Characterization of Mutant Neutrophil Elastase in Severe Congenital Neutropenia*

Feng-Qian Li and Marshall Horwitz†

From the Markey Molecular Medicine Center, Division of Medical Genetics, Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195

Severe congenital neutropenia is a heritable human disorder characterized by neutropenia and acute myelogenous leukemia. We recently determined that the majority of cases result from de novo or autosomal dominantly inherited heterozygous mutations in ELA2, encoding neutrophil elastase. Neutrophil elastase is a chymotryptic serine protease localized in granules of neutrophils and monocytes and is the major target of inhibition of the serpin α1-antitrypsin. The mutations causing severe congenital neutropenia consist of amino acid missense substitutions, in-frame deletion, splice donor mutation producing a deletion, splice acceptor mutation causing insertion of novel residues, and protein truncating mutations of the carboxyl terminus resulting from nonsense substitutions and deletions leading to frameshifts. We have expressed 14 mutant forms of neutrophil elastase in vitro and have characterized their biochemical properties. The mutations have variable effects on proteolytic activity, eliminating the possibility that the disease results from haploinsufficiency. There is no evidence that the mutant enzymes are cytotoxic. The mutant enzymes retain vulnerability to inhibition by α1-antitrypsin, but demonstrate variable avidity for interaction with this serpin. Somewhat surprisingly, the mutant enzymes inhibit the wild type enzyme when both are coexpressed within the same cell, suggesting the potential to interfere with normal subcellular trafficking or post-translational processing.

Commencing at birth, the human disorder severe congenital neutropenia (sometimes known as “Kostmann syndrome”) is typified by circulating neutrophil counts of less than 200 µl⁻¹ (normal >1,800 µl⁻¹) but generally normal quantities of other blood cells. Since neutrophils are the primary phagocyte of the blood responsible for defending against sepsis, affected individuals develop chronic infections. The bone marrow in SCN¹ is characterized by a promyelocytic maturation arrest (1). About 10% of individuals with SCN develop acute myelogenous leukemia or myelodysplasia (2), often with characteristic bone marrow cytogenetic abnormalities comprised of monosomy 7 and trisomy 21.

SCN was first described in consanguineous Swedish families, where it results from autosomal recessive inheritance (1). More commonly, however, the illness results from apparent autosomal dominant inheritance or arises sporadically in the absence of a family history, presumably as a consequence of new dominant mutations (3). SCN was once thought to be caused by constitutional mutations of the gene encoding the GCSF receptor (GCSFR) (4), but it is now known that GCSFR mutations are not the cause of SCN, instead occurring somatically in the bone marrow of some patients in association with leukemia (5–9). We recently found that both cyclic neutropenia (10, 11), an autosomal dominant illness with a 21-day cycle of oscillating neutropenia, and about 75% of cases of SCN (11) result from heterozygous, autosomal dominant mutations in ELA2, encoding neutrophil elastase.

Neutrophil elastase (EC 3.4.21.37) is a monomeric polypeptide of about 25 kDa synthesized in promyelocytes and promonocytes and predominantly localized in azurophilic cytoplasmic granules (reviewed in Refs. 12 and 13). It possesses activity toward a wide variety of proteins, including matrix components, clotting factors, immunoglobulins, and complement. Released from neutrophils migrating to sites of inflammation, it is thought responsible for tissue destruction in pulmonary emphysema, rheumatoid arthritis, and the adult respiratory distress syndrome, among other illnesses. Consequently, its activity is counteracted by a variety of endogenous inhibitors, including the serpins α1-antitrypsin and ELANH2 and the nonserpin elafin.

Neutrophil elastase was once purified as “medullasin” based on its cytokine activity. It induces activated killer lymphocytes (14), cleaves aminoacyl-tRNA synthetase to yield one polypeptide with interleukin-8-like activity and another promoting leukocyte and monocyte chemotaxis (15), and further modulates the expression of components of the inflammatory response, including ICAM-1 (16).

Given the plurality of its activities and hence myriad potential pathogenic mechanisms, we here undertake to biochemically characterize the properties of the variety of mutant forms of neutrophil elastase causing SCN. We have considered three genetic hypotheses compatible with the autosomal dominant inheritance of SCN resulting from mutant forms of neutrophil elastase. First, it is possible that the mutations merely disable protease activity, and the disease is a result of haploinsufficiency. We have therefore measured enzymatic activity of each of the mutants. Second, the mutant enzymes could gain a novel function. We examine two potential gain of function activities, toxic gain of function and loss of inhibition by α1-antitrypsin. Third, the mutant enzymes may have the potential to be a...
dominant negative inhibitor of the wild type protease; we address this hypothesis through coexpression of both wild type and mutant enzymes.

**EXPERIMENTAL PROCEDURES**

*cdNA, Mutagenesis, and Construction of Cell Expression Vectors—* CDNAs of neutrophil elastase and dipetidyl aminopeptidase I (DPP1) were reverse transcribed from total RNA of human HL-60 leukemia cells (10). By designing appropriate polymerase chain reaction primers, the Kozak consensus leader sequence for maximum translational efficiency was introduced, and the flanking restriction enzymes EcoRI and XhoI were included for subsequent subcloning into the PCS2+ expression vector (17, 18) containing a simian cytomegalovirus promoter/enhancer. Individual clones were isolated and sequenced to verify the integrity of reading frame and confirm that polymerase chain reaction introduction did not mutate. The α,-antitrypsin cDNA (19) was a gift of Dr. Andre Lieber (University of Washington, Seattle, WA). Site-directed mutagenesis employing oligonucleotide cassettes was used to construct each of the neutrophil elastase mutations and the M358R α,-antitrypsin negative control.

