Improved Potency of Hyperactive and Actin-resistant Human DNase I Variants for Treatment of Cystic Fibrosis and Systemic Lupus Erythematosus*

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Clark Q. Pan‡, Tony H. Dodge§, Dana L. Baker§, William S. Prince§, Dominick V. Sinicropi¶, and Robert A. Lazarus††

*From the ‡Departments of Protein Engineering and §BioAnalytical Technology, Genentech, Inc., South San Francisco, California 94080

The ability of recombinant human DNase I (DNase I) to degrade DNA to lower molecular weight fragments is the basis for its therapeutic use in cystic fibrosis (CF) patients and its potential use as a treatment for systemic lupus erythematosus (SLE). To increase the potency of human DNase I, we have generated and characterized three classes of mutants: (a) hyperactive variants, which have from one to six additional positively charged residues (+1 to +6) and digest DNA much more efficiently relative to wild type; (b) actin-resistant variants, which are no longer inhibited by G-actin, a potent inhibitor of DNase I, and (c) combination variants that are both hyperactive and actin-resistant. For DNA scission in CF sputum where the DNA concentration and length are large, we measured a ~20-fold increase in potency relative to wild type for the +3 hyperactive variant 9R/E13R/N74K or the actin-resistant variant A114F; the hyperactive and actin-resistant combination variant was ~100-fold more potent than wild type DNase I. For digesting lower concentrations of DNA complexed to anti-DNA antibodies in human serum, we found a maximal enhancement of ~400-fold over wild type for the +2 variant E13R/N74K. The +3 enzymes have ~4000-fold enhancement for degrading moderate levels of exogenous DNA spiked into human serum, whereas the +6 enzyme has ~30,000-fold increased activity for digesting the extremely low levels of endogenous DNA found in serum. The actin resistance property of the combination mutants further enhances the degree of potency in human serum. Thus, the human DNase I variants we have engineered for improved biochemical and pharmacodynamic properties have greater therapeutic potential for treatment of both CF and SLE.

In addition to its fundamental role of storing genetic information, DNA is also an important component in the pathogenesis of several diseases. In patients with cystic fibrosis (CF), a defective CF transmembrane conductance regulator gene results in viscous airway secretions containing high concentrations of DNA, which is derived from leukocytes in response to the persistent bacterial infections associated with this disease (1–3). Recently, CF patients have been treated clinically with recombinant human deoxyribonuclease I (DNase I), which is inhaled into the airways where it degrades DNA to lower molecular weight fragments, thus reducing the viscoelasticity of CF sputum and improving lung function (4–6).

The presence of anti-nuclear antibodies and immune complexes containing anti-DNA antigens is the hallmark of systemic lupus erythematosus (SLE), an autoimmune disease that affects many organs such as kidney, skin, joints, and central nervous system, resulting in chronic tissue damage (7, 8). Anti-DNA antibodies, in particular those against double-stranded DNA, have been implicated in inducing some of the disease manifestations of SLE, especially nephritis, and are generally present at elevated serum levels in clinically active disease (9). In SLE patients, anti-DNA antibodies are associated with glomeruli in stained kidney sections and are lower than expected in the urine, suggesting retention in the kidneys. DNase I may potentially block the progression of SLE by hydrolysis of the DNA component of membrane-deposited DNA-anti-DNA immune complexes, facilitating clearance of the anti-DNA antibodies and reducing glomerular nephritis. Alternatively, DNase I could hydrolyze circulating and/or antibody-complexed DNA, reducing the antigen load and subsequent deposition of immune complexes. In a recent study in NZB/W mice, systemic administration of recombinant murine DNase I was found to delay the progression of SLE and demonstrated beneficial effects on renal function and histopathology (10).

