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Identification of defensin binding to C1 complement

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Abstract In human serum we found strong defensin binding to the complexes of activated C1 complement ($C\bar{1}$) and C1 inhibitor (C1i). Purified C1q, activated C1 tetramer ($\bar{r}_2\bar{s}_2$) and C1i did not bind defensin. When $\bar{r}_2\bar{s}_2$ was dissociated by EDTA, only the activated C1s ($C\bar{1}s$) bound defensin. Binding of defensins to $C\bar{1}$ complement represents a newly recognized bridge between the complement- and phagocyte-mediated host defenses, and a potential mechanism for protecting infected tissue from cytotoxic injury by defensin.

Key words: Defensin; C1 complement; C1 inhibitor; Inflammation

1. Introduction

Human defensins or human neutrophil peptides, HNP, are variably cationic, 3.5 kDa antimicrobial, cytotoxic and pro-inflammatory peptides, containing six disulfide-paired cysteines. Defensins are abundant in human neutrophils and small intestinal Paneth cells [1]. Neutrophil activation leads to the secretion of up to 10% of their defensin content [2], accounting for the defensin concentrations over 100 μg/ml in the plasma of some septic patients [3]. Unless inactivated, extracellular defensins may injure host tissues [1]. What mechanisms prevent defensin cytotoxicity? Previously we documented defensin binding to methylamine-activated 'fast' α2-macroglobulin (α2M) in human plasma [4]. In this study, we describe defensin binding to C1 complement.

C1 complement is a calcium-dependent multimolecular complex that initiates the classical pathway of complement activation [5]. C1 is composed of one recognition subunit, C1q and one tetrameric protease subunit abbreviated as r_2s_2. Binding of aggregated IgG and other stimuli to C1 leads to autocatalyzed cleavage of its subcomponent proenzyme C1r to active protease $C\bar{1}r$ (the bar above indicates an active enzyme). $C\bar{1}r$ in turn cleaves and activates proenzyme C1s into protease $C\bar{1}s$. The resulting proteolytically active form of C1 ($C\bar{1}$) is regulated by C1 inhibitor (C1i) [5], a protein belonging to the serine protease inhibitor (serpins) superfamily [6]. C1i binds to $C\bar{1}$ and dissociates the $C\bar{1}$ multimer. When only C1r is activated, C1i releases two molecules of C1i–$C\bar{1}r$–C1s [7]. When both C1r and C1s are activated, two molecules of C1i–$C\bar{1}r$–$C\bar{1}s$–C1i are released [8]. In the current paper we identify defensin-binding C1 forms.

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Abbreviations: α2M, α2-macroglobulin; C1i, C1 inhibitor; HNP, human neutrophil peptide, defensins; NHS, normal human serum; NHP, normal human plasma; PAGE, polyacrylamide gel electrophoresis; r_2s_2, C1 tetramer, catalytic subunit of C1.

2. Materials and methods

2.1. Reagents and materials

Human defensin HNP-1 and pig neutrophil peptide protegrin, PG-1, were isolated from human and pig neutrophils [9,10]. Tachyplesin, TP-1, was purchased from Bachem (King of Prussia, PA). Human C1 subcomponents were isolated by IgG-Sepharose and DEAE chromatography [11], C1i was purified by the method of Sim and Reboul [12]. Goat anti-C1q, -C1r, -C1s and -C1i IgG were purchased from Atlantic Antibodies (Scarborough, ME). Control goat IgG and human serum albumin (HSA) were from Sigma (St. Louis, MO). Rabbit anti-goat IgG alkaline phosphatase conjugate was from Bio-Rad (Richmond, CA). Iodobead and Iodo-gen iodination reagents were supplied by Pierce (Rockford, IL). Sodium iodide-125 was purchased from Du-Pont-NEN (Boston, MA). BioBlot nitrocellulose (NC) membranes, 0.45 mM pore size, were from Costar (Cambridge, MA).

Blood was obtained from volunteer blood donors, and used to prepare normal human serum (NHS), or normal human plasma (NHP) containing 5 mM EDTA. To minimize the in vitro discharge of cellular constituents into serum or plasma, the blood cells were immediately removed by centrifugation [4].

