Expression and Characterization of a DNase I-Fc Fusion Enzyme*

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Recombinant human deoxyribonuclease I (DNase I) is an important clinical agent that is inhaled into the airways where it degrades DNA to lower molecular weight fragments, thus reducing the viscoelasticity of sputum and improving the lung function of cystic fibrosis patients. To investigate DNases with potentially improved properties, we constructed a molecular fusion of human DNase I with the hinge and Fc region of human IgG1 heavy chain, creating a DNase I-Fc fusion protein. Infection of Sf9 insect cells with recombinant baculovirus resulted in the expression and secretion of the DNase I-Fc fusion protein. The fusion protein was purified from the culture medium using protein A affinity chromatography followed by gel filtration and was characterized by amino-terminal sequence, amino acid composition, and a variety of enzyme-linked immunosorbent assays (ELISA) and activity assays. The purified fusion contains DNase I, as determined by a DNase I ELISA and an actin-binding ELISA, and an intact antibody Fc region, which was quantified by an Fc ELISA, in a 2:1 stoichiometric ratio, respectively. The dimeric DNase I-Fc fusion was functionally active in enzymatic DNA digestion assays, albeit about 10-fold less than monomeric DNase I. Cleavage of the DNase I-Fc fusion by papain resulted in a specific activity comparable to the monomeric enzyme. Inhibition by wild type monomeric DNase I but actually enhanced the activity of the dimeric DNase I-Fc fusion. The DNase I-Fc fusion protein was also less Ca2+-dependent than DNase I itself. These results are consistent with a higher affinity of the dimeric fusion protein to DNA than monomeric DNase I. The engineered DNase I-Fc fusion protein described herein has properties that may have clinical benefits.

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 patients (1). It is inhaled into the airways where it reduces the viscoelasticity of cystic fibrosis sputum, resulting in improved lung function and fewer respiratory exacerbations (2, 3). In addition, the use of DNase I in a murine model of systemic lupus erythematosus has been investigated with encouraging results (4). The pharmacological and clinical significance of DNase I has led us recently to engineer actin-resistant variants of the human enzyme that are no longer inhibited by actin (5) as well as hyperactive variants that alter the functional mechanism of DNA cleavage (6, 7); characterization of these variants under biologically relevant ex vivo conditions has shown a marked improvement in potency compared with wild type (8). Furthermore, we have recently addressed the functional importance of various residues of human DNase I at the DNA binding interface by extensive mutagenesis (9).

DNase I is an endonuclease that catalyzes the hydrolysis of double-stranded DNA predominantly by a single-stranded nicking mechanism under physiological conditions when both Ca2+ and Mg2+ ions are present (10). The hyperactive variants have been shown to use a more efficient functional mechanism involving processive nicking which yielded a greater number of double-stranded breaks as a result of higher affinity for DNA (6, 7). The hyperactive variants were selected from a mutational scanning analysis of the DNase I-DNA interface where Arg or Lys replacements resulted in attractive interactions with the negatively charged phosphates on the DNA backbone. In addition, the tighter binding to DNA also eliminated the significant inhibition of DNase I by physiological saline.

In the present study, we have expressed and characterized a DNase I-Fc fusion, resulting in a dimeric form of DNase I (Fig. 1). We hypothesized that dimerization of DNase I using the hinge and Fc portion of IgG1 could impart several interesting properties. Dimerization could result in an enzyme with enhanced ability to degrade DNA having increased levels of “cutting” relative to “nicking” if each active site of the dimer bound to the same molecule of DNA. Furthermore, the potential enhanced binding affinity of a dimeric versus a monomeric form of DNase I could also lead to an enzyme that is more resistant to inhibition by salt. The strategy we chose to dimerize DNase I has also been used on many other ligands to make protein-IgG chimera termed immunoadhesins (11, 12); these often have a longer pharmacokinetic half-life, similar to humanized monoclonal antibodies, which may be desirable for therapeutic use. Although many immunoadhesins have been characterized, to our knowledge, this is only the third example of an immunoadhesin that is an active enzyme (13, 14).