DNase I is an endonuclease that catalyzes the hydrolysis of double-stranded DNA predominantly by a single-stranded nicking mechanism under physiological conditions (11). To improve the biochemical activity and potentially the efficacy of DNase I, we have recently assessed the importance of various residues at the DNA-binding interface and engineered hyperactive variants that utilize a more efficient functional mechanism involving processive nicking of double-stranded DNA (12, 13). The hyperactive variants were created by introducing basic amino acids into DNase I at the DNA binding interface to generate attractive interactions with the negatively charged phosphates on the DNA backbone. Based on this principle, we constructed and characterized a series of hyperactive variants having from one to six (+1 to +6) basic residue substitutions with a slash between the individual mutations such as E13R/N74K/T205K or 13R/74K/205K. The term +1 variant, +2 variant, . . . , +6 variant refers to human DNase I mutants having one, two, . . . , six additional positively charged residues compared with wild type.
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(14). We found that the degree of hyperactivity was inversely proportional to both DNA concentration and length as well as salt concentration. Under pseudophysiological metal ion and salt conditions, these variants displayed maximal activities ranging from several- to thousands-fold greater than wild type depending on the concentration and length of DNA. In addition to improving the intrinsic DNA-degrading activity of DNase I, we have also recently engineered actin-resistant DNase I variants that no longer bind to G-actin, a potent inhibitor of DNase I having a Kᵋ of ~1 nM (15). The act-resistant variants were 10–50-fold more potent than wild type in reducing viscoelasticity as determined in sputum compaction assays, implicating G-actin as a significant inhibitor of DNase I in CF sputum. Because the DNA and actin binding regions are distinct (Fig. 1), we reasoned that combining these mutations into one enzyme should result in variants that are both hyperactive and actin-resistant and thus even more potent. In the present work, we have characterized hyperactive, actin-resistant, and combination variants of human DNase I in several biologically relevant assays to determine their relative potency and pharmacodynamics in CF sputum and human serum. These results have significant implications for their therapeutic potential for treatment of both CF and SLE.

MATERIALS AND METHODS

Actin Inhibition Assay—Supercoiled pBR322 plasmid DNA (New England Biolabs) at 29 μg/ml was treated with diluted culture media from human 293 cells transfected with wild type or variant DNase I (final DNase I concentration of 1 ng/ml as measured by a human DNase I ELISA (13, 15)) preincubated with increasing amounts of G-actin for 24 min in the presence of 25 mM Hapes, pH 7. 100 μg/ml bovine serum albumin, 1 mM MgCl₂, and 2.5 mM CaCl₂ at room temperature. After 18 min of DNA digestion, the reaction was quenched with 25 μM EDTA, 6% glycerol, xylene cyanol, and bromphenol blue and loaded directly on a 0.8% agarose gel. The gel was run overnight at ~1 V/cm in TBE (90 mM Tris borate and 2 mM EDTA), stained with ethidium bromide, and digitized on a Molecular Dynamics model 575 FluorImager. Mock-transfected media displayed no background activity. CF Sputum DNA Degradation—DNase I degradation of CF sputum DNA was measured by pulsed-field electrophoresis as described previously (15). The sputum collected from a single patient contained 2.24 mg/ml was treated with diluted culture media at 37 °C for 2 h; the reaction was quenched with 25 mM EDTA. A volume of low melting agarose was added to each sample and maintained at 37 °C until loading onto a 1% agarose gel prior to pulsed-field electrophoresis. After the reaction mix plugs solidified in the wells, the gel was run for a 20-h cycle and stained in 0.5 μg/ml ethidium bromide. Gels were scanned with a FluorImager, followed by quantitation of the intensities found in the 23,000 bp or greater region. The decrease in intensity in this higher molecular weight region over that of the control (no DNase added) is presented as the percent DNA hydrolyzed. Experiments were performed in duplicate. No detectable activity was found using the mock-transfected media.

[²⁵P]DNA Digestion—The [³²P]DNA digestion assay follows the extensive degradation of DNA in isotonic buffer or serum (17); [³²P]DNA is an attractive alternative to [²⁵P]DNA because of its longer half-life and lower energy emission. Double-stranded [³²P]-labeled M13 DNA was digested with 100 nM enzyme with specific radioactivity of 0.8 μCi/μg by the addition of salmon testes DNA (Sigma). Five μl of this mixture at 81 μg/ml total DNA concentration was added to 90 μl of buffer or human serum followed by addition of 5 μl of diluted DNase I culture media at room temperature for 2 h. The reaction was stopped by sequential addition of 100 μl of ice-cold 50 mM EDTA and 100 μl of ice-cold 20% trichloroacetic acid, followed by centrifugation at 12,000 × g for 15 min at 4 °C. Fifty μl of the supernatant, which contains radiolabeled DNA fragments that are ~20 bp or less in size, was counted in a scintillation counter. Again, mock-transfected media had no background activity.