2.2. Detection of defensin binding in human blood

HNP-1, PG-1 and TP-1 were iodinated by the Iodo-gen or Iodobeads method [13] to a specific activity of 10 μCi/μg. Trace amounts of radiolabeled HNP-1, PG-1 or TP-1 was incubated with NHS, NHP, and purified C1 subcomponents for 30 min at 37°C. Resulting complexes were followed by native or rate PAGE at pH 5.7 [14] or at pH 8.8 [15]. After electrophoresis toward the anode in a Minigel apparatus (Hoeffer Scientific Instruments, San Francisco, CA) at 4°C, gels were Coomassie stained, dried and exposed to X-OMAT AR films (Eastman Kodak Co., Rochester, NY) at −70°C for 1–3 days.

2.3. Immunoprecipitation

Goat IgG to C1 subcomponents, or control IgG were diluted in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS) and incubated for 1 h at room temperature with equivalent volumes of NHS ± EDTA. Samples were further incubated with trace amounts of radiolabeled HNP-1 ([^{125}I]HNP) for 30 min at 37°C and directly subjected to 5% native PAGE, pH 8.8, and autoradiography. Resulting autoradiograms were analyzed using the Speedlight gel documentation system (B/T Sci Tech, Carlsbad, CA) and Qgel software (Kendrick Lab. Inc., Madison, WI).

2.4. Immunoblotting

Samples containing radiolabeled defensin in NHS or NHS + aggregated IgG (NHS + aggr IgG) were used for 5% native PAGE, pH 8.8. To 'activate' NHS, human IgG, 30 mg/ml in TBS was aggregated at 56°C for 30 min, and 10 μl of this solution was added to 50 μl of NHS for 30 min at 37°C. Half of each sample of NHS and NHS + aggr IgG was incubated with 10 mM EDTA for 30 min on ice

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to chelate Ca^{2+}. Following PAGE, the gels were electrotransferred onto NC in 25 mM Tris-HCl, 192 mM glycine buffer, pH 8.2, 10% methanol for 1 h at a constant current 250 mA. The same NC membranes were first used for autoradiography and then for immunoblotting with goat anti-C1q, -C1i, -C1r, -C1s or control IgG [4].

3. Results

3.1. Defensin binding in human blood is calcium-dependent

Defensin interactions with α2M [4] could not explain the complex pattern of defensin binding in human blood. In a search for the additional defensin-binding proteins we noticed that defensin binding depended on the presence of Ca^{2+}. Chelating of Ca^{2+} with EDTA resulted in the shift of defensin binding (Fig. 1). The effect of EDTA was reversed by excess Ca^{2+}. Excess Ca^{2+} by itself did not change defensin binding in NHS. Since native PAGE, represented in Fig. 1 and the following figures, separates anionic plasma proteins according both to their molecular weight and charge, we used purified 'slow' and 'fast' α2M, transferrin, HSA, C1 subcomponents and Coomassie staining to 'calibrate' the gels in each experiment. Unbound, cationic defensin did not enter the native gels [3].

3.2. Identification of defensin-binding C1 forms

Based on the Ca^{2+}-dependence, we suspected that defensin may interact with C1 complement, since (i) coagulation and complement multienzyme cascades are the two major Ca^{2+}-dependent systems in blood, (ii) in the absence of calcium, C1 complement breaks into the individual subcomponents, and (iii) during native PAGE the C1s subcomponent migrates in the α_2 region adjacent to transferrin [16].

Preliminary immunoprecipitation experiments suggested defensin binding to $C\bar{1}$–C1i complexes, containing $C\bar{1}s$, $C\bar{1}r$ and C1i in NHS, and to $C\bar{1}r$–C1i dimers and monomeric C1s in NHS + EDTA (not shown). $C\bar{1}$–C1i interactions in NHS are known to result in the formation of the C1i–$C\bar{1}r$–C1s and C1i–$C\bar{1}r$–$C\bar{1}s$–C1i complexes [7,8]. The first complex increases in NHS after prolonged incubation at 37°C, and the second appears after activation with aggregated IgG [7]. To identify composition of defensin-binding $C\bar{1}$–C1i complexes, we compared defensin redistribution in NHS and NHS + aggr IgG. Alignment of defensin-containing bands on autoradiograms with the bands stained by anti-C1 antibodies on the immunoblots allowed us to demonstrate defensin binding to C1i–$C\bar{1}r$–C1s in non-activated NHS, and to both C1i–$C\bar{1}r$–C1s and C1i–$C\bar{1}r$–$C\bar{1}s$–C1i in NHS + aggr IgG (Fig. 2A). Immunoblotting identified defensin interaction with $C\bar{1}s$–C1i as well as $C\bar{1}r$–C1i in NHS + aggr IgG + EDTA and C1s and $C\bar{1}r$–C1i in NHS + EDTA (Fig. 2B). Defensin bound only to the more anionic (lower) C1s band, present in NHS + EDTA (Fig. 2B, lanes αC1s), but not in NHS (Fig. 2A, lanes αC1s). Since the cationic C1q subunit did not enter the native gels, immunoblots probed with anti-C1q antibodies yielded the same background staining as non-immune goat IgG (not shown).