MATERIALS AND METHODS

Construction of AcNPV.DNase I-Fc Expression Vector—Standard recombinant DNA techniques were used for the construction of recombinant transfer vectors based on the vector pVL1393 (PharMingen) (15, 16). The pVL1393-derived plasmid pPhH.Vis was linearized with NcoI and SmaI and treated with shrimp alkaline phosphatase. The Fc portion of the human IgG1 was obtained as a 700-base pair fragment by restriction digestion using NdeI and subsequent treatment with Klenow and Ncol of another pVL1393-derived plasmid pVL1393.IgG. Following ligation, competent Escherichia coli XL-1 Blue was transformed, and bacteria were selected for the correct recombinant plasmid (pVL1393.Fc) by DNA sequence analysis. Then, pVL1393.Fc was linearized with BamHI and StuI and treated with shrimp alkaline phosphatase. The linearized vector was then ligated with a DNase I cDNA insert. The full-length human DNase I cDNA insert contained within the plasmid pRK.DNase (17) was obtained as a 1 kilobase fragment by restriction enzyme digestion using BamHI and StuI. Following ligation, competent E. coli XL-1 Blue was transformed, and bacteria were selected for the correct recombinant plasmid (termed pVL1393.DNase...
conjugated rabbit anti-human DNase I antibody; proteins were then detected using the ECL system (Amersham Pharmacia Biotech). An aliquot of the purified DNase I-Fc fusion was also treated with PNGase F (Roche Molecular Biochemicals) and run on a nonreducing gel as described above.

**Gel Filtration Chromatography and Sulphydryl Analysis**—An aliquot of the purified DNase I-Fc fusion was run at 0.35 ml/min on an S-200 column in 25 mM Hepes, pH 7.2, 1 mM Ca<sup>2+</sup>, and 150 mM NaCl. Fractions were analyzed by SDS-PAGE under nonreducing conditions. Purified DNase I-Fc fusion was treated with 0.1 mM DTNB in 25 mM Hepes, pH 7.2, 1 mM Ca<sup>2+</sup> to detect any free cysteines; 1-Cys was used as a standard curve by measuring the absorbance at 412 nm.

**Protein Sequencing**—DNase I-Fc purified from the infected Sf9 cell supernatant was subjected to SDS-PAGE and then transferred to a PVDF membrane. Electroblotting onto Millipore Immobilon-P (48) membranes was carried out for 1 h at 250 mA constant current in a Bio-Rad Trans-Blot transfer cell (19). The PVDF membrane was stained with 0.1% Coomassie Blue R-250 in 50% methanol, for 0.5 min, and destained for 2–3 min with 10% acetic acid in 50% methanol. The membrane was thoroughly washed with water and allowed to dry before storage at -20 °C. The DNase I-Fc bands at about 120 kDa were cut, and the first 12 residues were sequenced using a 494A Applied Biosystems sequencer equipped with an on-line PTH analyzer. Peaks were integrated with Justice Innovation software using Nelson Analytical software packages (20).

**Amino Acid Analysis**—Samples were hydrolyzed using 6 M HCl containing 0.1% phenol at 110 °C for 24 h. Amino acids were analyzed on a Beckman 6300 amino acid analyzer using post-column detection with ninhydrin.

**DNase I**—DNase I concentrations for the DNase I-Fc fusion protein were determined by ELISA using a rabbit anti-DNase I polyclonal antibody coat and detecting with a goat anti-DNase I polyclonal antibody conjugated to horseradish peroxidase (5). Multiple sample dilutions were compared with standard curves of purified recombinant human DNase I (Pulmozyme®, Genentech) to determine concentrations of DNase I for the DNase I-Fc fusion proteins. The concentration of the DNase I-Fc protein was calculated taking into account the molecular weights and molarities.