Degradation of Chromatin Anti-DNA Immune Complexes in Serum—Preparation of human anti-DNA IgG from SLE patient sera complexed to human chromatin from normal human blood has been described (17). Briefly, 1 volume of the immune complex added to 2 volumes of normal human serum was digested by 1 volume of diluted DNase I transfection media at 37 °C for 2 h; the reaction was quenched with 25 mM EDTA. Actin was present in the preparation of chromatin; the final concentration of total actin in this serum was ~13 μg/ml as determined by quantitative Western analysis (data not shown). For the gel-based assay, the stopped reaction mix was treated with 2% SDS and 1.0 mg/ml proteinase K (Boehringer Mannheim) overnight at 50 °C, followed by electrophoresis on 2.7% MetaPhor agarose (FMC)/0.2% SDS gels in TBE buffer. The gels were stained with 1.0 μg/ml ethidium bromide and scanned with the FotoAnalyst system (Fotodyne). For the ELISA-based assay, the quenched reaction mix was diluted and added to a plate coated with anti-histone monoclonal antibody (Boehringer Mannheim), followed by detection with peroxidase-conjugated goat anti-human IgG, Fc-specific (17).

DNase I Digestion of Endogenous DNA in Serum—Extracellular DNA was extracted from normal human serum pool using the QIAamp 96 spin blood kit (Qiagen) with modifications in a 96-well format. The purified endogenous serum DNA was then characterized by quantitative PCR and dig-lamers and a dual-labeled probe specific for the human Alu gene. Briefly, the fluorescence of one label is quenched by the other label (18). Degradation of the hybridization probe during PCR amplification produces a decrease in fluorescence quenching that is quantified in an Applied Biosystems model 7700 Sequence Detector. The PCR cycle where a fluorescence increase is detected (threshold cycle) is directly proportional to the concentration of target in the sample (19). In this study, using the Alu gene as the target, human DNA concentration was interpolated from a calibration curve generated with a human chromatin standard.

RESULTS

Actin-resistant and Hyperactive DNase I Variants—Previous protein engineering of human DNase I at the actin-binding interface revealed several key positions involved in actin binding (15). In particular, single point mutations introducing charged, aliphatic, or aromatic residues at Ala₁₁₄ of the mature enzyme resulted in actin-resistant variants that had >10,000-fold reduced affinity for G-actin (Fig. 1). In the present work, we chose to characterize the A₁₁₄F mutant, which might potentially reduce any immunogenicity, because phenylalanine is found at position 114 in several other naturally occurring human DNase I-like enzymes, at least one of which has been shown to be actin-resistant (20–22). We have further combined the A₁₁₄F mutation with ones that make DNase I hyperactive to generate several combination variants (14), including Q₉R/E₁₃R/ N₇₄K/A₁₁₄F and Q₉R/E₁₃R/H₄₄K/N₇₄K/T₂₀₅K/A₁₁₄F, and tested them in an actin-binding ELISA (15) and a plasmid-based actin inhibition assay (Fig. 2). The DNA hydrolytic activity of wild type DNase I is inhibited by actin at concentrations as low as 3 nM, with complete inhibition observed at ~3 μM. In contrast, the A₁₁₄F and combination variants were insensitive to actin at concentrations as high as 3 μM (Fig. 2), consistent with their lack of detectable actin binding in the ELISA (data not shown). In the absence of actin, the A₁₁₄F variant showed similar activity and plasmid DNA digestion patterns as wild type DNase I; the lack of a linearized band in the A₁₁₄F lane (Fig. 2) is not significant and only reflects a slightly lower amount of enzyme added relative to wild type.