3.3. Defensin interactions with purified C1 tetramer

To confirm defensin interactions with C1, we used purified C1 tetramer r_2s_2, which was 90–95% activated as was shown by it dissociation into heavy and light chains in SDS-PAGE under reducing conditions [5]. Preincubation of $\bar{r}_2\bar{s}_2$ with an excess of C1i resulted in defensin binding to C1i–$C\bar{1}r$–$C\bar{1}s$–C1i and to a lesser extent, to C1i–$C\bar{1}r$–C1s, corresponding to the degree of

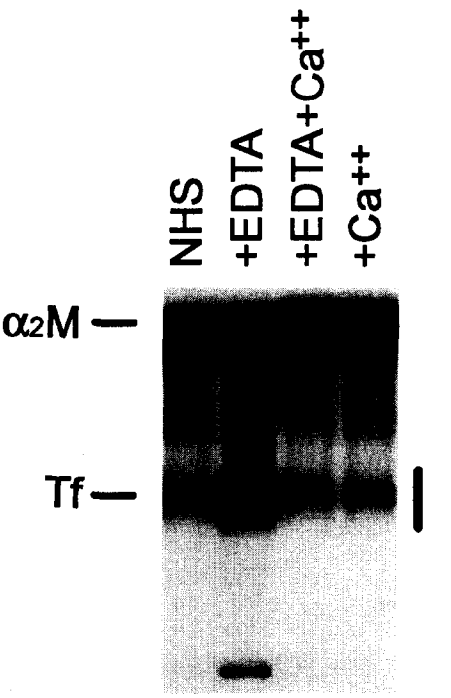

Fig. 1. The effect of calcium on defensin binding in normal human serum. An autoradiogram after 10% native PAGE, pH 5.7. Each lane containes 1.5 μl of NHS and 0.5 ng of [^{125}I]HNP. As indicated, NHS was preincubated with 5 mM and/or 10 mM Ca^{2+} for 30 min at 4°C. Lines labeled α2M and Tf mark the positions of 'slow' α2M and transferrin on the Coomassie stained gel. The bar on the right indicates the defensin-binding zone in NHS ± EDTA.

$\bar{r}_2\bar{s}_2$ activation (Fig. 3). When EDTA was added to $\bar{r}_2\bar{s}_2$ + C1i, defensin binding shifted into the lower molecular weight zone of $C\bar{1}r$–C1i and $C\bar{1}s$–C1i dimers. The addition of EDTA to $\bar{r}_2\bar{s}_2$ resulted in defensin binding to monomeric $C\bar{1}s$, and subsequent addition of C1i moved defensin binding up to $C\bar{1}r$–C1i and $C\bar{1}s$–C1i and to some extent to C1i–$C\bar{1}r$–$C\bar{1}s$–C1i. In general, the radioactive bands that resulted from defensin binding to individual products of $\bar{r}_2\bar{s}_2$ interaction with C1i and EDTA aligned very well with defensin-binding bands in NHS, NHS + EDTA and NHS + aggr IgG (Fig. 3). Defensin did not bind to $\bar{r}_2\bar{s}_2$ in the absence of C1i, and did not bind to purified C1i and C1q (not shown). Although defensin bound equally well to purified $C\bar{1}s$ and $C\bar{1}s$–C1i complexes, defensin did not bind to purified $C\bar{1}r$, unless it was reacted with C1i (not shown).

