Cloning of cDNA for Proteinase 3: A Serine Protease, Antibiotic, and Autoantigen from Human Neutrophils

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Summary

Closely similar but nonidentical NH₂-terminal amino acid sequences have been reported for a protein or proteins in human neutrophils whose bioactivities is/are diverse (as a serine protease, antibiotic, and Wegener's granulomatosis autoantigen) but that share(s) several features: localization in the azurophil granules, a molecular mass of ~29 kD, reactivity with diisopropylfluorophosphate, and the ability to degrade elastin. We previously purified one such entity, termed p29b. Using a monospecific antibody, we have cloned from human bone marrow a cDNA encoding the complete p29b protein in its mature form, along with pre- and pro-sequences. The predicted amino acid sequence agrees closely with the NH₂-terminus sequence obtained previously from purified p29b, as well as with sequences newly obtained from CNBr fragments. The primary structure is highly homologous to elastase, cathepsin G, T cell granzymes, and other serine proteases, and shares both the catalytic triad and substrate binding pocket of elastase. Hybridization of the full-length cDNA with restriction enzyme digests of human genomic DNA revealed only one fragment. This suggests that the closely related species described previously are the same, and can be subsumed by the term used for the first-described activity, proteinase 3. Proteinase 3 is more abundant in neutrophils than elastase and has a similar proteolytic profile and specific activity. Thus, proteinase 3 may share the role previously attributed to neutrophil elastase in tissue damage, and has the potential to function as an antimicrobial agent.

The azurophil granules of human neutrophils (PMN) contain two ~29-kD proteins, cathepsin G and elastase, that are active both as neutral serine proteases and as antibiotics (1–4). These molecules are thought to help PMN form abscesses and damage other inflamed tissues (5), and may participate in killing microorganisms. Recently, we purified another azurophil granule protein of ~29 kD, termed p29b, whose abundance is intermediate between that of cathepsin G and elastase, and whose NH₂-terminal amino acid sequence is highly homologous to theirs (3, 4). Like the latter proteins, p29b is bifunctional, displaying broad-spectrum antimicrobial as well as elastase-like enzymatic activity (3, 4). The proteolytic profile of p29b resembled that of a diisopropylfluorophosphate (DFP)-inhibitable protease of ~29 kD purified by Kao et al. (6) from azurophil granules and shown to cause emphysema after intratracheal instillation in hamsters. The latter enzyme was termed proteinase 3 (PR-3), because it shared the reported properties of an organic ester hydrolyase that Baggiolini et al. (7) had so designated after separating it from cathepsin G and elastase on nondenaturing SDS-PAGE. The NH₂-terminal sequence of PR-3 (8) matched that of p29b in only 9 of 14 residues, leaving their relationship unclear.

Interest in p29b and/or PR-3 was heightened by the discovery that autoantibodies characteristic of Wegener's granulomatosis activate PMN (9), stain PMN granules, and can be used to affinity purify a 29-kD, DFP-binding protease, termed p29, ACPA, or C-ANCA (10–13), whose NH₂-terminal sequence, as first reported (11, 12), was related but not identical to those of p29b (3) or PR-3 (8). Anti-PR-3 mAbs could block the staining of PMN by sera from patients with Wegener's granulomatosis (13). Meanwhile, a cDNA cloned from differentiating HL-60 promyelocytic leukemia cells was predicted to encode a serine protease, termed myeloblastin (14), whose six NH₂-terminal residues matched residues 15–20 in p29b. Addition of a myeloblastin antisense oligode-
oxy nucleotide construct to HL-60 cells induced their myeloid differentiation (14). Very recently, a preliminary report by Jenne et al. (15) has revised the NH2-terminal sequence for the Wegener's autoantigen (12), such that it now matches all 20 NH2-terminal residues reported for p29b (3), except for Gln19 in place of Glu19. Based on PCR analysis, Jenne et al. (15) also proposed a revision of the 5' region of the myeloblastin cDNA, according to which the deduced NH2-terminal 20 residues of myeloblastin would likewise match those of the Wegener's autoantigen and p29b.