**Fc ELISA**—Concentrations for the DNase I-Fc protein were determined by ELISA using a rabbit anti-human Fc polyclonal antibody coat and were detected with a rabbit anti-human Fc polyclonal antibody conjugated to horseradish peroxidase. As above, multiple sample dilutions were compared with standard curves of human Fc (Sigma) to determine concentrations of Fc for DNase I-Fc. The concentration of DNase I-Fc protein was calculated taking into account the molecular weights and molarities.

**Actin-binding ELISA**—The actin-binding ELISA for DNase I or DNase I-Fc was carried out essentially as described previously (5). In addition to detecting with an anti-human DNase I polyclonal antibody conjugated to horseradish peroxidase (5), a duplicate plate was assayed using 100 μg/ml of an anti-human Fc rabbit polyclonal antibody conjugated to horseradish peroxidase (19 ng/ml). Color was developed by adding 100 μl/well of Sigma Fast α-phenylenediamine and urea/H<sub>2</sub>O<sub>2</sub> reagent and stopped by adding 100 μl/well 4.5 mM H<sub>2</sub>SO<sub>4</sub>. The A<sub>492</sub> was measured and plotted versus the DNase I-Fc concentration. Recombinant human DNase I (Pulmozyme®, Genentech) was assayed as a negative control. The sigmoidal curves were fit to a four-parameter regression equation by nonlinear regression analysis (21); the E<sub>50</sub> value is the DNase I-Fc or DNase I concentration that produced a half-maximal signal.

**Plasmid Digestion Assay in Native Agarose Gel**—Dilutions of baculovirus infected cell culture media or purified DNase I-Fc at room temperature in the presence of 25 μM Hepes, pH 7.0, 100 μg/ml bovine serum albumin, 1 mM MgCl<sub>2</sub>, 25 mM CaCl<sub>2</sub> with different NaCl concentrations were incubated with 160 μl of 30 μg/ml supercoiled pBR322 plasmid DNA (New England Biolabs) or 160 μl of 30 μg/ml EcoRI-linearized pBR322 (purified by phenol/chloroform extraction and ethanol precipitation). For reactions with Mg<sup>2+</sup> or Mn<sup>2+</sup> without Ca<sup>2+</sup>, 1 μM of the metal ion and 0.1 mM EGTA was included in the reaction mix. At selected time intervals, 1 μl aliquots of the reaction mix were quenched with 25 mM EDTA, 6% glycerol, xylene cyanol, and bromphenol blue and loaded onto 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 quantitated with a Molecular Dynamics model 575 FluorImager. The overall activity was estimated from the initial rate of disappearance of supercoiled or linear substrate. The linear to relaxed ratio was also determined from the initial rates of appearance based on 30 sulfhydryl groups per molecule.

**Protein Sequencing—**

**Amino Acid Analysis—**

**Actin-binding ELISA—**

**Plasmid Digestion Assay in Native Agarose Gel—**

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1 The abbreviations used are: AcNPV, *Autographa californica* nuclear polyhedrosis virus; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay.
of linear and relaxed products. Mock-infected media with similar dilutions showed no background activity.

**p-Nitrophenyl Phenylphosphonate Assay**—The catalytic activity by either wild type DNase I or the DNase I-Fc fusion on the hydrolysis of a small molecule substrate, p-nitrophenyl phenylphosphonate (Sigma), was determined by monitoring the increase in A_{405} as a result of the generation of p-nitrophenol. Reactions were carried out with 10 mM p-nitrophenyl phenylphosphonate incubated with culture media in the presence of 60 mM Hepes, pH 7.0, 50 mM MgCl_2, and 50 mM CaCl_2.

**Papain Digestion of DNase I-Fc**—Papain digestion of purified DNase I-Fc was performed by incubation at 37 °C for 30 min. The final mixture (150 μl total volume) contained 36 μg/ml DNase I-Fc, 3.3 mM cysteine, 0.67 mM EDTA, 42 μg/ml papain (Worthington 3126-PAP, 22 units/mg) in 10 mM sodium phosphate, 0.15 mM NaCl, pH 7.3. The reaction was quenched by addition of 6 μl of freshly prepared 130 mM iodoacetic acid at pH 6.8 and incubated at room temperature for 30 min. The digestion was evaluated by SDS-PAGE using a NOVEX 4–20% gradient gel with and without 0.15 mM β-mercaptoethanol.