Table 1
Forms of C1 and their defensin binding

C1 form	Form present in serum	Form present in serum + EDTA	Defensin binding
C1r	–	+	–
C1s	–	+	+
C1q	–	+	–
C1 tetramer r_2s_2	–	–	–
C1	+	–	–
C1i	+	+	–
$C\bar{1}r$–C1i	–	+	+
$C\bar{1}s$–C1i	–	+	+
C1i–$C\bar{1}$–C1s	+	–	+
C1i–$C\bar{1}r$–$C\bar{1}s$–C1i	+	–	+

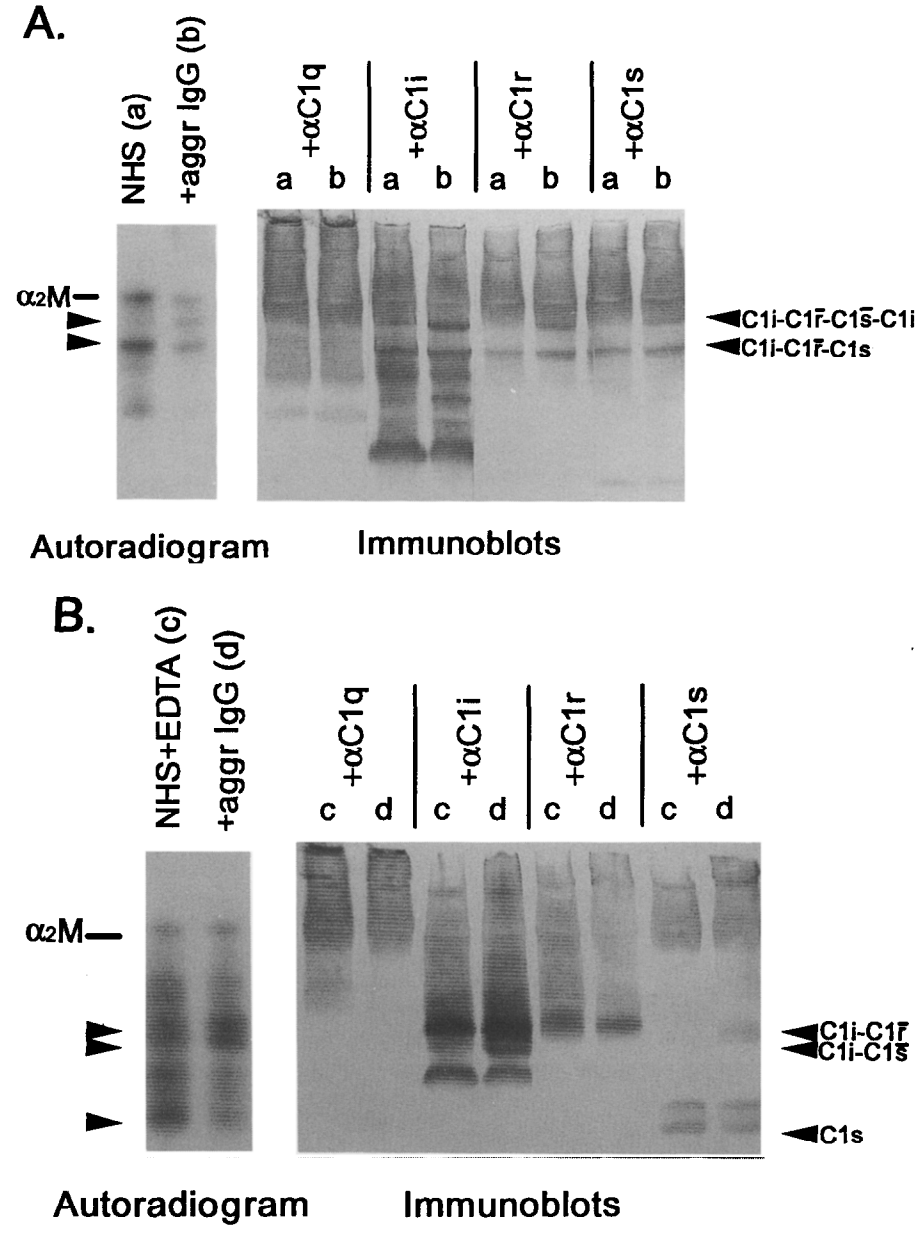

Fig. 2. Identification of defensin–C1 complexes in NHS (A) and NHS + EDTA (B). Comparision of autoradiograms and corresponding immunoblots after 5% native PAGE, pH 8.8. Each lane contains 2 ng of [^{125}I]HNP and 1.5 μl of NHS ± aggr IgG ± EDTA. Anti-C1 goat IgG (αC1q, αC1i, αC1r, αC1s) were incubated with NC strips, containing NHS (a) and NHS + aggr IgG (b), or NHS + EDTA (c) and NHS + aggr IgG + EDTA (d). The line labeled α2M marks the positions of 'slow' α2M on the Coomassie stained gel. Arrows on the left indicate defensin-binding proteins on autoradiograms, identified by immunoblotting (labeled arrows on the right).

