-resistant variants, which still catalyze DNA hydrolysis but are no longer inhibited by globular actin (G-actin) and active site variants, which no longer catalyze DNA hydrolysis but still bind G-actin. Actin-resistant variants with the least affinity for actin, as measured by an actin binding ELISA and actin inhibition of [32P]DNA hydrolysis, resulted from the introduction of charged, aliphatic, or aromatic residues at Ala-114 or charged residues on the central hydrophobic actin binding interface at Tyr-65 or Val-67. In CF sputum, the actin-resistant variants D53R, Y65A, Y65R, or V67K were 10- to 50-fold more potent than wild type in reducing viscoelasticity as determined in sputum compaction assays. The reduced viscoelasticity correlated with reduced DNA length as measured by pulsed-field gel electrophoresis. In contrast, the active site variants H252A or H343A had no effect on altering either viscoelasticity or DNA length in CF sputum. The data from both the active site and actin-resistant variants demonstrate that the reduction of viscoelasticity by DNase I results from DNA hydrolysis and not from depolymerization of filamentous actin (F-actin). The increased potency of the actin-resistant variants indicates that G-actin is a significant inhibitor of DNase I in CF sputum. These results further suggest that actin-resistant DNase I variants may have improved efficacy in CF patients.

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MATERIALS AND METHODS

Mutagenesis and Expression. Site-directed mutagenesis was carried out by the method of Kunkel et al. (21); mutations were verified by dyeode sequencing (22). Variant DNA was purified from 500 ml cultures of transformed Escherichia coli XL1 Blue MRF' (Stratagene) using Qiagen-tip-500 columns (Qiagen, Chatsworth, CA). Human embryonic kidney 293 cells (ATCC CRL 1573) were grown in serum containing media in 150 mm plastic Petri dishes and log phase cells were transiently cotransfected with 22.5 μg DNase variant DNA and 17 μg adenovirus DNA (23). About 16 h after transfection, cells were washed with phosphate-buffered saline (PBS) and the medium was changed to serum free media; cell culture supernatant was harvested after 96 h. Harvests were concentrated ~10-fold using Centriprep 10 concentrators and generally contained from 10 to 100 μg of DNase I variant.

DNase Activity Assays. The methyl green assay was used to measure DNA hydrolytic activity of DNase I (24). DNase I concentrations were determined by ELISA by using a goat anti-DNase I polyclonal antibody coated and detecting with a rabbit anti-DNase I polyclonal antibody conjugated to horseradish peroxidase. In both assays, multiple sample dilutions

Abbreviations: CF, cystic fibrosis; F-actin, filamentous actin; G-actin, globular actin.

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were compared with standard curves of purified recombinant human DNase I (Pulmozyme; Genentech) to determine concentrations. The relative specific activity is defined as the DNase I concentration determined in the methyl green assay divided by the DNase I concentration determined in the DNase I ELISA; the data are normalized to wild-type DNase I.

Actin Binding ELISA. MaxiSorp plates (Nunc) were coated with 100 μl human Gc globulin (Calbiochem) at 10 μg/ml in 25 mM Hepes (pH 7.2), 4 mM MgCl₂, and 4 mM CaCl₂ at 4°C for 16–24 h. After discarding the Gc globulin, excess reactive sites were blocked with 200 μl buffer A (25 mM Hepes, pH 7.5/4 mM CaCl₂/4 mM MgCl₂/0.1% BSA/0.5 mM ATP/0.01% thimerosal/0.05% Tween 20). Buffer A was used in all dilution steps unless otherwise noted; incubations were at room temperature for 1 h. The wash buffer was PBS containing 0.05% Tween 20. Rabbit skeletal muscle G-actin [1 mg/ml; prepared by the method of Pardee and Spudich (25) or obtained from Sigma] was dialyzed overnight at 4°C against 5 mM Hepes (pH 7.2), 0.2 mM CaCl₂, 0.5 mM ATP, and 0.5 mM 2-mercaptoethanol. After centrifugation at 13,000 × g for 5 min, the amount of G-actin-ATP was quantitated by measuring the absorbance at 290 nm, using ε₂₉₀ = 28.3 mM⁻¹ cm⁻¹ (19). Following the addition of 100 μl of 50 μg of G-actin per ml in buffer A, the plates were incubated and washed, and 100 μl of cell culture harvest at various dilutions was added. After incubation and washing, 100 μl of anti-human DNase I rabbit polyclonal antibody–horseradish peroxidase conjugate (19 ng/ml) was added. Following incubation and washing, color development was initiated by adding 100 μl per well of Sigma Fast o-phenylenediamine and urea/H₂O₂ reagent (prepared according to Sigma) and stopped by adding 100 μl per well 4.5 M H₂SO₄. The A₄₉₉ was measured and plotted versus the DNase I concentration. The resultant sigmoidal curves were fit to a four parameter equation by nonlinear regression analysis (26); the EC₅₀ value is the DNase I concentration that produced a half-maximal signal.

