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Limulus factor D, a 43-kDa protein isolated from horseshoe crab hemocytes, is a serine protease homologue with antimicrobial activity

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Abstract **A glycoprotein (M_r = 43 000) from horseshoe crab hemocytes with antimicrobial activity against Gram-negative bacteria was purified. The internal peptide sequences coincided exactly with the deduced amino acid sequence of a cDNA clone, designated limulus factor D, which was isolated by screening a hemocyte cDNA library with an anti-human plasminogen antibody. The open reading frame codes for a precursor of factor D of 394 amino acid residues, including an NH_2-terminal signal sequence. The COOH-terminal domain of factor D has significant sequence homology with the catalytic domain of mammalian serine proteases, in particular with human tissue plasminogen activator (32% identity), except for the substitution of Ser of the active site triad to Gly. Factor D has a unique NH_2-terminal domain with weak sequence homology with part of the mammalian interleukin-6 receptor α-chain. Factor D is likely to have an important role in host defense mechanisms.**

Key words: Antibacterial protein; Serine protease; Serprocidin; Host defense

1. Introduction

Invertebrates have characteristic host defense systems differing from those of the mammalian immune system and can eliminate foreign substances and invading microorganisms even without immunoglobulins. In the horseshoe crab, one of the major defense systems is carried by hemolymph, which contains two types of hemocytes, granular and non-granular cells [1–5]. The granular cells comprise 99% of total hemocytes, which contain two populations of secretory granules in the cytosol known as large and small granules [6]. The cells are highly sensitive to bacterial endotoxins, lipopolysaccharides (LPS), a major outer membrane component of Gram-negative bacteria, and respond by degranulating and secreting granular components from large and small granules after LPS stimulation, the result being clot formation. These responses are considered to be important for host defense in engulfing and killing invading microbes, in addition to preventing leakage of hemolymph. We purified and characterized serine protease zymogens participating in the clotting cascade [1–5], protease inhibitors [7–10], lectins [11,12], and antibacterial substances [13,14], all of which are stored in the two types of granules. In the course of studies on these host defense molecules, a 43-kDa protein was co-purified during isolation procedures of factor G, a (1,3)-β-D-glucan-sensitive serine protease zymogen [15]. The partial amino acid sequence of the 43-kDa protein indicated identity to the protein product of a cDNA clone, designated limulus factor D, which was isolated by screening a hemocyte cDNA library with an anti-human plasminogen antibody. We now report that the 43-kDa protein is a serine protease homologue with antimicrobial activity and probably belongs to the family of serprocidins [16,17].

2. Materials and methods

2.1. Materials

The lysate of hemocytes from Japanese horseshoe crabs (*Tachypleus tridentatus*) was prepared as described [18] and large and small granules of hemocytes were separated using our documented methods [19].

2.2. Antimicrobial activity

Antimicrobial activity was assayed as described previously [14].

2.3. Peptide preparation, sequencing, and amino acid analysis

The purified protein was reduced, *S*-alkylated with iodoacetamide, then digested with lysylendopeptidase (E/S=1/100, w/w) and the resulting peptides were separated by reversed-phase HPLC [14]. Amino acid sequences of the purified peptides were determined using Applied Biosystems gas-phase sequencer model 473A. For amino acid analysis, samples were hydrolysed in 6 M HCl in evacuated and sealed tubes at 110°C for 20 h. The hydrolysates were analyzed using a PICO-TAG system (Waters-Millipore, Milford, MA).

2.4. Computer-assisted analysis of sequence data

The sequence of factor D was compared with all entries in the SWISS-PROT Database (release 17, February 1996) with the Gene Works system (IntelliGenetics, Mountain View, CA).

2.5. SDS-PAGE and immunoblotting

SDS-PAGE was performed in 12% slab gels according to Laemmli [20]. The gels were stained with Coomassie brilliant blue R-250. An antiserum against factor D was raised in rabbits, using a synthetic adjuvant, TiterMax (Vaxcel, GA), and given intradermally [9]. For immunoblotting, gels were transferred to nitrocellulose membranes and the membranes were treated with the antiserum and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized with 4-chloro-1-naphthol [8].