Both enzymes cleave DNA via a nicking mechanism as evidenced by their low linear to relaxed product ratio (14). In contrast, the products of DNA scission by the combination variants are almost exclusively linear DNA, suggesting a fundamental shift in the functional mechanism. In the absence of NaCl, the overall nicking activity of the combination variants,

² D. L. Baker and D. V. Sinicropi, manuscript in preparation.
and the Alα114 position resulting in actin resistance upon substitution with a basic residue are colored in orange. We first tested them in an therapeutic potential of these three classes of DNase I variants, and hyperactive. We find that the combination variants are both actin-resistant at low DNA concentrations and size (14). Consistent with the variants are indeed more active than wild type, especially at the molecular weight substrates to fragments of 1400 bp in size (data not shown). Reaction with either wild type or the hyperactive variant E13R/N74K/T205K is 100-fold more potent than wild type, whereas that of the +3 to +6 variants are less than 3-fold lower (Table I). In isotonic buffer, the actin-resistant A114F mutation had no effect on the activity of wild type or the hyperactive variant Q9R/E13R/N74K in the [32P]DNA digestion assay (Fig. 4C and Table I). Both the +3 hyperactive and the +3 combination mutants were ~500-fold more potent than either wild type or A114F DNase I. Components in serum inhibited the actin-resistant and the +3 combination variants by only 5.7- and 1.4-fold, respectively, which was 33% and 52% lower than the -fold inhibition observed for the wild type and the +3 hyperactive variant, respectively (Table I). The effect of actin resistance on DNA scission activity in serum is minimal when comparing wild type and A114F DNase I; however, a significant enhancement of 2.4-fold for actin resistance was found in the background of the +3 hyperactive variant. These results suggest the presence of relatively low levels of G-actin in human serum that can inhibit activity effectively only at lower DNase I concentrations (Fig. 4D).

Digestion of Chromatin-anti-DNA Immune Complexes in Serum—Chromatin DNA prepared from normal white blood cells was incubated with SLE serum-derived IgG in normal human serum to form immune complexes. DNase I degradation of these complexes, which have a final DNA concentration of 120 μg/ml and a total actin concentration of ~13 μg/ml, was assessed by an agarose gel-based assay and an ELISA. As shown in Fig. 5, most of the immune complex DNA is >1,000 bp in size prior to any DNase I treatment. Pulsed-field electrophoresis with higher molecular weight standards revealed that most of the DNA in the immune complexes was 7,000–23,000 bp in length (data not shown). Reaction with either wild type or the +3 hyperactive variant E13R/N74K/T205K converts the high molecular weight substrates to fragments of ~180 and ~360 bp in length, consistent with cleavage at the linker region of chromatin, which has a nucleosome repeat length of ~190 bp and ~150 bp of core region (23); intermediate digestion products are also consistent with cleavage between nucleosomes (Fig. 5). DNA scission by the native enzyme is undetectable up to ~3 ng/ml with total digestion occurring at 1 μg/ml DNase I concentration. The +3 variant E13R/N74K/T205K is ~100-fold more potent than wild type, completely degrading DNA at ~10 ng/ml.

Degradation of immune complexes in serum was also determined by ELISA, which yields similar results to those of the gel-based assay. For example, the wild type enzyme has no activity at 3 ng/ml and 100% activity at 1 μg/ml (Fig. 6A); the EC50 for DNA scission determined from the ELISA is 32 ng/ml (Table I). The EC50 of the +1 variant N74K is ~10-fold lower than wild type, whereas that of +2 variant is 400-fold lower.
Further addition of positively charged residues decrease the potency so that the 16 variant is only 35-fold more active than the native DNase I. The actin-resistant variant A114F is 20-fold more active than wild type, consistent with the detection of actin in the immune complex preparation. Furthermore, the actin resistance and the hyperactivity in the combination variant Q9R/E13R/N74K/A114F results in the most active enzyme, having ~650-fold increased potency relative to wild type (Fig. 6B; Table I).

DISCUSSION

We have assessed the relative potency and therapeutic potential of three classes of human DNase I variants by testing them in several biologically relevant assays. For DNA scission in CF sputum, where the DNA and actin concentrations are relatively high, an increase in potency over wild type of ~20-fold was found for either the hyperactive variant Q9R/E13R/N74K or the actin-resistant variant A114F; the combination mutant Q9R/E13R/N74K/A114F was even more potent, resulting in an improvement of ~100-fold. The increase in potency by the hyperactive variants was much greater for degrading DNA in human serum, although the degree of hyperactivity was highly dependent upon the assay conditions. For the digestion of endogenous serum DNA present at extremely low concentrations, the degree of hyperactivity climbs with each additional positive charge, culminating with the 16 variant being ~30,000-fold more potent than wild type. Actin resistance eliminates any inhibition by endogenous G-actin under these conditions, making the +3 variants even more active.