*Cell Lines and Cultivation—* The rat basophilic/mast cell leukemia line RBL-1 (20) was purchased from ATCC. Mouse 32D myeloblast-like cells (21) were the gift of Dr. Joel Greenberger (University of Pittsburgh Pittsburgh, PA), by way of Drs. Laurie Milner (Fred Hutchinson Cancer Research Center, Seattle, WA) and Thalia Papayannopoulos (University of Washington, Seattle, WA) for RBL-1 cells. 32D cells were grown in McCoy’s 5A medium with 15% FBS, with the addition of 1% essential amino acids, 0.5% nonessential amino acids, 1% sodium pyruvate, 1% glutamine, 15 mg/ml L-asparagine, 5 mg/ml L-serine, 0.4% minimal essential medium vitamins, 1% penicillin/streptomycin, and 0% WEHI-3-conditioned medium was grown by growing WEHI-3 cells, a gift of Dr. Papayannopoulos, in RPMI medium containing 2% FBS. 

*Transient Transfection—* 10⁶ or half that quantity of cells were seeded onto 10-cm cell culture dishes for enzymatic assays and immunoprecipitation or microscope coverslips in 6-cm cell culture dishes for immunofluorescent staining and apoptosis assays, respectively, then transiently transfected by LipofectAMINE Plus reagent (Life Technologies). After 24 h following transfection, cultured cells were washed in phosphate-buffered saline (PBS), and the cell pellet was resuspended in 150 μl of ice-cold lysis buffer containing 20 mM Tris-HCl, pH 8.0, 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol followed by a 10-s sonication. Cell lysates were cleared by centrifugation at 15,000 × g for 30 min at 4 °C, normalized for protein concentration, and incubated using 2 μg of rabbit polyclonal anti-neutrophil elastase antisera (Calbiochem) at 4 °C for 1 h on a rotating platform, followed by addition of 30 μl of protein A-Sepharose slurry (30 μM) and an additional 1-h incubation. Immunoprecipitates were recovered by brief centrifugation and then washed three times with 1 ml of ice-cold lysis buffer and resolved on 12.5% SDS-PAGE. Western blot conditions described above were then used for detection.

**Immunofluorescence Staining and Photomicroscopy—** Cells were fixed and stained as described previously (22, 24) following transfection using 1:500 of rabbit polyclonal anti-neutrophil elastase (Calbiochem) as a primary antibody and 1:200 dilution of rhodamine-conjugated secondary anti-rabbit serum (Jackson Immunoresearch Laboratories) and 1:1,500 dilution of peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia Biotech) using a prior protocol (23) was used to prepare cell extracts. 10⁶ transfected cells were washed in phosphate-buffered saline (PBS), and the cell pellet was re-suspended in 150 μl of 100 mM Tris-Cl, pH 8.5, 1 M NaCl, 500 mM MgCl₂, 0.1% Triton X-100 and mixed with 20 μl of substrate: 100 mM suc-Ala-Ala-Pro-Val-pNA (Bachem) (26), or 1 mM MeOsuc-Ala-Ala-Pro-Val-pNA (Bachem) (27), or 1 M of the selective inhibitor MeOscu-Ala-Ala-Pro-Val-chloromethyl ketone. Fluorescence intensity was measured following incubation of the reaction in the dark for 1 h at room temperature at excitation/emission of 485/530 nm.

**Western Blotting Analysis—** Western polyacrylamide gel electrophoresis (SDS-PAGE) with transfer to nitrocellulose membranes was used for separation of cell extracts. Primary antibodies were 1:1,000 murine monoclonal antibody 4G10 (Calbiochem) or 1:600 murine monoclonal antibody AHN-10 to α,-antitrypsin (Research Diagnostics) diluted with 10 ml Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, plus 5% nonfat dry milk. Secondary detection used a 1:10,000 dilution of peroxidase-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratory) or 1:1,500 dilution of peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia Biotech) using ECL (Amersham Pharmacia Biotech). The blot was stripped and reprobed with mouse monoclonal antibody 6C5 to glyceraldehyde-3-phosphate dehydrogenase (Biodiagnostics) to standardize for variations in protein loading.

**Coimmunoprecipitation—** Following transient cotransfection of either wild type or mutants of neutrophil elastase with α,-antitrypsin expression constructs, cells were lysed in 400 μl of ice-cold lysis buffer containing 20 μl Tris-HCl, pH 8.0, 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol followed by a 10-s sonication. Cell lysates were cleared by centrifugation at 15,000 × g for 30 min at 4 °C, normalized for protein concentration, and incubated using 2 μg of rabbit polyclonal anti-neutrophil elastase antisera (Calbiochem) at 4 °C overnight on a rotating platform, followed by addition of 20 μl of protein A-Sepharose slurry (30 μM) and an additional 1-h incubation. Immunoprecipitates were recovered by brief centrifugation and then washed three times with 1 ml of ice-cold lysis buffer and resolved on 12.5% SDS-PAGE. Western blot conditions described above were then used for detection.

**Apoptosis Assay—** RBL-1 cells were grown on poly-l-lysine-coated glass slides and then transiently transfected with wild type or mutant neutrophil elastase constructs. The ApoAlert DNA fragmentation kit (CLONTECH) was used with these modifications; cells were fixed in 4% paraformaldehyde in PBS at 4 °C for 25 min and permeabilized by incubation for 5 min in ice-cold 0.2% Triton X-100 in PBS. After incorporation of fluorescein-dUTP at the 3'-hydroxyl termini of the fragmented DNA from apoptotic cells, staining was performed using 1:500 diluted rabbit polyclonal antibody to human neutrophil elastase (Calbiochem) and 1:200 dilution of rhodamine-conjugated secondary antibody (Jackson Immunoresearch Laboratory) in PBS. The ApoAlert Annexin V apoptosis kit (CLONTECH) was used following manufacturer's directions.