Actin Inhibition Assays. Varying concentrations of G-actin were preincubated in duplicate for 15 min at room temperature with 0.54 mM DNase I variant in buffer A containing 0.5 mM 2-mercaptoethanol and 150 mM NaCl. Reactions were initiated by the addition of 33P-labeled M13 DNA and salmon testes DNA (Sigma) to a final concentration of 4.1 μg/ml, incubated at room temperature for 2 h, and quenched with 25 mM EDTA and cold TCA (6.7% final concentration). After 10 min on ice samples were centrifuged at 9300 × g for 5 min at 4°C and the acid-soluble counts determined. The plots of the fractional activity (cpm inhibited/cpm uninhibited) versus actin concentration were fit by nonlinear regression analysis using KALEIDOGRAPH version 3.0.1 (Synergy Software, Reading, PA) to the following equation to determine the Kᵢ value.

Fractional activity =

\[1 - \frac{[E_0] + [I_0] + K_i - \sqrt{[E_0] + [I_0] + K_i} - (4 \cdot [E_0] \cdot [I_0])}{2 \cdot [E_0]},\]

where [E₀] is the DNase I concentration and [I₀] is the total G-actin concentration.

CF Sputum Compaction Assays. Assays using CF sputum collected from four patients and stored at −80°C prior to use were carried out as described (27); sputa were incubated in triplicate with DNase I samples at various concentrations for 20 min at room temperature.

Pulsed-Field Gel Electrophoresis. Pulsed-field gel electrophoresis was carried out in 1% agarose gels; after electrophoresis, gels were stained with ethidium bromide and imaged in a Molecular Dynamics Fluorimager.

RESULTS AND DISCUSSION

**Protein Engineering.** Selected mutations were made based on the x-ray crystal structures of bovine DNase I complexed with either G-actin or DNA (28, 29). A model of a ternary complex of DNase I, actin, and DNA, constructed by superimposing the DNase I structures (28–30), shows that the DNA and actin binding sites are adjacent yet distinct; the inhibition of DNase I by actin due to steric hindrance is apparent (Fig. 1). Bovine pancreatic DNase I shares 78% identity and superimposes with an overall rms deviation for main chain atoms of 0.56 Å with the human enzyme, recently solved at 2.2-Å resolution (30). The contact residues at the actin binding interface as well as those at active site are conserved. Both human and bovine DNase I have been found to reduce the viscosity of purulent sputum (8, 13).

The binding of DNase I to actin involves hydrophobic, electrostatic, and hydrogen bonding interactions over a buried surface area of 1849 Å² (17, 28); the actin binding interface is shown in Fig. 2. Main-chain interactions result from the parallel β-strands formed by Tyr-65, Val-66, and Val-67 of DNase I and Gly-42, Val-43, and Met-44 of actin. The DNase I side-chains forming the core of the interface are hydrophobic and include Tyr-65, Val-67, and Ala-114; interactions peripheral to this central hydrophobic region are polar in nature and involve His-44, Asp-53, and Glu-69. Initially, alanine substitutions for these residues were made to assess their relative contribution to actin binding. Further mutagenesis lead to actin-resistant variants that were created by introducing charge, charge reversals, or steric bulk. Active site variants were created by alanine substitution at residues His-134, Ala-114, Thr-67, and Gly-42.

**FIG. 1.** Model of a ternary complex of human DNase I with G-actin and a DNA oligonucleotide. Ribbon diagram of the DNase I–actin complex superimposed with the DNase I–d(GGATACCC)₂ complex [Brookhaven Protein Data Bank 1ATN (28) and 1DNK (29), respectively] and human DNase I (30). Residues at the active site (His-134, Gla-78, His-252, and Asp-212) are in yellow and those at the actin binding interface (His-44, Asp-53, Tyr-65, Val-67, Glu-69, and Ala-114) are in pink. The figure was made by using MIDASPLUS (31).
His-252, Asp-212, and Glu-78; these residues have been implicated in the general acid-base catalysis mechanism that occurs at the scissile phosphate bond (29, 32). In addition, the more conservative substitution of Gln for His at position 134 or 252 was also tested.

