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 NH2-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 NH2terminal 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 Gramnegative bacteria, and respond by degranulating and secreting granular components from large and small granules after LPS stimulation, the result being clot formation. These responses

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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.

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 43kDa 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 $12\sqrt[5]{}$ 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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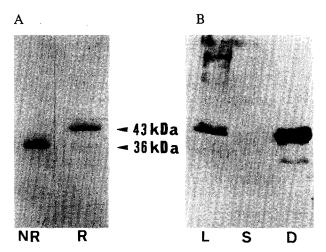


Fig. 1. SDS-PAGE of purified 43-kDa protein and its subcellular localization. A: SDS-PAGE under nonreducing (NR) and reducing (R) conditions. B: Large granules (L, 1.7 μ g), small granules (S, 1.3 μ g), and factor D (D, 1.4 μ g) were subjected to SDS-PAGE, under reducing conditions, and factor D was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

viously [21]. One positive clone with a 1.3-kb insert was subcloned into the EcoRI 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 NH₂-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 NH₂-terminal signal sequence with a typical hydrophobic core. A peptide with the amino acid composition corresponding to the NH2-terminal Gln19 to Lys61 was also obtained from the digest, and the result suggest that the α -amino group of NH₂-terminal Gln¹⁹ in the mature protein had been cyclized (data not shown). There was a potential N-linked glycosylation site at Asn²⁸⁰ and amino acid sequence analysis of the peptide yielded no PTH-Asn at that position, indicating that Asn²⁸⁰ 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).

GTORCAAGAACTUSCTTIGTOCOGTIGATINGGTIATIAAGGANGGACTITTTTOCGAAGGAAATTCTIACGCNGGTINGTOCOCTCAAAACCUGHTAGGACAATGAAAGTIGTIGTTGTTGGTGGCUTTT M K V L L L V A F	120 9
	240
LLGTTLAYPODDDGPVWGGSSNDNDDGGISSRVGNPOSGF	49
TGEIRACIGIGAGIGIGIRACCITATITATITATIGIRAACENTAATAATAATAATAATAATAATAATAATAATAATAATAA	360
G N C E C V P Y Y L C K D N N I I I D G S G L L D P R K K P V A S K E P K L S A	89
	05
	480
<u>RLGPEGP</u> SGCGPF(B)VCCIAPETSTVKPYTHQCGFRNVNGI	129
CANCARGENETITISAGEOCIATIOSIAAACAICENTCISAGENTIGGAGAGACGUSIATIGAACACCARGENAACHUCHOCAGIGIOSIOCOSITCT	600
N K <u>R I L S P N G</u> K D L S E F G E W P W Q G A V L K V E G K V N I F Q C G A V L	169
TATAGACASTINATCACTENCITACIOINGOCCATEGOCITERCANSTITACCACTEGOANACCOCTEGAANGEAGACTOCCAGAEGAGACCOCAAAAGACCAAUGACTACT	700
I D S Y H L L T V A H C V Y K F T L E N A F P L K V R L G E W D T Q N T N E F L	720 209
1951	209
CARCENTED AT TAXABLE AND A CONTRACTOR AND A CONTRACTOR AND AN AND AN AND AN AND AN AND AN AND AN AND AND	840
K H E D Y E V E K I Y I H P K Y D D E R K N L W D D I A I L K L K A E V S F G P	249
TCACATICACACTATICTICCCTACCAAAAAAACCAACAACACTICTICCACAGUGUGGTTACTICGTTACCAAAAAAACCAAAAAACCAAAAAACAAAAAAAAA	960
<u>H I D T I C L P N N O E H F</u> A G V Q C V V T G W G K N A Y K <u>N G S Y S N V L R E</u>	289
•	
	1080
<u>VHVPVITNDB</u> CQELLRK <u>TRLSEWYVLYENFICAGGES</u> NAD	329
CTCTTCCMCCCACCCCCCCCCCCCCCCCCCCCCCCCCCC	1200
SENED GEFETEW KEDETIGEAGEVSWGINCGSPNVPGVY	369
TOTATTINGDOM NOT TANDARD AND TRANSPORTED AND TRANSPORTANT AND TRANSPORTANCE CONTRACTOR TRANSPORTMENT IN TANDARD	1320
V R V S N Y L D W I T K I T G R P I S D Y W P R S *	394
TTTTTTTTT 1329	

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.