**RESULTS**

*Mutations in SCN—* The ELA2 mutations responsible for SCN are depicted in Fig. 1. We list here an additional person with the G185R mutation, whom we have not previously reported. Among 22 unique patients we have identified 17 different mutations, predicted to result in 16 distinctive mutant neutrophil elastase polypeptides, comprising eight single-base amino acid missense substitutions (A28T, I31T, C42S, V72M, V161-F170), and a 5-base insertion. The intensity was measured following incubation of the reaction in the dark for 1 h at room temperature at excitation/emission of 485/530 nm.
Mutant Neutrophil Elastase in SCN

Expression of Mutant Neutrophil Elastase Enzymes in RBL-1 Cells—We have elected to express the mutant proteins in vitro, rather than purifying the enzymes from patient neutrophils, because the mutations occur heterozygously, in which case it would prove difficult to purify the mutant away from the wild type protein, and because many of the patients with this rare disease are no longer living or reside in geographically disperse locales. The rat basophilic/mast cell line RBL-1 does not demonstrate endogenous neutrophil elastase activity and has previously been used successfully to express human neutrophil elastase (23, 28). We engineered expression vectors for the wild type enzyme—

Neutrophil elastase is a highly processed enzyme that undergoes extensive subcellular trafficking and compartmentalization. We therefore also attempted to determine whether the mutant forms of the enzyme were intracellularly mislocalized by examining the transfected cells with confocal microscopy. Although it can be seen that the wild type enzyme translocates into the granules of RBL-1 cells (Fig. 1B), as has been reported previously (23, 28), light microscopic resolution proves inadequate to determine conclusively whether the mutants were similarly correctly localized (data not shown). We are addressing this problem in more extensive, pending experiments employing biochemical methods to subfractionate the cellular compartments.

**Test of the First Hypothesis (Haploinsufficiency): Neutrophil Elastase Mutations Do Not Uniformly Abrogate Proteolytic Activity**—The first hypothesis is that the effect of the neutrophil elastase mutations is to merely inactivate enzymatic activity,
Mutant Neutrophil Elastase in SCN

A. Anti-Neutrophil Elastase

WT
ΔV145-C152
A28T
ΔV161-F170
I31T
G181V
C42S
G185R
V72M
G192ter
Q93+PQ
G199ter
S97L
P205fs
P110L
S173A

B.

Fig. 2. Expression of wild type and mutant human neutrophil elastase in RBL-1 cells by immunofluorescent staining. A, low power conventional fluorescence photomicrographs. B, photomicrograph of higher power confocal microscope image of wild type (WT) neutrophil elastase expression. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (and false-color imaged in green); three cells are apparent, only one of which expresses neutrophil elastase, in a granular distribution.

thereby causing the SCN phenotype through haploinsufficiency. To test this possibility, we transfected the panel of mutant neutrophil elastase constructs into RBL-1 cells and assayed their activity in a post-nuclear supernatant on peptide substrates.

We first tested activity (Fig. 3A) on suc-Ala-Ala-Ala-pNA (25), as it is perhaps the most commonly employed experimental substrate. As an internal control, we cotransfected a lacZ expression vector, simultaneously assayed for β-galactosidase activity, and normalized neutrophil elastase activity to β-galactosidase activity. (Given relatively uniform β-galactosidase activity, normalization did not significantly change the specific activity of the mutants, however.) Most of the mutations result in reduced levels of proteolytic activity, but two mutations (G199ter and P205fs) retain near wild type levels of activity, while others (S97L and V72M) demonstrate about a third as much activity, and still others (C42S) have levels of activity no different from the S173A negative control. To confirm this result, we repeated the transfection and cell extract preparation, but tested the activity on a second, more-sensitive neutrophil elastase substrate, suc-Tyr-Leu-Val-pNA (29), that differs at all three amino acid positions in the peptide. The profile of activities (Fig. 3B) across the panel of mutations was similar to that observed on the first substrate, with the G199ter mutation demonstrating activity equivalent to that of the wild type enzyme.

Three residues in the active site of neutrophil elastase, His11, Asp88, Ser173, comprise a “charge-relay” system in which His11 and Asp88 transiently bind protons from Ser173 to facilitate nucleophilic attack on peptide bonds undergoing hydrolysis (12, 13). The first two substrates that we examined are tripeptides. Extended substrates more efficiently form specific interactions with the transition site and can more fully engage the catalytic machinery of the charge-relay system (12). In order therefore to determine whether the mutations have differing effects on the efficiency of proteolysis of a substrate interfacing with the active site of the enzyme in a somewhat different manner, we tested the activity of the panel of mutants on MeOsuc-Ala-Ala-Pro-Val-pNA (Fig. 3C). The results are largely similar to that seen with the prior two substrates, suggesting that, collectively, the effect of the mutations is not substrate-dependent.

Among proteases with elastase activity (i.e., neutrophil elastase, pancreatic elastase, and macrophage elastase), there is a variable relationship between enzymatic activity on model substrates and activity toward elastin. This is a result of the fact that elastin is largely insoluble; neutrophil elastase inefficiently forms the productive complexes required for surface proteolysis (30). In order therefore to further determine whether the effect of the SCN mutations is substrate-dependent, we tested activity on the substrate DQ-elastin. Because DQ-elastin can also be digested by proteases other than neutrophil elastase, a selective inhibitor of elastase, MeOsuc-Ala-Ala-Pro-Val-chloromethyl ketone (31), was used to confirm the identity of the enzyme responsible for hydrolysis of this substrate. Once again, the panel of mutations tested similarly on this substrate (Fig. 3D), and the activity was selectively inactivated by MeOsuc-Ala-Ala-Pro-Val-chloromethyl ketone. The mutations causing SCN do not appear to have differing effects on the ability of neutrophil elastase to form productive catalytic complexes on elastin.