2.6. cDNA cloning of factor D

One million independent clones of the λgt 11 cDNA library were screened with anti-human plasminogen antibody as described pre-

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Abbreviations: LPS, lipopolysaccharides; PTH, phenylthiohydantoin

The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBank/EMBL Data Bank with accession number D87214.

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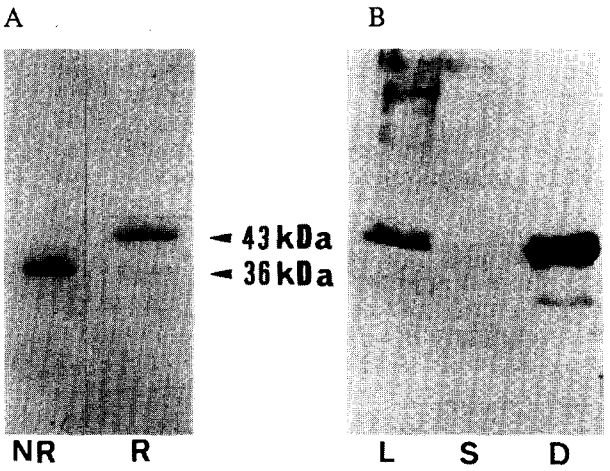

Fig. 1. SDS-PAGE of purified 43-kDa protein and its subcellular localization. A: SDS-PAGE under nonreducing (NR) and reducing (R) conditions. B: Large granules (L, 1.7 μg), small granules (S, 1.3 μg), and factor D (D, 1.4 μg) were subjected to SDS-PAGE, under reducing conditions, and factor D was identified by immunoblotting.

viously [21]. One positive clone with a 1.3-kb insert was subcloned into the *Eco*RI site of pBluescript II SK$^+$ and the nucleotide sequence on both strands was determined [8].

3. Results

3.1. Purification of a 43-kDa protein

A 43-kDa protein was isolated from a subfraction obtained during purification procedures of horseshoe crab factor G. Factor G has been purified from hemocyte lysate by three steps of chromatography – dextran sulfate-Sepharose CL-6B, Con A-Sepharose, and Sephacryl S200 [15]. A major peak with fractions 30–45 on the gel filtration of Sephacryl S200 contained a protein with an apparent molecular weight of 43000 on SDS-PAGE as a main component (data not shown). The pooled fraction was dialyzed against 50 mM sodium acetate, pH 5.5, containing 0.05 M NaCl, and applied to a CM Sepharose column (3×20 cm), equilibrated with the same buffer. After washing with the equilibration buffer, the 43-kDa protein was eluted with a linear gradient of 0.05–0.5 M NaCl in the same buffer and it gave a single protein band on SDS-PAGE, under reducing and non-reducing conditions (Fig. 1A). Thus, the 43-kDa protein from 100 g of hemocytes (wet weight) was purified with a yield of about 5 mg.

3.2. Peptide and nucleotide sequencing of the 43-kDa protein

No major PTH-amino acid derivative was obtained by Edman degradation of the purified 43-kDa protein, an event suggesting a blocked NH_2-terminus. Peptides derived from the 43-kDa protein were prepared by lysylendopeptidase digestion, and nine were sequenced. The resulting sequence data coincided exactly with the deduced amino acid sequence of a cDNA clone, designated limulus factor D, which was isolated by screening a hemocyte cDNA library with anti-human plasminogen antibody (Fig. 2). The cDNA included 1329 nucleotides with an open reading frame of 1182 nucleotides and encoded a protein of 394 amino acid residues, including an NH_2-terminal signal sequence with a typical hydrophobic core. A peptide with the amino acid composition corresponding to the NH_2-terminal Gln^{19} to Lys^{61} was also obtained from the digest, and the result suggest that the α-amino group of NH_2-terminal Gln^{19} in the mature protein had been cyclized (data not shown). There was a potential *N*-linked glycosylation site at Asn^{280} and amino acid sequence analysis of the peptide yielded no PTH-Asn at that position, indicating that Asn^{280} had been modified by the *N*-linked sugar chain. In fact, amino acid analysis on the mature 43-kDa protein indicated the presence of glucosamine residues (data not shown).