**RESULTS**

**Expression and Purification of DNase I-Fc**—High Five spinner cultures were infected with the recombinant baculovirus AcNPV.DNase I-Fc. Expression of the secreted DNase I-Fc protein was confirmed by adsorption of tissue culture supernatant to protein A-conjugated beads followed by SDS-PAGE under reducing conditions. A protein band that was immunoreactive against both an anti-DNase I antibody and an anti-Fc antibody was detected (data not shown). To purify the DNase I-Fc fusion, the baculoviral infected High Five supernatants were harvested 72 h after the initial infection. After removal of the High Five cells by centrifugation, the supernatant was dialyzed overnight against 25 mM Hepes, pH 7.2, 1 mM CaCl_2. The dialyzed sample was filtered and loaded onto a Hi-Trap protein A column. After elution with 3.5 mM MgCl_2, the DNase I-Fc-containing fractions, which were determined by A_{280} and SDS-PAGE, were desalted using a PD-10 column and concentrated using Centriplus-30 concentrators. Dialysis of the cell culture medium prior to purification on protein A was essential for eliminating the aggregation observed in the absence of this step; no evidence of higher order aggregates for the purified fusion were found by gel filtration chromatography or nonreducing SDS-PAGE and Western blotting analysis (data not shown). In addition, no free cysteines were detected using DTNB, implying that all of the disulfides are formed.

**Gel Electrophoresis, Amino Acid Sequence, and Composition of DNase I-Fc**—Purified DNase I-Fc was analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 2). Under nonreducing conditions, Coomassie Blue staining revealed a predominant band and a minor band with a molecular mass of −120 kDa (Fig. 2A). Amino-terminal sequencing of each of these bands confirmed the DNase I sequence. Under reducing conditions, a single predominant band of −62 kDa was found. A minor band migrating at ~45 kDa, which was unidentifiable by amino-terminal sequencing, is perhaps due to a reduced heavy chain of bovine IgG, which commonly has a blocked amino terminus. Immunoblot analysis using either an anti-Fc antibody (Fig. 2B) or an anti-DNase I antibody (C). DNase I is in lanes 8 and 10; the DNase I-Fc fusion is in lanes 9 and 11.

**Characterization of Actin Binding Activity**—The binding affinity of DNase I-Fc 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. Using the anti-human DNase I polyclonal antibody for detection, the affinity of DNase I-Fc for G-actin was assessed by an ELISA and DNase I ELISA. After accounting for the amount of Fc or DNase I proteins in the DNase I-Fc fusion, amino acid analysis determined the concentration of 28.5 μg/ml; the Fc ELISA and DNase I ELISA determined the concentrations of 27.8 and 17.5 μg/ml, respectively.