Hyperactivity as a Function of DNA Concentration—We previously characterized a series of hyperactive DNase I variants having from one to six additional positively charged residues relative to wild type in several in vitro assays (14). The level of hyperactivity was found to be inversely proportional to DNA concentration. For example, the ~2 variant E13R/N74K was ~20-fold more active than wild type for nicking 10 ng/ml of linear plasmid DNA, but was ~3-fold less active when the substrate concentration was elevated to 30 µg/ml. Our present ex vivo data in human serum and CF sputum is entirely consistent with the tendency of a greater degree of hyperactivity associated with lower DNA concentration. The DNA concentr-
tion in CF sputum is typically very high and is correlated with the severity of pulmonary disease; we have measured DNA concentrations as high as 25 mg/ml in CF sputum (data not shown). For the particular CF sputum sample we tested, which had a DNA concentration of 2.24 mg/ml, the +3 variant Q9R/E13R/N74K was -20-fold more active than wild type. Variants with four or more additional positively charged residues than wild type are not expected to further enhance the potency for CF sputum DNA digestion, since their degree of hyperactivity is actually lower than the +3 variant in assays containing relatively high DNA concentrations, presumably because of a reduction of the turnover rate as a result of binding to the DNA too tightly (14).

The measured hyperactivities in the three serum-related assays are much greater than those determined in the CF sputum DNA digestion assay as a result of much lower DNA

Fig. 4. [33P]DNA digestion in isotonic buffer and normal human serum by human DNase I variants. The percent [33P]DNA solubilized represents the percent of DNA that is converted from the 7000-bp M13 plasmid to fragments that are 20 bp or less. A (buffer) and B (serum) illustrate the effect of additional positive charges on the degree of hyperactivity relative to wild type; C (buffer) and D (serum) show the effects of the hyperactive, actin-resistant, and combination variants relative to wild type. The variants shown are E13R (+1), E13R/N74K (+2), E13R/N74K/T205K (+3), Q9R/E13R/N74K/T205K (+4), and Q9R/E13R/T14K/H44R/N74K/T205K (+6).

TABLE I
DNA digestion activity of human DNase I variants

| Variants | Exogenous radiolabeled DNA | Serum | Chromatin/IC | Endogeneous DNA |
|----------|---------------------------|-------|--------------|-----------------|
| WT       | 1 ± 0.03                  | 1 ± 0.06 | 8.5        | 1 ± 0.04        | 1 ± 0.03 |
| E13R (+1) | 15 ± 1                    | 15 ± 2  | 8.6        |                 |        |
| N74K (+1) | 16 ± 1                    | 9 ± 0.1 | 15.4       |                 |        |
| N74R (+1) | 23 ± 3                    | 25 ± 0.1 | 7.8        |                 |        |
| E13R/N74K (+2) | 211 ± 8           | 374 ± 22 | 4.8        | 403 ± 55       | 320 ± 40 |
| Q9R/E13R/N74K (+3) | 466 ± 22     | 1381 ± 124 | 2.9        | 220 ± 10       | 690 ± 60 |
| E13R/N74K/T205K (+3) | 1336 ± 92    | 3809 ± 236 | 3.0        | 92 ± 7         | 1700 ± 60 |
| Q9R/E13R/N74K/T205K (+4) | 370 ± 19     | 1414 ± 51  | 2.2        |                 |        |
| E13R/T14K/N74K/T205K (+4) | 374 ± 36    | −1800 ± 1   | 1.7        | 102 ± 12       | 12,000 ± 4000 |
| Q9R/E13R/T14K/H44R/N74K/T205K (+6) | 116 ± 8     | 341 ± 28   | 2.9        | 35 ± 3         | 28,000 ± 1000 |
| A114F    | 0.8 ± 0.06                | −1.2   | 5.7        | 20 ± 2         | 5 ± 1  |
| Q9R/E13R/N74K/A114F (+3) | 555 ± 31    | 3292 ± 333 | 1.4        | 654 ± 106      | 5500 ± 400 |
| E13R/N74K/T205K/A114F (+3) |                 |        |            | 6200 ± 100     |        |
| Q9R/E13R/H44R/N74K/T205K/A114F (+5) | 208 ± 9     | 19,000 ± 1000 |            |               |        |