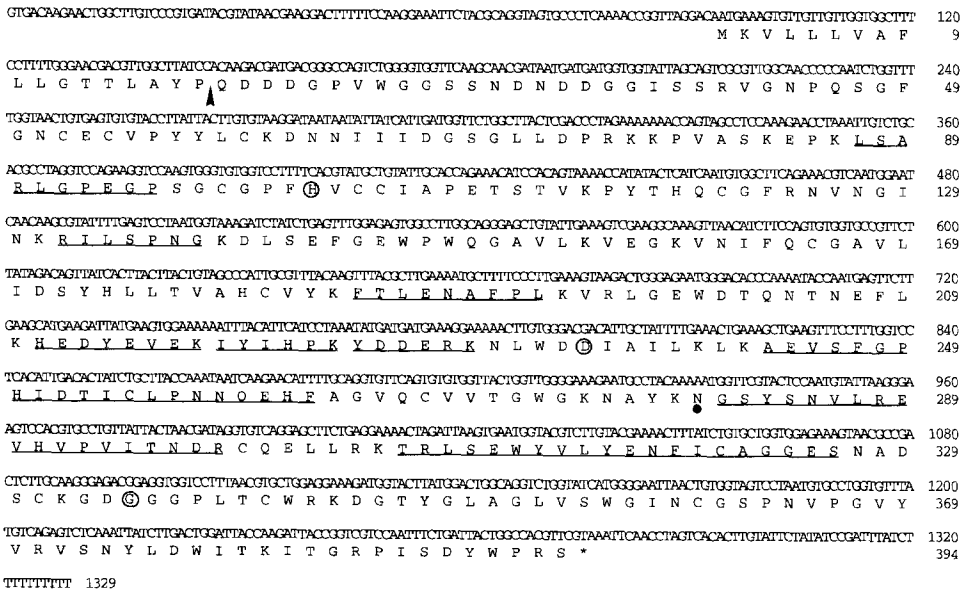

Fig. 2. Nucleotide and deduced amino acid sequences of factor D. The putative cleavage site for the signal peptide is indicated by the arrowhead. The *N*-glycosylation site is indicated by the closed circle. The amino acid residues corresponding to the catalytic triad of serine protease are circled. The stop codon is indicated by the asterisk. Amino acid residues confirmed by sequencing the isolated peptides are underlined. Numbering of amino acid residue starts with the initiation Met.