**Supercoiled Plasmid Digestion Assay**—To compare the DNA hydrolytic activity of DNase I-Fc and DNase I, supercoiled plasmid was used as a substrate for DNA cleavage under different metal ion conditions. A single nick on one of the two supercoiled DNA strands results in the formation of a relaxed circle that can subsequently be converted to linear DNA if it is nicked on the other strand at the same position. In the presence of Mg^{2+} and Ca^{2+}, monomeric wild type human DNase I cleaves double-stranded DNA via a nicking mechanism as evidenced by its ability to yield the linear DNA product only after nearly complete conversion of the supercoiled substrate to the relaxed circle intermediate (Fig. 3). In the presence of Mn^{2+}, human DNase I employs more of a cutting mechanism as demonstrated by a dramatic increase in the linear to relaxed product ratio, consistent with our previous studies (9). Under both metal ion conditions, the overall activity corresponding to the rate of disappearance of the supercoiled substrate for the dimeric DNase I-Fc fusion is ~10-fold lower than that of the native monomeric DNase I; however, the dinemic enzyme appears to generate a slightly higher linear to relaxed product ratio. As compared with the optimal conditions of Mg^{2+} and Ca^{2+}, the removal of Ca^{2+} or the addition of 150 mM NaCl results in ~10-fold lower activities for the monomeric DNase I but not for the dimeric fusion (Table 1). As a result, under these suboptimal conditions for DNase I, DNase I-Fc has approxi-
DNase I-Fc fusion is optimal from 50 to 100 mM NaCl and effects on the DNA cleavage activity in more detail, Eco
activities of 1.6 and 2.1 corrected for the concentration of active sites, displayed similar an-
should remain intact when using a small molecule substrate
zyme itself, we treated the DNase I-Fc fusion with papain to
dimerization is not due to some expression artifact of the en-
that the reduction in the overall plasmid nicking activity upon
DNase I-Fc fusion should remain intact when using a small molecule substrate analog. Indeed, wild type DNase I and DNase I-Fc, when cor-
activated from 0 to 50 mM. The linear DNA digestion activity of the
DNase I-Fc fusion is optimal from 50 to 100 mM NaCl and
becomes inhibited only at higher salt concentrations (Fig. 4).

**Effect of Salt on DNA Cleavage Activity**—To address the ionic

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**DISCUSSION**

We have fused human DNase I to the Fc region of an IgG heavy chain which can be expressed in the baculovirus system and secreted as a disulfide-bonded homodimer (Fig. 1). The DNase I-Fc fusion can be purified substantially in a single step using affinity chromatography on protein A (Fig. 1). The DNase I-Fc fusion binds quantitatively the same as wild type DNase I (5). Therefore it appears that the baculoviral expression of the fusion with the Fc heavy chain does not alter the structural integrity of DNase I. Detection with the Fc antibody also con-
in the presence and absence of papain. Supercoiled pBR322 substrate with a low level of relaxed circle background (1st lane, C for control) was incubated with a mixture of 3 pm wild type DNase I and 3 pm Fc or 15 pm DNase I-Fc dimeric fusion (30 pm monomer) in the presence and absence of papain.

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**TABLE I**

Supercoiled plasmid DNA nicking activity of DNase I and DNase I-Fc

| Metal ions | DNase I | DNase I-Fc |
|------------|---------|-----------|
| Mg^{2+} | 96      | 8         |
| Ca^{2+} | 11      | 12        |
| Mg^{2+}, Ca^{2+} | 13 | 14        |
| Mg^{2+}, Ca^{2+}, 150 mM NaCl | 90 | 7         |

* The overall plasmid nicking activities are reported in nm DNA min^{-1} nm^{-1} DNA nicking I. Note that 1 nm DNase I-Fc is equivalent to 2 nm DNase I.

rus expression system has been observed previously, where protein disulfide isomerase has enhanced solubility and facilitated recovery (24). Another possibility for this heterogeneity are different glycosylation patterns in the fusion, since there are two potential N-linked sites in DNase I and one in the Fc region, and both bands contain the amino-terminal sequence of DNase I. However, this is disfavored since treatment of the DNase I-Fc fusion with PNGase F, which should remove the carbohydrates, did not alter the molecular mass on a nonreduc-
gel (data not shown).

The actin binding experiments showed that the DNase I-Fc fusion binds quantitatively the same as wild type DNase I (5). Therefore it appears that the baculoviral expression of the fusion with the Fc heavy chain does not alter the structural integrity of DNase I. Detection with the Fc antibody also con-
figuration of DNase I is correctly folded in the fusion is

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**FIG. 4.** NaCl effect on DNA digestion by DNase I and DNase I-Fc. EcoRI-linearized pBR322 DNA was treated with 150 pm wild type DNase I or 400 pm DNase I-Fc dimeric fusion (800 pm monomer) in the presence of increasing NaCl concentrations.