To assure that there were neither significant differences in transfection efficiency or differences in the stability of the mutant enzymes, we used Western blots, rather than cotransfection with a lacZ standard, as an independent internal control, for the experiments in which the elastin substrate was tested. Western blots (Fig. 3E) reveal no gross differences in the levels of expression across the panel of neutrophil elastase mutations and are themselves internally controlled for total protein concentration by reprobing with anti-glyceraldehyde-3-phosphate dehydrogenase, endogenously expressed by RBL-1 cells.

Neutrophil elastase expressed in RBL-1 cells is biochemically indistinguishable from the enzyme purified from neutrophils (23, 28). Nevertheless, to control for the possibility that the activity in the panel of mutants arises artifically as a result of unique properties of RBL-1 cells, we assayed activity in promyelocytes. For this purpose we used the murine myeloblast-like cell line 32D, which has previously been employed for the in vitro expression of the homologous neutrophil granule serine protease, proteinase 3 (31). Activity in 32D cells toward the substrate suc-Ala-Ala-Ala-pNA (Fig. 3F) is generally indistinguishable from that observed in RBL-1 cells, and we con-
FIG. 3. **Activity of neutrophil elastase mutations causing SCN.** Cell extracts from the indicated transiently transfected cells were prepared and assayed against the indicated peptide substrate. β-Galactosidase was cotransfected as an internal control of transfection efficiency, and neutrophil elastase activity was normalized to β-galactosidase activity. The **black bars** represent neutrophil elastase activity (**left vertical axis**), and the **diagonally striped bars** represent β-galactosidase activity (**right vertical axis**). Each panel represents the mean of three different experiments. **Error bars** indicate the standard error of the mean. **WT**, wild type construct; **S173A**, engineered negative control. A, neutrophil elastase activity assay using substrate suc-Ala-Ala-Ala-pNA in RBL-1 cells. B, neutrophil elastase activity assay using substrate suc-Tyr-Leu-Val-pNA in RBL-1 cells. C, neutrophil elastase activity assay using substrate MeOsuc-Ala-Ala-Pro-Val-pNA in RBL-1 cells. D, neutrophil elastase activity assay using fluorescence-conjugated substrate DQ-elastin in RBL-1 cells (**black bars**) and in presence of inhibitor MeOsuc-Ala-Ala-Pro-Val-chloromethyl ketone (**spotted bars**). E, Western blot detection of transiently transfected neutrophil elastase in RBL-1 cells using a polyclonal antibody against neutrophil elastase. The blots were reprobed with monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal loading of sample. F, neutrophil elastase activity assay using substrate suc-Ala-Ala-Ala-pNA in 32D cells.
clude that the differences in proteolytic activity in the panel of mutant enzymes is unlikely to result from cell-specific factors.

In summary, no consistent effect of the mutations was observed when the collection of mutants was assayed on four different substrates, using two different cell types for expression, and when controlling for levels of expression by three different standards. The SCN phenotype is therefore unlikely to result from haploinsufficient reductions in proteolytic activity toward native substrates.

Test of Second Hypothesis (Gain of Function Activity): Mutant Neutrophil Elastase Is Not Intracellularly Toxic and Retains Sensitivity to Inhibition by and Interaction with α1-Antitrypsin—An alternate genetic explanation for the dominant inheritance of SCN is that the mutant neutrophil elastase enzymes acquire a new activity. One possibility is a so-called “toxic gain of function,” in which the enzymes prove toxic to cells expressing them. In particular, it has been reported that chymotrypsin and other proteases can induce apoptosis (32) and that “accelerated apoptosis” in the bone marrow might be one mechanism for SCN (33). Cytotoxicity is somewhat unlikely because, by transient transfection (Fig. 2) or stable transfection (data not shown), the mutant enzymes were expressed as efficiently as the wild type enzyme and a β-galactosidase control. Nevertheless to determine whether the mutant enzymes induced apoptosis, we subjected transfected RBL-1 cells to the terminal dUTP nick-end labeling assay (34) for apoptosis, in which fragmented DNA is labeled at the 3'-hydroxyl termini by deoxynucleotidyl transferase mediated fluorescein-conjugated dUTP incorporation. As shown in Fig. 4, transfected cells were detected by indirect immunofluorescent staining of neu-
Mutant Neutrophil Elastase in SCN
trophil elastase with rhodamine (red) and apoptotic cells identified by fluorescein (green) stain. There is a low background level of apoptosis, but the merged images (yellow) indicate that neither the wild type nor the indicated mutant enzymes induce significant apoptosis; these data are representative of that obtained with the other mutants (data not shown). Similar results (data not shown) were observed by using another marker of apoptosis, annexin V (35). At least under the simple cell culture conditions employed in this assay, then, there is no evidence for a toxic gain of function activity in the mutants, which would lead to an increased rate of cell death.

A second potential novel activity that the mutants could acquire is invulnerability to inhibition. To determine the likelihood of this hypothesis, we assayed the mutant enzymes for their ability to be inhibited by the serpin α1-antitrypsin. Under normal conditions, α1-antitrypsin affords protection to tissues, particularly the lung in pulmonary emphysema, by inhibiting the neutrophil elastase discharged from neutrophils participating in the inflammatory response. Among the mutant enzymes with residual proteolytic activity, the ability to be inhibited by α1-antitrypsin in a concentration-dependent manner remains similar to that observed for the wild type (Fig. 5A). As expected, the inactive enzymes remain inactive following the addition of α1-antitrypsin.