We have used a monospecific anti-p29b IgG to clone a cDNA that encodes the entire mature protein. Below, we provide the sequence of this clone, compare its deduced amino acid sequence with the observed sequence of proteolytic fragments of p29b, note the extensive homology of this molecule to cathepsin G, elastase, and the granymes of T cells, and demonstrate that only one human genomic DNA fragment appears to hybridize with the p29b cDNA. Thus, it is likely that the cDNA presented here encodes a single PMN azurophil granule protein that has the potential for elastolysis, microbial killing, and the regulation of myeloid differentiation, and serves as an autoantigen.

Materials and Methods

Amino Acid Sequence of CNBr Fragments. 50 μg of purified p29b (3, 4) was incubated with a 500-fold excess (with respect to methionine content) of CNBr in 0.1 N HCl for 48 h at room temperature in the dark. The samples were subjected to SDS-PAGE. Resolved peptides were transferred to polyvinylidene difluoride membranes (Millipore Continental Water Systems, Bedford, MA) (16) and sequenced directly by automated Edman degradation on a gas-phase sequenator (470; Applied Biosystems, Inc., Foster City, CA).

cDNA Cloning. A λgt11 bone human marrow cDNA library (Clontech, Palo Alto, CA) was plated on Escherichia coli Y1090 (17). Nitrocellulose filter lifts saturated with isopropyl-β-D-thiogalactoside (IPTG; Sigma Chemical Co., St. Louis, MO) were dried, blocked with 5% nonfat dry milk and 0.05% Tween 20 in PBS, rinsed, probed with a 1:500 dilution of monospecific rabbit anti-p29b IgG (4) that had been preabsorbed with E. coli Y1090, rinsed, and treated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Plaques positive by a colorimetric phosphatase reaction were purified to homogeneity, as monitored by rescreening with the antibodies. Plate cultures were lysed in chloroform, clarified by centrifugation, and rinsed with bacterial DNA with DNAase (Pharmacia Fine Chemicals, Piscataway, NJ). Phage were collected by ultracentrifugation (89,500 g, 2 h, 4°C), purified on spin columns of DEAE cellulose (3-Prime 3-Prime, Westchester, PA) (18), and the DNA was isolated according to the manufacturer's instructions. The cDNA insert was recovered by restriction with EcoR1 (Boehringer Mannheim Biochemicals) and ephrinesorhesis in low melt/low gelling agarose (Bio-Rad Laboratories, Richmond, CA) as visualized with long-wavelength UV light. The appropriate gel slice was melted and extracted with phenol and ether, and the DNA was precipitated with ethanol. pBluescript II SK+ (Stratagene, La Jolla, CA) was digested with EcoR1 and dephosphorylated ( calf intestinal alkaline phosphatase; Boehringer Mannheim Biochemicals) for insertion of the p29b cDNA with T4 DNA ligase (Stratagene). The resultant plasmid was used to transform competent E. coli XL1 Blue (Stratagene) (19). Recombinants were screened by blue/white color (β-galactosidase) selection on luria broth agar with ampicillin, 20 mM IPTG, and 80 μg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside. After transformation of DH5 cells (20), the recombinant plasmids were amplified and purified (21). cDNA was sequenced by the dideoxy nucleotide chain termination method using double-stranded plasmid DNA as a template (22) with a kit from U.S. Biochemical Corp. (Cleveland, OH) and deoxycytidinose 5'-(3P)Triphosphate (New England Nuclear, Boston, MA). After initial use of universal primers, sequencing was continued independently on both strands with sequentially constructed 20-25-mer oligonucleotides so as to obtain sequences in regions overlapping by 20-40 bp. Results were analyzed by DN.ASIS and PROSIS software (Hitachi America, Ltd., San Bruno, CA). Alignment to other proteases was scored according to Lipman and Pearson (23) with the program FASTP. Structural predictions were obtained as described (24).