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**FIG. 5.** Plasmid DNA nicking by DNase I and DNase I-Fc in the presence and absence of papain. Supercoiled pBR322 substrate with a low level of relaxed circle background (1st lane, C for control) was incubated with a mixture of 3 pm wild type DNase I and 3 pm Fc or 15 pm DNase I-Fc dimeric fusion (30 pm monomer) in the presence and absence of papain.
DNase I-Fc fusion was ~10-fold less active in the plasmid nicking assay than wild type DNase I. This was independent of the linker length between the Fc and DNase I. This reduction in activity was initially somewhat surprising and unanticipated. However, we can propose two possible explanations for this result. The first is that the second DNase I on the dimer somehow inhibits the activity of the first, most likely by imparting adverse steric interactions. An alternative related and more likely possibility is that binding of both DNases of the dimer to a given DNA at the same time induces a bend in the DNA that alters the geometry of the scissile bonds such that they are no longer optimal for catalysis. This is consistent with crystallographic studies on DNA complexed to DNase I where significant conformational change occurs, resulting in significant DNA bending and a widening of the minor groove (25, 26). The width of the minor groove is a major factor that affects DNA cleavage rates (27). In addition, the stiffness and flexibility of DNA also contribute significantly to catalytic rates of hydrolysis (28, 29). Helix parameters such as tilt, rise, and twist could also affect cutting rates. A discussion of DNase I induced conformational changes, and other factors that determine the catalytic efficiency of DNase I has been recently reviewed (30).

Assuming that distortion of the DNA occurs upon binding of one of the DNases in the DNase I-Fc fusion, perhaps only one of the two active sites can contact the DNA substrate in a manner that permits optimal hydrolysis of phosphodiester bond. However, having two DNases linked together by the Fc fusion may result in a greater frequency of nicking on both strands of the DNA by each monomer, leading to the somewhat higher linear to relaxed product ratio. The ability of a DNase I dimer to create increased levels of cutting relative to nicking could be due to an increased concentration of active sites locally on a given piece of DNA. Given that one monomer can properly interact with DNA to allow DNA cleavage, the other monomer could partially interact with DNA, resulting in greater protein-DNA stability in an analogous fashion as compared with the hyperactive DNase I, engineered by the introduction of additional positively charged residues into the DNA-DNase I interface (6, 7). Consequently, the DNase I-Fc fusion and hyperactive DNase I variants share certain characteristics such as greater salt resistance and lower Ca$^{2+}$ dependence. Like hyperactive DNase I, the fusion dimer is also much less sensitive to salt, with a salt titration profile similar to that of the hyperactive E13R/N74K variant (6). Both engineered proteins display optimal activity at ~100 mM NaCl instead of 0 mM for the wild type monomeric DNase I. The stabilization of the DNA complexed to the first DNase I monomer by the second monomer may replace the role of the protein-stabilizing Ca$^{2+}$ ion; therefore, in the absence of this metal ion, the activity of DNase I-Fc is not reduced as compared with wild type DNase I (Table I). Finally, under certain conditions, both the DNase I-Fc fusion and the hyperactive variants can have lower catalytic activity than wild type, presumably under conditions where dissociation is rate-limiting (6, 7).

In addition to the biochemical properties of the DNase I-Fc fusion described herein, the possibility that this strategy could impart different pharmacological properties has potentially important consequences. DNase I, albeit at relatively high concentrations, has recently been shown to have some beneficial response in a murine model of systemic lupus erythematosus (4). The pharmacokinetics of DNase I following a single bolus intravenous injection in rats shows a relatively fast dose-dependent clearance followed by a much slower phase, likely modulated by a serum-binding protein (31). The DNase I-Fc fusion, like other Fc-protein fusions termed immunoadhesins (11, 12), could have a much longer pharmacokinetic half-life than wild type DNase I, similar to humanized monoclonal antibodies, which may be desirable for therapeutic use. Future studies are planned to address the physiological aspects of the DNase I-Fc fusion.

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