**DNA Hydrolysis and Actin Binding Assays.** The relative specific activity of DNase I variants was assessed by comparing the specific DNA catalytic activity of the variant to that of wild type. DNase I concentration was determined by ELISA; reaction with the anti-DNase I antibodies also implied that the mutants were correctly folded. None of the active site variants catalyzed DNA hydrolysis, confirming the critical role of these residues in the enzyme mechanism. In contrast, the actin binding site variants all had similar specific activity compared with wild type, demonstrating that changes at the actin binding interface do not affect the active site (Fig. 3 A and C).

The binding affinity of DNase I variants for G-actin was assessed by an ELISA where actin was bound to immobilized Gc globulin, a protein whose affinity for actin is unaffected by DNase I (33–35); the EC\textsubscript{50} values are plotted in Fig. 3 B and D. The active site variants bound with the same high affinity to G-actin as wild-type DNase I, again confirming the independent nature of these two sites. However, a wide range of reduced affinities for actin was found for the actin binding site variants (Fig. 3D).

Selected variants were also assayed for actin inhibition of DNase I catalyzed hydrolysis of \textsuperscript{32}P-labeled DNA in solution. The DNase I variants and their respective \( K_i \) values are reported in Table 1. The inhibition of DNase enzymatic activity by actin in solution correlated well with the relative actin binding affinity determined in the solid-phase actin binding assay (Fig. 3D and Table 1).

**DNase I Variants at the Actin Binding Interface.** Single point mutations introducing charged, aliphatic, or aromatic residues at Ala-114 resulted in actin-resistant variants that were reduced in actin binding by over 10,000-fold relative to wild-type DNase I (Fig. 3D). This large effect is likely due to steric hindrance, since Ala-114 resides on an internal \( \beta \)-strand and lies at the bottom of a pocket with its side chain in van der Waals contact with that of Val-45 of actin (Fig. 2). The introduction of charge on the central hydrophobic interface at Tyr-65 and Val-67 (Y65R, V67K, V67D) also greatly impaired actin binding (Figs. 2 and 3D). The fact that Y65R and V67K are much more actin resistant than Y65W or V67M implicates charge rather than steric hindrance as the critical factor. Moderate reductions in actin binding were noted upon removal of the aromatic side-chain at Tyr-65 (Y65A) and introduction of a charge reversal at Asp-53 (D53R), which should disrupt the salt bridge formed with Arg-39 and the hydrogen bonds to His-40 of actin (Fig. 3D and Table 1). Mutations at His-44, which interacts with Thr-203 and Glu-207 in actin, and Glu-69, which forms a salt bridge with Lys-61 of

![Figure 2](image_url)  
**FIG. 2.** Structure of DNase I complexed with G-actin. The structure (1ATN) was taken from the Brookhaven Protein Data Bank (28); DNase I is represented in grey and actin is depicted in blue. Residues at the actin binding interface that were altered are highlighted in pink. The figure was made by using MIDASPLUS (31).

![Figure 3](image_url)  
**FIG. 3.** Relative specific activity and actin binding affinity of active site (A and B) and actin binding site (C and D) DNase I variants. The relative specific activity for DNA hydrolysis, as defined in Materials and Methods, is normalized to wild type. The fold reduction in actin binding affinity \([EC_{50} \) (variant)/\( EC_{50} \) (wild type); mean ± SD, \( n = 2 \)] was determined by the actin binding ELISA and is normalized to wild type. The relative actin binding affinity is plotted on a logarithmic scale for the actin binding site variants (D); bars labeled with an asterisk (*) represent a lower limit due to protein expression.
Table 1. Inhibition constants ($K_i$) for G-actin inhibition of 33P-labeled DNA-catalyzed hydrolysis by DNase I variants

<table>
<thead>
<tr>
<th>DNase I variant</th>
<th>$K_i$, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type DNase I</td>
<td>1.3</td>
</tr>
<tr>
<td>H44A</td>
<td>3.9</td>
</tr>
<tr>
<td>D53A</td>
<td>24</td>
</tr>
<tr>
<td>D53R</td>
<td>47</td>
</tr>
<tr>
<td>Y65A</td>
<td>36</td>
</tr>
<tr>
<td>Y65R $&gt;$1000</td>
<td></td>
</tr>
<tr>
<td>V67K</td>
<td>161</td>
</tr>
<tr>
<td>E69R</td>
<td>3.5</td>
</tr>
<tr>
<td>A114R $&gt;$10,000</td>
<td></td>
</tr>
</tbody>
</table>

The inhibition constants ($K_i$) were calculated using the known concentrations of DNase I variant and G-actin, the fractional activity, and fitting the data by nonlinear regression analysis to the equation for tight binding reversible inhibition as described in Materials and Methods.

actin, had minimal effects (Fig. 3D and Table 1), suggesting that their contributions to the binding energy of the complex are negligible.