Southern Hybridization. Human placental genomic DNA (10 μg; Clontech) was digested to completion with EcoRI, HindIII, BamHI, PstI, or BglII (Boehringer Mannheim Biochemicals). The digests were transferred onto a nylon membrane (Schleicher & Schuell, Inc., Keene, NH) and hybridized with p29b cDNA [32P] labeled by random priming (25). Prehybridization (2 h in 5× Denhard's solution) and hybridization (18 h in 10% dextran sulfate) were performed at 42°C (high stringency) or 32°C (low stringency) in 50% (vol/vol) formamide in 6× SSC/0.5% SDS with 100 μg/ml sonicated salmon sperm DNA. Filters were washed twice for 15 min at room temperature with 6× SSC/0.5% SDS, twice at 37°C and once at 65°C with 1× SSC/0.5% SDS (high stringency), or twice at room temperature with 2× SSC/0.1% SDS and twice at 37°C with 0.25× SSC/0.1% SDS (low stringency).

Results

Cloning. A human bone marrow cDNA expression library in λgt11 was screened with monospecific anti-p29b IgG (4). One of the positive clones, containing an insert of 1,014 bp, was purified, subcloned in pBluescript SK+, and sequenced (Fig. 1). The open reading frame of 762 bp encodes a 254-amino acid polypeptide, including a 26-residue NH2-terminal peptide and a 228-residue mature protein. The latter corresponds to p29b, based on the following evidence. First, the deduced amino acid sequence for residues 1-20 (numbering for the mature protein) matches exactly the sequence obtained from the NH2 terminus of the purified protein (Glu19 was originally observed [3], but Gln19 was detected in the CNBr peptide sequenced in the present study and conforms to the cDNA). Second, two peptides from CNBr cleavage of purified p29b provided the sequence of 55 residues, and a [3H]DFP-binding tryptic fragment of p29b reported by Wilde et al. (26) provided another 22 residues; of these 77 residues, 70 were identical to the deduced sequence. Third, the deduced amino acid composition corresponded closely to that determined for purified p29b (not shown).

Features of the Predicted Structure. The 5'-end of the cDNA encodes a hydrophobic stretch (residues -22 to -9) (Fig. 2) typical of a signal sequence followed by a consensus signal peptidase cleavage site (Ala-X-Ala) (27) at -5 to -3 (Fig. 1). A 24-residue pre-signal peptide (Arg-26 to Ala-27) 50% homologous to the pre-sequence in the elastase cDNA (28) is followed by a two-residue pro-peptide (Ala-28,Glu-29), as
Figure 1. Nucleotide and deduced amino acid sequences of p29b (PR-3). The nucleotide sequence is numbered from the first base of the cDNA insert after the EcoR1 linker site. The deduced amino acid sequence (single-letter code) is numbered from the NH2-terminal residue of the mature protein (+1) (23). Regions for which amino acid sequence have been determined directly are underlined. These include residues 1-20, as determined earlier from the intact protein (3), 16-44 and 129-154, as determined in this study from CNBr fragments, and 169-196, as determined by Wilde et al. (26) from a cryptic peptide; the latter contained gaps corresponding to positions 182 and 189 of p29b. The following discrepancies are discussed in the text (giving the position, the deduced amino acid, and the assignment from amino acid sequencing): 29, C for I; 31, G for P; 35, H for L/V; 37, S for A/V; 44, H for I; 130, W for P; 147, N for R.

Components of the catalytic triad are boxed. Cysteines are circled.

Figure 2. Hydrophobic plot of p29b (PR-3) according to the algorithm of Kyte and Doolittle (42) with a window of n = 6. The lower panel indicates the distribution of basic (+) and acidic (-) residues.

Potential N-linked glycosylation sites (Asn-X-Ser/Thr) are present, and may account for the apparent isoforms (3, 4).