3.4. C1 complement also binds non-defensin cationic peptides

As illustrated on Fig. 4, PG-1 and TP-1, small cationic peptides from pig neutrophils [10] and horseshoe crab amebocytes [17], also bound to C1i–$\overline{C1r}$–C1s in NHS. While defensin, in addition to C1i–$\overline{C1r}$–C1s, interacted with 'slow' and 'fast' α2M, C1i–$\overline{C1r}$–C1s was the main PG-1-binding protein in NHS. There was little binding of PG-1 and TP-1 to 'fast' or 'slow' α2M.

4. Discussion

We found defensin interactions with C1 complement and identified defensin-binding C1 forms (Table 1). Inactivation of $\overline{C1}$ by C1i was necessary for the binding. The interaction of C1i with $\overline{C1}$ complement is known to change its calcium-binding properties and antigenicity [5,18]. It is likely that C1i interactions with $\overline{C1}$ also 'opens' defensin-binding sites. In additional experiments we found defensin binding to the purified N-terminal α-fragment of C1s (not shown). We did not see defensin binding to the more cationic (higher) C1s band in NHS + EDTA (Fig. 2B, lanes αC1s), which appears as a result of proteolytic cleavage of the N-terminal part of the heavy chain of C1s [19]. Since the α-fragment of C1s participates in calcium-dependent formation of C1 tetramer and whole C1 molecule [20], its defensin binding site(s) are likely to be

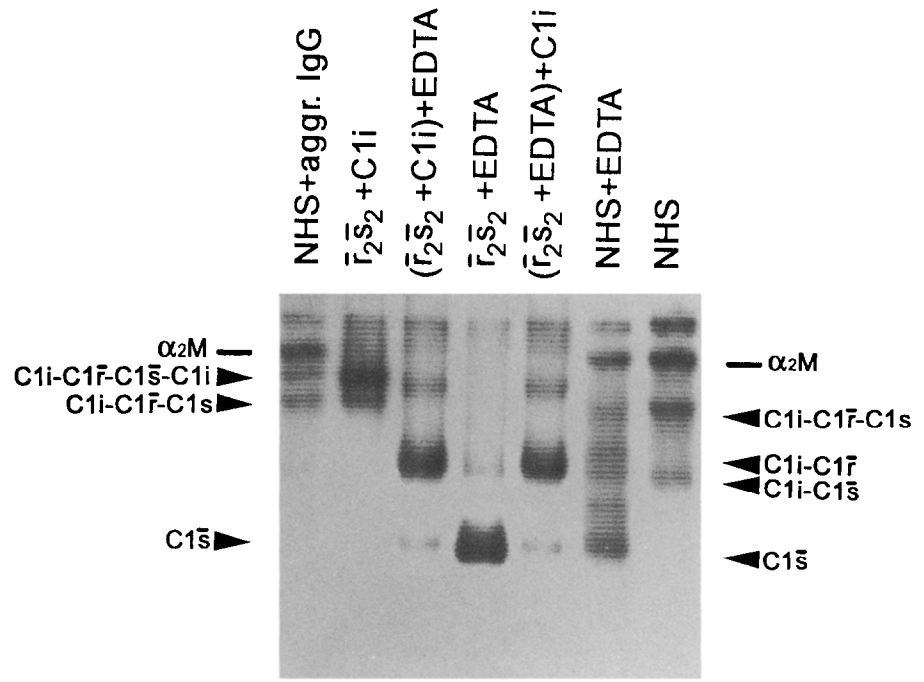

Fig. 3. Defensin interactions with purified $C\bar{1}$ tetramer. An autoradiogram after 5% native PAGE, pH 8.8. Samples contain 0.5 ng of [^{125}I]HNP added to 1.5 μl of NHS ± aggr IgG ± EDTA or 4 μg of $\bar{r}_2\bar{s}_2$ ± 6 μg C1i ± EDTA. The line labeled α2M marks the positions of 'slow' α2M on the Coomassie stained gel. Arrows indicate defensin-binding C1 forms.

masked. This may explain why we did not observe defensin binding to purified $\bar{r}_2\bar{s}_2$, or $C\bar{1}$ complement reconstituted from purified $\bar{r}_2\bar{s}_2$ and C1q.