Activity of DNase I Variants in CF Sputum. Sputum compaction assays were used to measure the relative viscoelasticity of CF sputum after incubation with wild-type and selected DNase I variants (27); the percent change in compaction is shown in Fig. 4. The active site variants (H134A, H252A), which no longer catalyze DNA hydrolysis but still bind G-actin and could therefore depolymerize F-actin, did not reduce sputum viscoelasticity. However, both wild type and the actin-resistant variants tested (D53R, Y65A, Y65R, V67K) decreased viscoelasticity in a dose-dependent manner. The data from both sets of mutants demonstrate that the reduction in sputum viscoelasticity by DNase I results from DNA hydrolysis and not actin depolymerization. The hydrolysis of DNA in CF sputum and subsequent reduction in DNA length by wild type and the actin-resistant variant V67K was confirmed using pulsed-field gel electrophoresis (Fig. 5); no reduction in DNA length was seen with the active site variant H134A (D.S., unpublished results).

Most importantly, the actin-resistant variants were about 10 to 50-fold more potent in both reducing sputum viscoelasticity and in reducing DNA length compared with wild-type DNase I (Fig. 4 and 5). Based on these results, we conclude that G-actin is a significant inhibitor of DNase I in CF sputum, and that the actin-resistant variants are not subject to this inhibition.

Fig. 4. Effect of DNase I variants on CF sputum compaction. The percent compaction of the sputum (mean ± SEM), which correlates with sputum viscoelasticity, is plotted versus the variant concentration: wild type (●); active site variants: H134A (○) and H252A (◇); and actin-resistant variants: D53R (■), Y65A (●), Y65R (▲), and V67K (▼).

FIG. 5. DNA length in CF sputum treated with wild-type DNase and the actin-resistant variant V67K. DNase concentrations and DNA molecular mass markers are indicated. C refers to a control sputum in the absence of DNase. Pulsed-field gel electrophoresis was carried out as described.

The actin-resistant variants tested, which had from about 30 to >1000-fold reduced affinity for actin, were equivalent in the compaction assay, suggesting that they were all uninhibited by actin. Using the equation for tight binding reversible inhibition in the materials and methods as well as assuming that free G-actin is present minimally at its critical concentration of 100 nM (6, 16) and a $K_i$ of 1.3 nM, we calculate that 99, 98, and 37% of wild-type DNase is inhibited at 0.1, 1, and 10 μg/ml DNase, respectively, consistent with the data in Fig. 4.

Biological Significance and Clinical Implications. The biological significance of DNase I inhibition by actin is not well understood; a potential role during mitosis and apoptosis has been recently proposed (36). DNase I from mammalian sources is highly conserved (37); however, not all mammalian DNases are inhibited by actin (38, 39), suggesting that actin inhibition of DNase I is not essential for cellular function. In fact, a gene encoding a novel human DNase, termed LS-DNase, which is expressed predominantly in the liver and spleen and has 46% amino acid sequence identity to human DNase I, has recently been cloned and expressed (W. F. Baron, C. Q. Pan, R.A.L., and K. P. Baker, unpublished data). Of particular interest is the fact that LS-DNase has DNA hydrolytic activity that is not inhibited by G-actin. The finding that a family of DNase I-like enzymes exists within the human genome raises additional questions as to the cellular biology and biochemistry of DNase as well as the role of actin.

The results presented here suggest that actin is a significant inhibitor of DNase I in CF sputum. F-actin is present over a range of concentrations in a limited number of CF sputum studied to date (7); however, the precise amount of actin capable of inhibiting DNase I is unknown. Although the concentration of actin, whether it exists as G-actin, F-actin, or actin in complex with actin binding proteins, in sputum throughout the CF patient population is not well defined, our results suggest that actin-resistant variants may have improved clinical efficacy, especially in patients with high sputum actin levels.

We thank C. Eigenbrot and C. Schiffer for helpful discussions.
regarding DNase structure; A. Spudich for helpful discussions on actin; Genentech’s Assay Services Group for technical assistance; L. O’Connell for tissue culture expertise; M. Vasser, P. Jhurani, and P. Ng for oligonucleotide synthesis; and K. Andow and D. Wood for graphics.