As another approach to determining whether there are disrupted interactions between the mutant forms of neutrophil elastase and α1-antitrypsin, we performed immunoprecipitation experiments in which both molecules were coexpressed in RBL-1 cells. As shown in Fig. 5B, coimmunoprecipitation with anti-neutrophil elastase antiserum brings down a complex detectable upon immunoblotting with anti-α1-antitrypsin antibody. Interestingly, the strongest interaction is evident for the wild type enzyme and the COOH-terminal mutants G199ter and P205fs, and the strength of the interaction generally parallels the residual activity that is observed for each of the mutant forms of neutrophil elastase. Confirmatory results were obtained both in 32D cells (data not shown) and through the use of a reverse set of antibodies (immunoprecipitation with anti-α1-antitrypsin and Western blot with anti-neutrophil elastase; data not shown). As a negative control, we engineered α1-antitrypsin “Pittsburgh” (M358R) (36), a naturally occurring α1-antitrypsin allele causing thrombosis, in which a methionine residue critical for correct interaction with neutrophil elastase is substituted; nevertheless, some interaction with wild type α1-antitrypsin is still apparent. (Purified α1-antitrypsin Pittsburgh, however, did not inhibit the mutants of neutrophil elastase causing SCN (data not shown).) A potential explanation for this observation is that the mutations induce a conformational change in neutrophil elastase that affects both catalytic activity and the ability to interact with serpins.

Test of Third Hypothesis (Dominant Negative): Co-expression of Mutant Neutrophil Elastase Inhibits Wild Type Activity—A third potential genetic explanation for the dominant inheritance of SCN is that the mutant enzyme is capable of dominantly negatively inhibiting the activity of the normal, wild type allele. To test this possibility, we performed two experiments.

First, we mixed the mutant enzyme with the wild type enzyme (both derived from RBL-1 cell extracts following transient transfection). The combined extracts were coincubated at 37 °C for 30 min and then assayed for activity on the substrate suc-Tyr-Leu-Val-pNA (Fig. 6A). The total activity appears to represent the sum of the activities of the two enzymes in isolation. For example, addition of extract from cells transfected with the inactive negative control S173A was largely indistinguishable from the addition of an extract obtained from cells transfected only with the empty expression vector. In contrast, combining preparations of mutant enzymes with near wild type levels of activity (G199ter or P205fs) led to a near doubling of activity. We conclude that, when mutant and wild type enzyme are added together, there is no inhibition of the wild type activity by the mutant.

Given that individuals with SCN resulting from mutations in the gene encoding neutrophil elastase are heterozygous, then the mutant and wild type enzyme will be coexpressed within the cell. There is thus a possibility of the two forms of protein aberrantly interacting within the context of the compartments.
in which subcellular trafficking and post-translation modification takes place. A well known example of such a situation involves liver toxicity and cirrhosis resulting from polymerization in the endoplasmic reticulum of the Z form of mutant α₁-antitrypsin in the human disorder involving deficiency of this protein (37). To determine whether the mutant enzyme could intracellularly inhibit the wild type activity, we cotransfected the wild type expression vector along with each of the mutants, such that each transfected cell would have an opportunity to simultaneously express both mutant and wild type protein. We again assayed activity on the substrate suc-Tyr-Leu-Val-pNA (Fig. 6B). In contrast to the results observed in Fig. 6A, coexpression of the mutant enzyme inhibits the activity of the wild type enzyme in a concentration-dependent manner, when compared with cotransfection with an empty expression vector (or a frameshifted wild type construct; data not shown). The degree of inhibition roughly parallels the proteolytic activity of the mutant. Generally, the mutants with the least activity are the most inhibitory in this assay, whereas the mutants with the greatest activity are the least inhibitory. The total activity most closely matches that of expression of the mutant enzyme alone. When both mutant and wild type are coexpressed within the same cell, there is thus evidence for dominant negative inhibition.

Normal Processing of the Amino Terminus of Neutrophil Elastase Is Required for Activity—Neutrophil elastase undergoes extensive post-translational processing in which it is synthesized as an inactive zymogen and fully activated by removal of the amino and COOH-terminal extensions (38). It is possible that the mutations could interrupt trafficking, processing, or folding events governed by either terminus. For example, the mutations could interfere with a protein-protein interaction mediated by either terminus and thereby prevent it from undergoing subsequent post-translational modifications, intracellular routing, or appropriately folding. If this situation were to hold, then it is possible that deletion of either terminus, to shortcut potentially aberrant processing events occurring in the mutants, could intragenically complement the mutation. To test for this possibility, we endeavored to genetically delete the amino and COOH-terminal extensions.

We first sought to delete the amino terminus from the wild type enzyme. Normal processing of the amino terminus of neutrophil elastase involves two steps (12, 38). First, the signal peptidase cleaves the signal peptide portion. Second, DPPI (also known as cathepsin C) cleaves the remaining two amino acid leader sequence, resulting in a mature protein with an isoleucine residue at the amino terminus. We prepared a construct in which the entire amino terminus was deleted, but note that translation initiation in the resulting protein necessarily must begin with a methionine and therefore differs from the mature protein resulting from normal processing. Expression of this construct resulted in no neutrophil elastase activity (data not shown), either because the addition of the amino-terminal methionine inactivates proteolytic function or because at least one of the two steps of amino-terminal processing is necessary for appropriate post-translational modification, folding, or subcellular localization of the enzyme. We then tested a construct in which we allowed the two peptide target for DPPI to remain in place (again with an amino-terminal methionine), but, again, this construct was not active (data not shown). Next, we inserted variable lengths of arbitrary protein-encoding DNA sequence into the above construct to distance the NH2-terminal methionine from the DPPI recognition sequence, in case it was interfering with the second processing step, but this also did not yield activity (data not shown). Finally, we coexpressed DPPI along with these neutrophil elastase con-
structs, but again failed to demonstrate activity (data not shown). We conclude that either the necessary presence of an amino-terminal methionine in all engineered forms of the enzyme lacking an amino terminus disables proteolytic activity or disrupts the second processing step by DPPI or, else, normal processing is required for correct post-translational modification, folding, or subcellular trafficking of neutrophil elastase. In any event, it is not possible to experimentally test for intragenic complementation of the SCN mutations through deletion of the amino terminus.