Α		_	
Factor D-	ser III.SPNGKIII.SEF		73
tPA-ser	IKGGLFACIASH	Ĩ₽₩ĊĠĂVLKVEGKV <mark>MIECĊĠŊĹĨŊŚŊĦĹĹĨŊĂĤĊŊŃĔ</mark> ŦĹĿŊĂĘĔĬĸŴŖĹĠĿŴŊĬŊŊŢ ₽₩Ċ <mark>ĂŊ</mark> IFAKHRRSPĠE ŖFĹĊĊĠĨĹĨŚĠĊŴĨĹŚŊĂŀĊ ŦŎĔŖĔ₽₽Ħ <u>ĦĹĨŊĨĹĠ</u> -RTŸŔVV	69
Plm-ser	VVGGCVAHPHSW	PWQVSLRTRFGMHE-CCGTLISPEWVLTAAHCLEKSPRESSYKVI	56
C1s-ser	IIGGSDADIKNF	PWQVFFDNPW-AGGALINEYWVLIAAHVMEGNREPTMYVGSTSVQTSRLAKS	63
Thr-ser	IVEGSDAEIGMS	PWOVMLFRKSPQELICGASLISDRWVLTAAHOLLYPPWDKNFTENDLUVRIGKHSR	68
		rinterchain	-1
Factor D-		ТІНЕККИДОВЕ-КІККТІ VIDIA ПІЛІТА ПІЛІТА КАРЕ VISEGEH ПІДТІ ССЕРИМОВЕНЕ ДІ	138
tPA-ser	PGEEEO-KFEVE-KY	IVHK-EFD-DDTYDNDIALLQLKSDSSRCAQESSVVRUVCLFPADLQLPDWTECEL	136
Plm-ser	LGAHOEVNLEPHVOE	TEVS-RLF-LEPTRIDIALLKISSPAWITDKVIPA-CLESPNYVVADRTECFI	121
C1s-ser	KMLTPEHVFIHPGWK	LLEV-PEG-RINFONDIALVRLKDPVKMGPTVSFICLFGTSSDYNLMDGDLG	128
Thr-ser	TRYERNIEKISMLEK	TYIHPRYNWRENTDRDIALMKLKKPWARSDYTHPVCLRDRETAASLLQAGYKGRV-	138
Factor D-		:::YSNVIREVEVPVITNCROELLEKTRLSEWYVLYENFLCAGGESNA	196
tPA-ser		SISHVILLEVIN	196
Plm-ser		FGAGLIKEAQLPVJENKVQNRYEFLNGRVQSTELCAGH-LAGGT	173
C1s-ser	LISGWORTEKRDR	AVRIKAARLPMAPLRKCKEVKVEKPTADAEAYMFTFMMICAGGEKGM	188
Thr-ser	T GWCNLKETWT	ANVGKGQPSVLQVVNLHIVERPVCKDSTRIRITDNMFCAGYKPDEGKR	197
	-		_
Factor D-		RKIGTYGLAGLVSWGINCGSPN/PGVYVRVSNYLDWITKITGRFISDYWPRS	262
tPA-ser		N-DG-RMTHNGI ISWGLQC <u>GO</u> KDVPGVIVKVSNILDWILDWIRDNM-R <u>P</u> (31.7%)	252
Plm-ser	-DSCOGIISCOPINGF	E-KI-KIILGOVISWOLOGARENKEGYYRVSRFVIWIEGVM-BANN (29.4%)	230
C1s-ser	-DSCKGDSGGAFAVO	DPNDKTKFYAAGLVSWOPOCOTYOLYTIRVKNYVDWIMKTMQENSTPRED (27.9%)	251
Thr-ser	CITACECTSCCEFVMK	SPFNN-RWYQMGIVSWGEGODRDGKYGFYTHYFRLKKWICKVIDQFGE (23.3%)	259
В			
D			
I	Factor D (N-terminal domain)	QDDDGFWWGGSSNDNDDGGISSRVGNPQSGFGNCECVP 38	
	Mouse IL6 receptor	LVLGSCRALEVANGTVTSLPGATVTLICPGKEAAGNVTIHWVYSGSQNRE 50	
	Rat IL6 receptor	LVLGSCRALEVANGTVTSLPGATVTLICPGKEAAGNATIHWVYSGSQSRE 50	
1	Human IL6 receptor	LAPRRCPAQEWARGVLTSLPGDSVTLTCPGVEPEDWATVHWVLRKPAAGS 50	
F	Factor D (N-terminal domain)	YYLCKDNVIIIDGSCTIDPRKKFMASK 65	
	Mouse IL6 receptor	WTTTGNTLVLRDVQLSDTGDYLCSINDHLVGTVFLLVDVPPE 92	
F	Rat IL6 receptor	WTTTGNTLVLRAVQVNDTGHYLCFLDDHLVGTVFLLVDMPPE 92	
F	Human IL6 receptor	HPSRWAGMGRRLLLRSVQLHDSGNYSCYRAGRPAGTVHLLVDYPPE 96	
Ţ	Factor D (N-terminal domain)	EPKLSA-RLCHEGPSCCGPFHVCCIAPETSTVKPYTHQCGFRNVNGINKR - 114	
	Mouse IL6 receptor	EPKLSCFRKNFLVNAICE- 110	
	Rat IL6 receptor	EPKLSCFRKNFLVNAFCE- 110	

Fig. 3. Alignment of the COOH-terminal domain of factor D with catalytic domains of human serine proteases (A) and alignment of the NH₂terminal 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.

EFALSCERKSELSNVVQE-

3.3. Sequence similarity to other proteins

Human IL6 receptor

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 Ser¹⁹⁵ (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%).

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On the other hand, the NH₂-terminal domain of factor D (114 residues) had weak sequence homology with the NH₂-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

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Table 1

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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 μ g/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 (IC₅₀) 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 NH₂-terminal and the COOH-terminal domains may be linked by a single interchain disulfide bridge, and the peptide bond cleavage between Arg¹³² and Ile¹³³ 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 COOHterminal 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 NH₂-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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