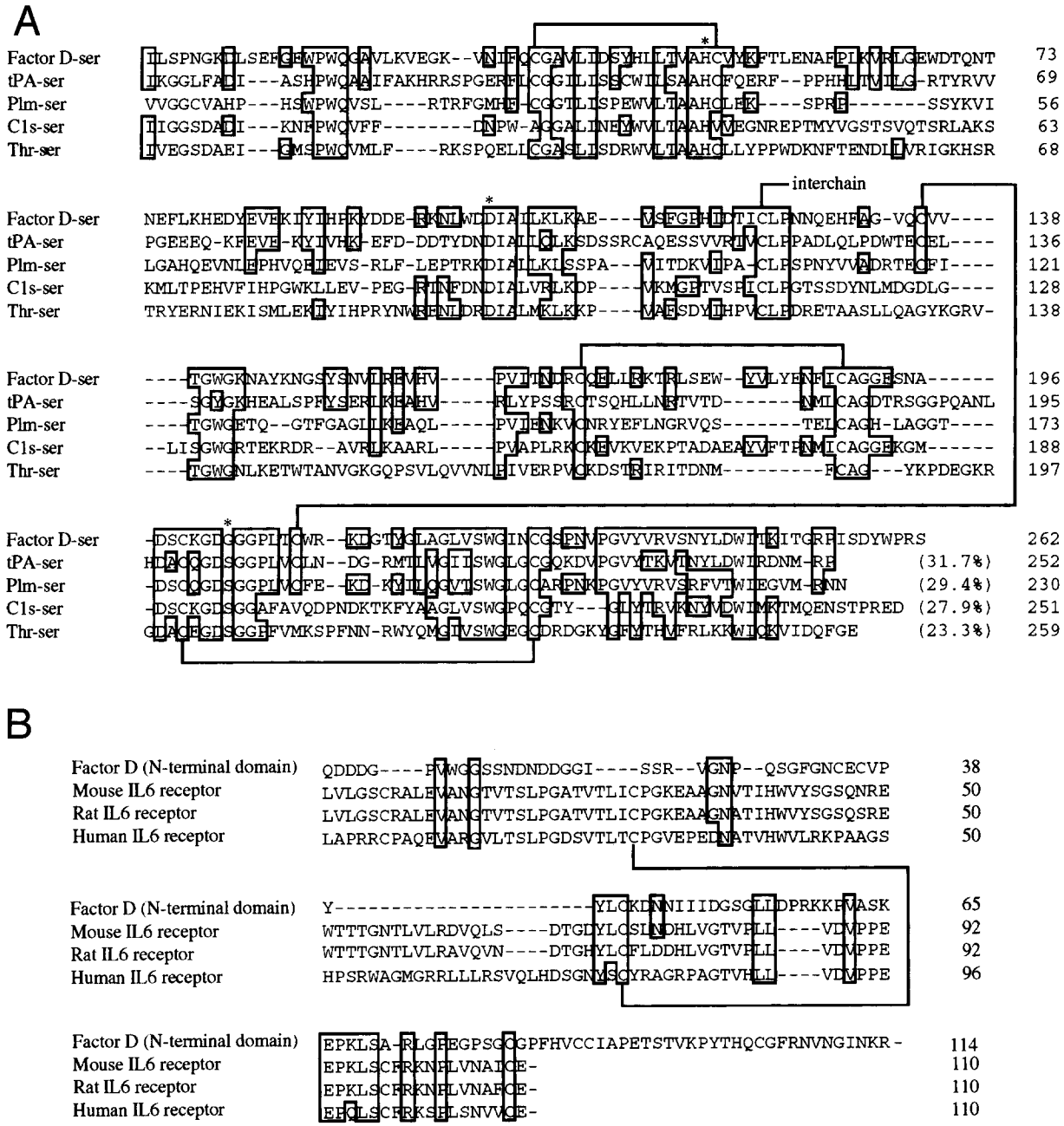

Fig. 3. Alignment of the COOH-terminal domain of factor D with catalytic domains of human serine proteases (A) and alignment of the NH_2-terminal region of factor D with those of mammalian interleukin-6 (IL6) receptor α-chains (B). A: The protease domains of tissue plasminogen activator (tPA-ser), plasmin (Plm-ser), complement factor C1s (C1s-ser), and thrombin (Thr-ser) are compared with that of the COOH-terminal domain of factor D (Factor D-ser). Residues identical to factor D are boxed. Residue numbering starts with the first amino acid residue of each catalytic domain and the residues that form the catalytic site in serine proteases are indicated by asterisks. B: Residue numbering starts with the first amino acid residue of each mature protein.

3.3. Sequence similarity to other proteins

A search of SWISS-PROT showed a striking similarity of the COOH-terminal domain of limulus factor D (262 residues) to the catalytic domain of mammalian serine proteases, except for substitution of a catalytic residue corresponding to the active site Ser^{195} (chymotrypsinogen numbering) to Gly (Fig. 3A). This pseudo-serine protease domain of factor D was more closely related to the catalytic domains of human tissue plasminogen activator [22] (31.7% identity) and also human plasmin [23] (29.4%) than to those of human C1s [24] (27.9%) and human thrombin [25] (23.3%). Among four serine proteases involved in the horseshoe crab coagulation cascade, the sequence of factor D showed the highest similarity with that of the clotting enzyme [26] (31.7%).