The Mutations Do Not Exert Their Effect through Processing of Folding Mediated by the Carboxyl Terminus—Proceeding similarly to the above, we sought to determine whether deletion of the portion of the gene encoding the COOH-terminal prodomain-deleted wild type neutrophil elastase was cotransfected with each of the constructs encoding the mutant neutrophil elastase, then the cell extracts were assayed on suc-Ala-Ala-Ala-pNA. The bars represent cotransfection of the full-length wild type construct (1.8 µg) with cotransfection with 0.9 µg (diagonally striped bars) or 1.8 µg (black bars) each of the mutant constructs. C, the experiments were similar to those in panel B except constructs (1.8 µg) encoding the COOH-terminal prodomain-deleted wild type neutrophil elastase were used instead of the full-length construct.
granules of RBL-1 cells (data not shown), in accord with prior reports that this region of the molecule is not necessary for enzymatic activity and granule localization (23).

We next tested whether deletion of the carboxyl terminus from the SCN mutations would restore wild type characteristics in the previously conducted assays of proteolytic activity, α1-antitrypsin inhibition and coimmunoprecipitation, and dominant negative inhibition. Deletion of the COOH-terminal extension had no effect on any of the biochemical properties of each of the tested mutant enzymes; the COOH-terminal deleted constructs (with "Δ C" appended to their names) have nearly equal levels of proteolytic activity in comparison to the respective intact sequences (Fig. 3, A–C). The COOH-terminal deletion constructs could be similarly inhibited by α1-antitrypsin (Fig. 5A) and demonstrated equal avidity for α1-antitrypsin by immunoprecipitation (Fig. 5B). Deletion forms of the COOH-terminal extension behaved similarly to constructs containing this region (Fig. 6C). It appears that the mutations responsible for SCN do not influence functions mediated by the COOH-terminal extension of neutrophil elastase, such as subcellular localization, post-translational modification, or protein folding. Indeed, given the lack of necessity of this tail for appropriate in vitro expression and the fact that it is poorly conserved both across species and between other members of the chymotryptic family (39), its function remains obscure.

**DISCUSSION**

Some of the biochemical consequences of the mutations occurring in SCN can be inferred from the distribution of the mutations with respect to the known crystal structure (Fig. 7) of neutrophil elastase and by sequence alignments with other serine proteases. First, the mutations are unlikely to have too disruptive of an effect upon protein structure; note that all five of the mutations causing premature protein truncation are clustered near the carboxyl terminus. This distribution suggests that the mutations cause the SCN phenotype only when a substantial portion of the amino terminus of neutrophil elastase, including the catalytic serine and the charge relay triad, is intact. Presumably, truncating mutations occurring in closer proximity to the amino terminus would not be capable of causing SCN and are therefore not observed. Second, in contrast to the mutations responsible for cyclic neutropenia (10, 11), the mutations causing SCN are generally spatially distant from the active site in the structure of the enzyme, and therefore might not be expected to have significant effects upon catalytic activity. Third, based on protein sequence homology (data not shown), the mutated amino acids tend to represent conserved, signature residues defining the chymotryptic-family of serine proteases. Collectively, these observations suggest that the mutations causing SCN might, in common, alter a core function of this family of serine proteases. We consequently sought to define biochemical abnormalities common to all forms of mutant neutrophil elastase. The first two predictions were largely borne out, as we found no consistent effects upon catalysis or vulnerability to proteolytic inhibition. The possibility of potentially disruptive effects of the mutants upon post-translational processing and subcellular trafficking is suggested by evidence of a dominant negative effect.

Haploinsufficient reductions of proteolytic activity largely can be excluded, based on the fact that not all of the mutations causing SCN reduce neutrophil elastase activity. A potential objection to the interpretation of the data is that, because of the broad range of proteolytic activity of neutrophil elastase, the
appropriate substrate was not examined. However, the panel of 16 mutant and control forms of neutrophil elastase individually demonstrated consistently similar relative activities on a variety of representative substrates, including those in which nucleophilic attack through the charge relay system proceeds via a single proton (suc-Ala-Ala- Ala-pNA and suc-Tyr-Leu-Val-pNA), two protons (MeOsuc-Ala-Ala-Prof-Val-pNA), or in which surface proteolysis occurs (elastin). Another potential objection to the interpretation of these experiments is that because activities were assayed in crude cell extracts, potential inhibitory effects of associating proteins cannot be excluded. In preliminary, unpublished experiments, however, we have detected the activities of the mutant enzymes by zymography conducted under SDS-PAGE. The mutant enzymes migrate according to their expected molecular weight while demonstrating reduced rates of catalysis toward elastin and casein; in isolation of other potential proteins, such as in a complex with a serpin, the mutant enzymes still seem to have diminished proteolytic activity. Exclusion of haploinsufficiency is compatible with reports that heterozygous or homozygous deficiency of neutrophil elastase in gene-targeted mice do not display neutropenia, but rather have a vulnerability to sepsis following exposure to fungi and enteroinvasive bacteria (40–42).

We similarly did not find much support for a gain of function activity arising from the SCN mutations. We specifically tested two potential novel functions. First, it has been proposed that the promyelocytic arrest characteristic of SCN might be attributable to an enhanced rate of cell death (33). Although our simple cell culture model does not fully reproduce events in the bone marrow, expression of wild type or mutant forms of neutrophil elastase appeared to have no toxic effects on the cells and specifically did not induce DNA fragmentation characteristic of apoptotic induction nor externalization of phosphatidylserine to the plasma membrane as revealed by immunoreactivity for annexin V (data not shown). As alternative explanations, it may be that the mechanism for SCN involves either deficient rates of production of mature neutrophils from myeloid precursors or possibly defects in the mobilization of myeloid cells from the bone marrow. Second, we considered the possibility that the mutations result in an aberrant interaction with α1-antitrypsin or a related serpin. α1-Antitrypsin is still capable of inhibiting the mutant forms of neutrophil elastase, and stable complexes between the mutant forms of neutrophil elastase and α1-antitrypsin were immunoprecipitated from cells coexpressing both proteins. Nevertheless, there is reduced formation of stable complexes with the mutants to a degree that relates directly to loss of proteolytic activity. The most likely explanation, given that serpin inhibition is an irreversible process thought to require cleavage of the serpin by the protease, is that the impairment of stable complex formation merely parallels the inconsistent effects of the mutations upon catalytic activity. One alternative possibility, given the number of serpin family members, is that we have not yet tested interactions between the mutants and the appropriate serpin. Indeed, in preliminary experiments, we have found that in a variety of myeloid cells neutrophil elastase interacts both with α1-antitrypsin and another protein whose properties are most compatible for those previously reported for the serpin ELANH2 (43).