* The number of additional positively charged residues relative to wild type DNase I are indicated in parentheses. The [33P]DNA digestion assay was carried out in isotonic buffer containing 150 mM NaCl or in normal human serum. Values in the first two columns are reported as -fold improvements over wild type which has a buffer EC_{50} of 130 ± 3 ng/ml and a serum EC_{50} of 1100 ± 64 ng/ml. The third column lists the -fold inhibition observed in serum as compared with buffer. The final two columns tabulate the -fold enhancements over wild type, which has an EC_{50} of 32 ± 1 ng/ml for degrading chromatin/immune complex in serum and an EC_{80} of 1700 ± 50 ng/ml for digesting endogenous DNA in serum, as determined by the Taqman assay. For the endogenous serum DNA assay, we reported the EC_{80} instead of EC_{50}, because we could not accurately determine the activity at low variant concentrations (Fig. 7).
concentrations used. The endogenous DNA concentration in human serum is extremely low, ranging from 25 to 250 ng/ml; the particular serum pool we tested was $\sim 180$ ng/ml DNA. Consequently, the degree of hyperactivity in the endogenous serum DNA digestion assay is much greater than that found in the CF sputum DNA digestion assay (Table I); a maximal improvement of 30,000-fold relative to wild type was found for the $+6$ variant. This correlation between the degree of hyperactivity and the number of engineered positive charges on DNase I was also found in a DNA nicking assay using a 32-bp DNA fragment at 6.3 ng/ml as substrate under isonic conditions in the presence of Mg$^{2+}$ and Ca$^{2+}$ ions (14). The maximal -fold improvement over wild type in the in vitro assay was $\sim 6000$-fold for the $+6$ variant. The larger -fold enhancement in the ex vivo versus the in vitro assay could be because of the presence of serum DNA binding proteins (27), which might inhibit the activity of wild type more than that of the hyperactive variants; this is consistent with the higher degree of inhibition seen for wild type in the $[^{32}P]$DNA digestion assay in serum relative to buffer (Fig. 4). It is possible that these DNA binding proteins could sequester some of the $[^{32}P]$ substrate, accounting for the observation that the activity appears to level off at $\sim 60\%$ in serum (Fig. 4, B and D). The DNA concentrations in the chromatin-immune complex DNA degradation assay and the $[^{32}P]$DNA cleavage assay were 120 and 4 $\mu$g/ml, respectively, intermediate between the mg/ml range found for the CF sputum DNA digestion assay and the ng/ml range for the endogenous serum DNA digestion assay. Not surprisingly, we also found intermediate maximal enhancements of hundreds- to thousands-fold over wild type (Figs. 4B and 6A), again consistent with the inverse correlation of the degree of hyperactivity with the DNA concentration.

Hyperactivity as a Function of DNA Length—Besides being inversely dependent on substrate concentration, the degree of hyperactivity also has an inverse correlation with DNA length (14). For example, the $+2$ variant is $\sim 20$-fold more active than wild type when nicking the 4361-bp linear plasmid pBR322, but $\sim 200$-fold more potent when the substrate was a 32-bp DNA fragment. In the present study, the larger difference between the hyperactive variants and the native DNase I measured in the $[^{32}P]$DNA cleavage assay as compared with that found in the immune complex assay may be explained by the difference in DNA size. In the former assay, the M13 plasmid used is 7000 bp in length, whereas the chromatin DNA used in the latter assay is mostly 7000–23,000 bp in size. In addition, the former only measures extensive DNA degradation down to 20 bp or less, whereas the latter is likely following digestion down to the size of nucleosome core, at $\sim 150$ bp. This association of greater hyperactivity with shorter DNA length supports the proposed processive DNA nicking mechanism attributed to the hyperactive variants (14). As the hyperactive DNase I processively digests DNA, it can slide off the ends of a smaller DNA fragment faster than a longer one, thus elevating the turnover rate. This shift in the functional mechanism of DNA cleavage could also explain the thousands-fold maximal improvements observed in the $[^{32}P]$DNA digestion assay as compared with $<10$-fold maximal enhancements found in the plasmid nicking assay (13), both performed in isonic buffer and in the presence of relatively similar DNA concentrations and substrate sizes. The difference between these two assays is that although the former follows extensive DNA digestion, the latter measures the rate of the initial DNA nick. Consequently, the expected faster generation of smaller molecular weight
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DNA fragments by processive DNA nicking with a hyperactive variant versus nonprocessive DNA nicking with native DNase I would result in a greater degree of hyperactivity determined in the [³²P]DNA digestion assay.