On the other hand, the NH_2-terminal domain of factor D (114 residues) had weak sequence homology with the NH_2-terminal region, an immunoglobulin-like domain, of mammalian interleukin-6 receptor α-chains in mouse [27], rat [28], and human [29] (Fig. 3B).

3.4. Antibacterial activity of factor D

Factor D had significant antibacterial activity against

Table 1
Antimicrobial activity of factor D

Bacteria	Factor D IC_{50} (μg/ml)
Gram-negative bacteria	
Escherichia coli (clinical isolate)	36
Escherichia coli 09; K39 (K^-)	290
Escherichia coli 09; K39 (K^+)	480
Salmonella typhimurium LT2 (S)	40
Salmonella minnesota R595 (Re)	70
Klebsiella pneumoniae (clinical isolate)	NI
Gram-positive bacteria	
Staphylococcus aureus	NI

NI, no inhibition at 400 μg/ml.

Gram-negative bacteria, including several *Escherichia coli* strains, *Salmonella typhimurium* LT2 (S), and *Salmonella minnesota* R595 (Re), but not the Gram-positive *Staphylococcus aureus*. The 50% inhibitory concentration (IC_{50}) of factor D for growth of these bacteria was determined using the microplate culture method [14], as summarized in Table 1.

3.5. Subcellular localization of factor D

Antiserum raised against purified factor D was used to identify the subcellular localization in hemocytes. Isolated large and small granules from hemocytes were subjected to SDS-PAGE, under reducing conditions for immunoblotting [8]. The anti-factor D antiserum recognized the 43-kDa factor D in the extract of large granules (Fig. 1B). However, immunoreactive materials in the extract of small granules with this antiserum were never observed, indicating that factor D locates in the large granule.

4. Discussion

The established sequence of limulus factor D indicates that the COOH-terminal domain of 262 residues has a significant sequence homology with the catalytic domain of serine proteases, except for an amino acid substitution. The higher sequence similarity of factor D to proteases found in the fibrinolytic system is consistent with its cross-reactivity against anti-human plasminogen antibody. Substitution of Ser in the active site triad to Gly occurs in the serine protease-like domain, despite conservation of two other catalytic residues (His and Asp), thereby indicating no endogenous proteolytic activity of factor D. In fact, the isolated factor D shows no amidase activity toward the synthetic peptide substrates tested (data not shown). Based on the predicted disulfide locations of factor D shown in Fig. 3A, the NH_2-terminal and the COOH-terminal domains may be linked by a single interchain disulfide bridge, and the peptide bond cleavage between Arg^{132} and Ile^{133} is presumed to form a two-chain factor D. However, at present we have no evidence for a specific protease that converts the single-chain factor D to the two-chain form.

Human neutrophils contain serine proteases (cathepsin G and elastase) and a serine protease homologue (azurocidin) in the azurophil granule, a specialized lysosome of the cells, in addition to defensins [16,17]. These have antimicrobial activities, and hence are referred to as 'serprocidins', a family of serine proteases and serine protease homologues with antimicrobial activity. Azurocidin also shows no demonstrable protease activity, as might be predicted from the two amino acid substitutions, His to Ser and Ser to Gly, in the active site of the molecule. Factor D, in this sense, may belong to serprocidins, although the sequence homology between the COOH-terminal domain of factor D and azurocidin is only 25.6%.

Factor D is present in large granules of hemocytes and may be co-released with other defense molecules stored in granules, in response to external stimulation of LPS. Factor D has a unique NH_2-terminal domain with weak homology to part of the interleukin 6 receptor α-chain, suggesting an important functional role of factor D in host defense systems, in addition to its antimicrobial activity. The role of limulus factor D in host defense mechanisms is the subject of ongoing study.

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