A third potential novel gain of function activity not addressed in this report is that of a change of proteolytic specificity. Given the distribution of the SCN mutations away from the catalytic site and with a tendency toward involving conserved residues of the chymotrypsin family, we think this to be a less likely possibility. Unfortunately, we have not been able to adequately address this hypothesis using the in vitro expression system described in this report, as there are other, nonelastase serine protease activities in RBL-1 and 32D cells that copurify in the cell extracts employed here. We are presently attempting to express and more cleanly purify the mutant enzymes in using the yeast species Pichia (44).

Somewhat to our surprise, there is the greatest support for the third of the hypotheses, that of dominant negative inhibition of the wild type enzyme by the mutant. Interestingly, the mutant enzyme appeared to have no effect when simply added to wild type preparations; the total activity was the sum of the two components. However, when the two were cotransfected such that both the mutant and the wild type are expressed within the same cell, then the total activity appears inhibited by the mutant. It is possible that the mutant and the wild type enzyme form misprocessed protein aggregates, in analogy to retention in the endoplasmic reticulum of Z forms of α1-antitrypsin causing cirrhosis (37) or that the two proteins compete for limiting concentrations of associating cofactors. In support of the latter possibility, it may be of relevance that SCN is a genetically heterogeneous disease, i.e., not all cases of this disorder can be attributed to mutations in the gene encoding neutrophil elastase (11). It is possible that the individuals whose disease is unaccounted for by ELA2 mutations could have defects in proteins interacting aberrantly with the mutant forms of neutrophil elastase.

To discriminate among potential pathogenic mechanisms leading to dominant negative inhibition, we sought to determine whether the mutations causing SCN could interrupt the activation of thezymogen to the mature form of neutrophil elastase in which both amino and COOH-terminal extensions have been removed. Specifically, we attempted to find out whether the mutant enzymes would demonstrate normal function if activation to the zymogen state were artificially abridged by deleting either terminal extension from the cDNA in the expression vector. A variety of constructs were engineered in which either the complete amino terminus or just the portion containing the signal peptide was removed from the wild type sequence, but in all cases expression resulted in a failure of proteolytic activity. Coexpression of the amino-terminal processing enzyme DPPI had no effect. Presumably the mandatory presence of an amino-terminal methionine residue in all such constructs interferes with proteolytic activity, interferes with DPPI-mediated cleavage, or else stepwise processing of the amino-terminal extension is required for appropriate intracellular post-translational modification, subcellular trafficking or folding. In any event, genetic deletion of the amino terminus is not an experimentally tractable approach. Deletion of the COOH-terminal extension produced a more clear-cut result. The wild type enzyme demonstrates full proteolytic activity and correct subcellular localization when expressed from a cDNA in which this domain has been deleted. Removal of this domain from each of the mutants failed to complement the observed defect in proteolysis, inhibition by or avidity of association with α1-antitrypsin, or dominant negative interference of wild type activity. The mutants are thus unlikely to have effects upon functions of the protein that are mediated by the COOH-terminal domain.

Any attempt to biochemically characterize the consequences of mutations in neutrophil elastase must ultimately be reconciled with the organismal biology of the phenotype. Key questions remain unanswered. Why are most patients responsive to treatment with GCSF? What distinguishing feature of the different ELA2 mutations accounts for hematopoietic oscillations in the allelic disorder cyclic neutropenia? How does bone marrow failure in this disorder ultimately lead to leukemic transformation? Continued molecular genetic and biochemical dissection of the mutant enzymes, in concert with the
development of transgenic and gene-targeted mouse models, under way in our laboratory, are likely to provide the best opportunity of answering these questions and defining normal hemopoietic mechanisms governing steady state levels of production of granulocytes.