Therapeutic Potential—Based on our ex vivo experiments, we suggest that the engineered variants of human DNase I could significantly increase the efficacy for the treatment of CF. Since the reduction of viscoelasticity of the CF sputum is likely because of moderate DNA degradation down to kilobase in size, a processive DNA nicking mechanism may not be such a dramatic advantage. However, the additional positively charged residues in the hyperactive variants also result in greater resistance to inhibition by physiological saline and perhaps endogenous DNA binding proteins present in CF sputum. The more or less additive effects of hyperactivity and actin resistance lead to a DNase I variant with ~100-fold enhancement in potency relative to wild type, which could prove valuable for CF therapy.

The hyperactive class of DNase I variants also has potential for treatment of SLE, where a more dramatic impact on DNA degradation in serum was found. In both normal and SLE sera, the DNA concentrations are very low. Furthermore, it may be necessary to reduce the DNA size down to less than 10–20 bp to prevent immune complex formation or destroy existing ones. The hyperactive variants are improved relative to wild type in addressing both of these properties, demonstrating an increase in potency of three to four orders of magnitude. DNase I has shown beneficial effects in a murine model of lupus nephritis, albeit at relatively high doses of 7.5 mg/kg/day (10). The observed increase in potency for the most active variants could overcome any dosage limitation of wild type DNase I and show improved efficacy.

The effect of actin-resistance was observed in the CF sputum and some of the serum assays; however, these results should be taken with appropriate caution because of the inherent difficulty of measuring the precise level of G-actin, which is the form of actin that inhibits DNase I. Complicating factors in a biological medium include the equilibrium between F- and G-actin, the presence of actin-binding proteins, and potential artifacts resulting from either cell lysis or proteolysis of actin during the course of the assays. Nonetheless, in the event that G-actin is present, the actin-resistant variants are indeed more potent, as we found in the CF sputum, immune complex, and endogenous serum DNA assays. In the [³²P]DNA digestion assay (Fig. 4B), the effect of actin resistance was somewhat attenuated. We attribute these differences to varying concentrations of G-actin in serum for the abovementioned reasons. We have measured total actin levels as high as 125 µg/ml in normal human serum; values ranging between 30 and 50 µg/ml in human plasma have been reported (28). Increased levels of an inhibitor of DNase I in human SLE serum, presumably G-actin, have also been reported (29). Finally, actin concentrations could be much higher locally either in CF sputum or in the glomerulus of lupus patients because of increased cell lysis.

The degree of increased activity for the DNase I variants relative to wild type that we have presented is quite substantial. However, extrapolation of ex vivo data to the in vivo environment is inherently speculative and should be taken with caution; in particular, the immunogenicity profile of our DNase I variants has yet to be investigated but could foreseeably pose some limitations to their applicability in the clinic. Nonetheless, the results presented herein suggest that the DNase I variants we have engineered for improved biochemical and pharmacodynamic properties may have improved clinical benefits as well.