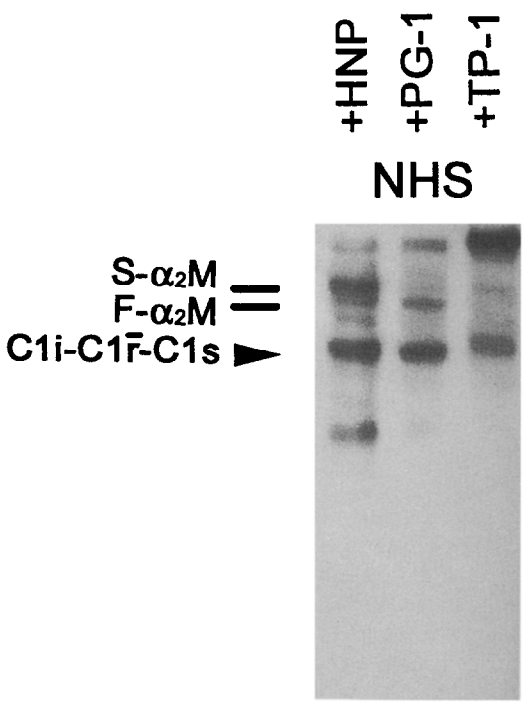

Fig. 4. Binding of protegrin and tachyplesin to $C\bar{1}$ complement. An autoradiogram after 5% native PAGE, pH 8.8. As indicated, samples contain 0.5 ng of [^{125}I]HNP, 4 ng of [^{125}I]PG-1 or [^{125}I]TP-1 in 1.5 μl of NHS. Lines labeled S-α2M and F-α2M mark the positions of 'slow' and 'fast' α2M on the Coomassie stained gel. Arrow indicates the position of C1i–$C\bar{1}$r–C1s complex in NHS.

C1 concentration in human blood approximates to 180 nM, and up to 5% of $C\bar{1}$ is inactivated [21], yielding 18 nM of $C\bar{1}$–C1i complexes. Assuming one binding site per complex, inactivated $C\bar{1}$ will bind 18–36 nM (60–120 ng/ml) of extracellular defensins. In normal human plasma we measured median defensin concentrations of 25 ng/ml [3]. $C\bar{1}$–C1i complexes can thus bind and dispose of defensins at their physiological level. Indeed, in our experiment with NHS and NHP and close to physiological amounts of defensin (Figs. 3 and 4), C1 forms, along with α2M, were the main defensin-binding compartments. At the high level found in the plasma of septic patients, defensins may chiefly bind to and be disposed of by α2M [3] the concentration of which in human blood (2–4 μM) is much higher than that of C1 complement [22]. Human serum albumin, the major anionic protein of human blood, did not compete with defensin binding to purified C1 subcomponents even when HSA was added in 100 fold excess, nor did HSA compete with defensin binding to $C\bar{1}$ in NHS or NHP (not shown).

Cationic peptides PG-1 and TP-1 also interact strongly with $C\bar{1}$–C1i complexes in NHS. Defensins, protegrins and tachyplesins constitute structurally distinct families of cysteine-rich antimicrobial peptides. Protegrins, discovered and characterized recently [10], have a molecular weight of approximately 2 kDa and contain four cysteine residues that form two intramolecular cysteine disulfide bonds. Tachyplesins resemble PG in molecular weight and amount of cysteine residues but have different primary structures and cysteine placement. Although defensins are almost twice as large as PG or TP and have six cysteine residues, they share some N-terminal similarities with protegrins [10]. The natural diversity of cysteine-rich antimicrobial peptides may be used to identify defensin site(s) involved in $C\bar{1}$ binding.

The uptake of HNP–$C\bar{1}$s–C1i complexes in our preliminary

experiment with human hepatoma cell line HepG2 (not shown) suggests that binding to $C\bar{1}$ could serve as a disposal or recycling pathway for defensins through the SEC receptors, responsible for the uptake of serpin–protease complexes [23]. Complement and phagocytes are the major interlinked systems of innate host defense. The current study suggests that activated complement may also serve to bind defensins released extracellularly during neutrophil activation. This mechanism may confine the activity of defensins to the phagolysosome, and may protect the surrounding tissue from cytotoxic damage.

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