REFERENCES

1. Kostmann, R. (1975) Acta Paediatr. Scand. 64, 362–368
2. Freedman, M. H., Bonilla, M. A., Fier, C., Bolyard, A. A., Scarlata, D., Boxer, L. A., Brown, S., Cham, L., Kannourakis, G., Kinsey, S. E., Mori, P. G., Cottle, T., Welte, K., and Dale, D. C. (2000) Blood 96, 429–436
3. Dale, D. C., Guerry, D. T., Wewerka, J. R., Bull, J. M., and Chusid, M. J. (1979) Medicine 58, 128–144
4. Dong, F., Brynes, R. K., Tidow, N., Welte, K., Lowenberg, B., and Touw, I. P. (1994) Blood 83, 847–848
5. Nomura, T., Ito, M., Noda, M., and Tateishi, Y. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2831–2835
6. Li, F. Q., Conrod, A., and Horwitz, M. (2000) Mol. Cell. Biol. 20, 5129–5139
7. Gullberg, U., Lindmark, A., Lindgren, G., Persson, A. M., Nilsson, E., and Olsson, I. (1994) J. Biol. Chem. 269, 25219–25225
8. Kagan, H. M., Crombie, G. D., Jordan, R. E., Lewis, W., and Franzblau, C. (1972) Biochemistry 11, 3412–3418
9. Gullberg, U., Lindmark, A., Hillmark, T., Gladh, M., Jogi, J., and Gullberg, U. (1997) J. Leukocyte Biol. 61, 113–123
10. Tidow, N., Pilz, C., Teichmann, B., Muller-Brechlin, A., Germeshausen, M., Kasper, B., Rauprich, P., Sykora, K. W., and Welte, K. (1997) Blood 89, 2369–2375
11. Tidow, N., Pilz, C., Kasper, B., and Welte, K. (1997) Stem Cells 15, 113–119
12. Nakajima, K., Powers, J. C., Biasi, G. (1991) J. Immunol. Methods 136, 125–131
13. Raynal, P., and Pollard, H. B. (1994) Biochim. Biophys. Acta 1197, 65–93
14. Stratigos, E., and Gettins, P. G. (1996) J. Biol. Chem. 271, 15582–15589
15. Bieth, J. G. (1998) in Methods Enzymol. 299, 28–32
16. Aprikyan, A. A. G., Liles, C. W., Person, R. E., Rodger, E., and Dale, D. C. (1999) J. Immunol. 161, 141–149
17. Aprikyan, A. A. G., Person, R. E., Bolyard, A. A., Scarlata, D., Boxer, L. A., Brown, S., Cham, L., Kannourakis, G., Kinsey, S. E., Mori, P. G., Cottle, T., Welte, K., and Dale, D. C. (2000) Blood 96, 429–436
18. Dale, D. C., Guerry, D. T., Wewerka, J. R., Bull, J. M., and Chusid, M. J. (1979) Medicine 58, 128–144
19. Dong, F., Brynes, R. K., Tidow, N., Welte, K., Lowenberg, B., and Touw, I. P. (1994) Blood 83, 847–848
20. Gullberg, U., Lindmark, A., Hillmark, T., Gladh, M., Jogi, J., and Gullberg, U. (1997) J. Leukocyte Biol. 61, 113–123
21. Tidow, N., Pilz, C., Kasper, B., and Welte, K. (1997) Stem Cells 15, 113–119
22. Nakajima, K., Powers, J. C., Biasi, G. (1991) J. Immunol. Methods 136, 125–131
23. Raynal, P., and Pollard, H. B. (1994) Biochim. Biophys. Acta 1197, 65–93
24. Stratigos, E., and Gettins, P. G. (1996) J. Biol. Chem. 271, 15582–15589
25. Bieth, J. G. (1998) in Methods Enzymol. 299, 28–32
26. Nakajima, K., Powers, J. C., Biasi, G. (1991) J. Immunol. Methods 136, 125–131
27. Raynal, P., and Pollard, H. B. (1994) Biochim. Biophys. Acta 1197, 65–93
28. Stratigos, E., and Gettins, P. G. (1996) J. Biol. Chem. 271, 15582–15589
29. Perlmutter, D. H. (1999) Lab. Invest. 79, 623–638
30. Dall’Acqua, W., Halin, C., Rodriguez, M. L., and Carter, P. (1999) Protein Eng. 12, 981–987
31. Takahashi, H., Nukiwa, T., Yoshimura, K., Quick, C. D., States, D. J., Holmes, M. D., Whang-Peng, J., Krutzen, T., and Crystal, R. G. (1988) J. Biol. Chem. 263, 14729–14747
32. Thorell, L. A., Brown, S., Cham, B., Kannourakis, G., Kinsey, S. E., Mori, P. G., Cottle, T., Welte, K., and Dale, D. C. (2000) Blood 96, 429–436
33. Bieth, J. G. (1998) in Methods Enzymol. 299, 28–32
34. Nakajima, K., Powers, J. C., Biasi, G. (1991) J. Immunol. Methods 136, 125–131
35. Raynal, P., and Pollard, H. B. (1994) Biochim. Biophys. Acta 1197, 65–93
36. Stratigos, E., and Gettins, P. G. (1996) J. Biol. Chem. 271, 15582–15589
37. Perlmutter, D. H. (1999) Lab. Invest. 79, 623–638
38. Dall’Acqua, W., Halin, C., Rodriguez, M. L., and Carter, P. (1999) Protein Eng. 12, 981–987
39. Takahashi, H., Nukiwa, T., Yoshimura, K., Quick, C. D., States, D. J., Holmes, M. D., Whang-Peng, J., Krutzen, T., and Crystal, R. G. (1988) J. Biol. Chem. 263, 14729–14747
40. Thorell, L. A., Brown, S., Cham, B., Kannourakis, G., Kinsey, S. E., Mori, P. G., Cottle, T., Welte, K., and Dale, D. C. (2000) Blood 96, 429–436
41. Nakajima, K., Powers, J. C., Biasi, G. (1991) J. Immunol. Methods 136, 125–131
42. Raynal, P., and Pollard, H. B. (1994) Biochim. Biophys. Acta 1197, 65–93
43. Stratigos, E., and Gettins, P. G. (1996) J. Biol. Chem. 271, 15582–15589
44. Perlmutter, D. H. (1999) Lab. Invest. 79, 623–638
45. Dall’Acqua, W., Halin, C., Rodriguez, M. L., and Carter, P. (1999) Protein Eng. 12, 981–987
46. Takahashi, H., Nukiwa, T., Yoshimura, K., Quick, C. D., States, D. J., Holmes, M. D., Whang-Peng, J., Krutzen, T., and Crystal, R. G. (1988) J. Biol. Chem. 263, 14729–14747
47. Thorell, L. A., Brown, S., Cham, B., Kannourakis, G., Kinsey, S. E., Mori, P. G., Cottle, T., Welte, K., and Dale, D. C. (2000) Blood 96, 429–436
48. Dall’Acqua, W., Halin, C., Rodriguez, M. L., and Carter, P. (1999) Protein Eng. 12, 981–987