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REFERENCES

1. Boat, T. F., Welsh, M. J., and Beaudet, A. L. (1989) in The Metabolic Basis of Inherited Disease (Scrivner, C. L., Beaudet, A. L., Sly, W. S., and Valle, D., eds) Vol. II, pp. 2649–2680, McGraw-Hill, New York
2. Collins, F. S. (1992) Science 256, 774–779
3. Quinton, P. M. (1990) PASER J. 4, 2709–2717
4. Ramsey, B. W. (1996) N. Engl. J. Med. 335, 179–188
5. Ramsey, B. W., Astley, S. J., Atitken, M. L., Burke, W., Colin, A. A., Dorkin, H. L., Eisenberg, J. D., Gibson, R. L., Harwood, I. R., Hahn, B. H., and Scriver, C. R., eds) Vol. II, pp. 507–628, McGraw-Hill, New York
6. Fuchs, H. J., Borowitz, D. S., Christiansen, D. H., Morris, E. M., Nash, M. L., Ramsey, B. W., Rosenstein, B. J., Smith, A. L., and Wohl, M. E. (1994) N. Engl. J. Med. 331, 637–642
7. Hahn, B. H. (1997) in Dubois’ Lupus Erythematosus (Wallace, D. J., and Hahn, B. H., ed) 5th Ed., pp. 69–75, Williams and Wilkins, Baltimore
8. Woods, V. L., Jr. (1993) in Textbook of Rheumatology (Kelley, W. N., Harris Jr., E. D., Ruddy, S., and Sledge, C. B., eds) Vol. I, 4th Ed., pp. 999–1016, W. B. Saunders Co., Philadelphia
9. Hahn, B. H. and Tsao, B. P. (1997) in Dubois’ Lupus Erythematosus (Wallace, D. J., and Hahn, B. H., ed) 5th Ed., pp. 407–422, Williams and Wilkins, Baltimore
10. Macanovic, M., Sinicropi, D., Shak, S., Baughman, S., Thiru, S., and Lachmann, P. J. (1996) Clin. Exp. Immunol. 106, 243–252
11. Campbell, V. W., and Jackson, D. A. (1980) J. Biol. Chem. 255, 3726–3735
12. Pan, C. Q., Ulmer, J. S., Herzka, A., and Lazarus, H. A. (1998) Protein Sci. 7, 628–636
13. Pan, C. Q., and Lazarus, R. A. (1997) Biochemistry 36, 6624–6632
14. Pan, C. Q., and Lazarus, R. A. (1998) J. Biol. Chem. 273, 11701–11708
15. Ulmer, J. S., Herzka, A., Toy, K. J., Baker, D. L., Dode, A. H., Sinicropi, D., Shak, S., and Lazarus, R. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5 D. L. Baker, T. H. Dodge and D. V. Sinicropi, unpublished results.
18. Livak, K. J., Flood, S. J., Marmaro, J., Giusti, W., and Deetz, K. (1995) *PCR Methods Appl.* 4, 357–362

19. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. (1996) *Genome Methods* 6, 986–994

20. Baron, W. F., Pan, C. Q., Spencer, S. A., Ryan, A. M., Lazarus, R. A., and Baker, K. P. (1998) *Gene (Amst.), in press*

21. Zeng, Z., Parmalee, D., Hyaw, H., Coleman, T. A., Su, K., Zhang, J., Gentz, R., Ruben, S., Rosen, C., and Li, Y. (1997) *Biochem. Biophys. Res. Commun.* 231, 499–504

22. Rodriguez, A. M., Rodin, D., Nomura, H., Morton, C. C., Stanislawa, W., and Schneider, M. C. (1997) *Genomics* 42, 507–513

23. Pruss, D., Hayes, J. J., and Wolfe, A. P. (1995) *Bioessays* 17, 161–170

24. Steinman, C. R. (1982) *Methods Enzymol.* 84, 187–193

25. McCoubrey-Hoyer, A., Okarma, T. B., and Holman, H. R. (1984) *Am. J. Med.* 77, 25–34

26. Deininger, P. L. (1989) in *Mobile DNA* (Howe, M., and Berg, D., eds) pp. 619–636, American Society for Microbiology Press, Washington, D. C.

27. Gardner, W. D., and Hoch, S. O. (1979) *J. Biol. Chem.* 254, 5238–5242

28. Mejean, C., Roustan, C., and Benyamin, Y. (1987) *J. Immunol. Methods* 99, 129–135

29. Frost, P. G., and Lachmann, P. J. (1968) *Clin. Exp. Immunol.* 3, 447–455

30. Wolf, E., Frenz, J., and Suck, D. (1995) *Protein Eng.* 8, (suppl.) 79

31. Weston, S. A., Lahm, A., and Suck, D. (1992) *J. Mol. Biol.* 226, 1237–1256

32. Kahsch, W., Mannherz, H. G., Suck, D., Pai, E. F., and Holmes, K. C. (1999) *Nature* 347, 37–44