Eight Cys residues (five clustered in the COOH-terminal third of the molecule) suggest that there may be four intramolecular disulfide bonds, as in elastase (32). His*29, Asp*P, and Ser*76 correspond to the active site triad typical of serine proteases (Fig. 1).

After a hydrophobic region (−22 to −9) corresponding to the presumed signal sequence, the NH2-terminal half of p29b contains six regions averaging 17 residues each, in which there is a marked, periodic alternation of hydrophilicity and hydrophobicity (Fig. 2). The mature protein is predicted by the Chou-Fasman algorithm (33) to contain three short stretches of α-helix totalling 8.3%, and 10 β-sheets totalling 40%. Remarkably, the β-sheets are all predominantly hydrophobic. With 27 basic and 15 acidic amino acids in the mature protein (Fig. 2, bottom), p29b has an overall calculated pI of 7.9. Regions of the molecule predicted to be surface exposed (34) correspond to residues 7-12, 63-72, 83-88, 113-118, 165-168; these 32 residues include four acidic and eight basic amino acids.

Fig. 3 compares the p29b sequence with that of other serine proteases, aligned to maximize homology. p29b exhibits the highest homology with elastase (54%). There is also considerable homology with the T cell enzymes human lymphocyte protease (36%) and granzyme B (33%), and with cathepsin G (35%), rat mast cell protease II (34%), and chymotrypsin (30%). Residues 9-16 of p29b align with the Cys residues of elastase; five align with corresponding residues in the other proteases listed. One of the potential N-linked glycosylation sites in p29b (Asn*72) aligns with a corresponding site in elastase; both enzymes have two such sites. The members of the catalytic triad (His, Asp, Ser) are located at homologous positions in all the aligned sequences, flanked by well-conserved peptides. The residues at −6, 15-17, and 28 relative to the active site serine are thought to determine the

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tein termed myeloblastin was also evident from its cDNA terminal sequences. High homology with the predicted pro-

### Discussion

Close relatedness among PR-3, p29b, and the cytoplasmic autoantigen of Wegener's granulomatosis has emerged from their subcellular localization, relative abundance, molecular mass, enzymatic activity, ability to bind DFP, and NH2-terminal sequences. High homology with the predicted protein termed myeloblastin was also evident from its cDNA sequence. However, apparent discrepancies in their partial amino acid sequences have left unclear the precise interrela-

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The predicted amino acid sequence of PR-3 differs in 79 residues from the sequences determined from the purified protein or its fragments. Six of the seven discrepant positions are highly conserved among serine proteases; in each case, the conserved amino acid is that predicted by the cDNA. The discrepancies may arise from ambiguities in amino acid sequencing. Alternatively, PR-3 may be polymorphic. The nonconservative nature of the substitutions militates against this; nonetheless, we plan to test for polymorphism by PCR techniques. Finally, we cannot exclude the remote possibility that an additional gene(s) is linked in tandem to PR3 without intervening sites for the restriction enzymes used in the Southern blotting experiments. Genomic cloning will resolve this issue.

The PR-3 cDNA encodes a precursor with signal (pre) and propeptides typical of serine proteases. The hydrophobic prepeptide of 24 or more residues (the cDNA may be incomplete at the 5' end) may target PR-3 to the endoplasmic retic-
Figure 4. Southern blot of human genomic DNA with p29b (PR-3) cDNA as a probe. Human placental genomic DNA was digested to completion with five restriction enzymes and subjected to electrophoresis in 0.7% agarose. (A) The gel was stained with ethidium bromide and photographed under short-wavelength UV light to show the extent of digestion of the genomic DNA and the migration of size markers (left lane) derived from phage λ DNA digested with HindIII (23, 9.6, 6.6, 4.4, 2.3, 2.0 kb). (B) The digests were transferred to a nylon membrane, hybridized with [32P]-labeled p29b (PR-3) cDNA under high stringency, and washed as described in Materials and Methods, then autoradiographed with an intensifying screen for 4 d at -70°C.

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