ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2015, Vol. 41, No. 6, pp. 597–601. © Pleiades Publishing, Ltd., 2015.
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Interaction of Arenicin-1 with C1q Protein[1]

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Received April 2, 2015; in final form, May 15, 2015

Abstract—The interaction of arenicin-1, an antimicrobial peptide from the lugworm *Arenicola marina* with the protein C1q of the human complement system has been analyzed using enzyme-linked receptor sorbent assay and ELISA. Arenicin-1 and C1q were shown to form a stable complex that persisted at elevated ionic strength (0.5 M NaCl). The ability of arenicin-1 to interact with C1q is comparable to that of the porcine cathelicidin protegrin-1, an antimicrobial peptide that has a spatial structure similar to that of arenicin (an antiparallel β-hairpin stabilized by disulfide bridges).

Keywords: antimicrobial peptides, arenicins, C1q, complement, protein-protein interactions, β *structure*

DOI: 10.1134/S1068162015060035

INTRODUCTION

Antimicrobial peptides (AMPs) are among the most important molecular factors of human and animal innate immunity. Many of these peptides possess a wide range of immunomodulatory properties [1–3], such as the ability to modulate the complement system, in addition to direct antimicrobial activity. The modulation is mediated by interactions with complement proteins, such as C1q. The first study in this area was reported by Panyutich et al., who had investigated the interaction of a range of AMPs having a secondary structure of the β type with the protein complex forming the C1 component of complement [4]. The interaction of three AMPs—human α-defensin HNP-1, porcine cathelicidin protegrin-1, and tachyplesin-1, a peptide from the hemocytes of the horseshoe crab *Tachypleus tridentatus* —with C1 was demonstrated. Binding of the peptides with C1r and C1s proteinases and the inhibitory protein C1i was demonstrated, while no immediate interactions with the peptides were reported for the protein C1q. The latter result contradicted the reports by other researchers who demonstrated the ability of both α-defensins [5–8] and β-defensins [8, 9] to form complexes with C1q. Data on the character of the effects of defensins on complement activation remain contradictory and vary depending on the procedure used to assess the activation.

We investigated the interactions of defensins with C1q and reported the formation of a complex between this protein and protegrin-1 [8]. Direct interaction with C1q leading to activation of the complement system on the surface of the TSU line of human prostate carcinoma cells was demonstrated for tachyplesin as well [10]. Defensins and protegrin exhibit a certain sequence homology, while neither of these peptides is homologous to tachyplesin. However, tachyplesin and protegrin share a range of structural characteristics: both are amphipathic cationic peptides forming an antiparallel β-hairpin structure stabilized by two disulfide bonds [11]. Analysis of the structurally related peptides with regard to their ability to interact with C1q provides information on the exact nature of structural characteristics underlying the capacity for interaction. The data on complex formation between tachyplesin and C1q allow for the assumption that arenicins, tachyplesin homologues from the celomocytes of the polychaete lugworm *Arenicola marina*, can also interact with this protein. This possibility was investigated in the present work.

Arenicins are short (21-residue) AMPs represented by three isoforms found in the celomocytes of the lugworm. Arenicins -1 and -2 were first described by the authors of the present study [12, 13], and arenicin-3 was discovered later [14]. Interestingly, arenicins-1 and -2 contain two cysteine residues each, and these residues form a single disulfide bond, while arenicin-3 contains four cysteine residues and two disulfide

Abbreviations: AMPs, antimicrobial peptides; PBS0 0.01 M phosphate buffered saline, pH 7.4, supplemented with 0.15 M NaCl.

[1] The article has been adapted from the report presented at the VIIth All-Russian symposium "Proteins and Peptides", Novosibirsk, 12–17 July, 2015.

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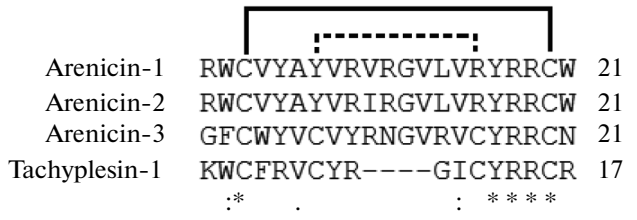

Fig. 1. Homology between arenicins and tachyplesin. The disulfide bonds are shown in the top part of the image: the bond found in all peptides presented in the figure is shown as a solid line, and the bond found in arenicin-3 and tachyplesin-1 only is shown as a dotted line.

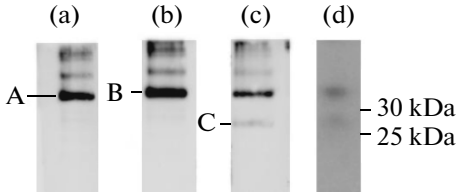

Fig. 2. Analysis of the C1q preparation obtained in the present study. (a), (b), and (c) Immunodetection with antibodies targeting A, B, and C subunits of C1q protein, respectively; (d) Coomassie G-250 staining.

bonds, similarly to tachyplesins (Fig. 1). Similarly to tachyplesin and protegrin, arenicins form antiparallel β-hairpins [15–17].

The ability of arenicin-1 to form a complex with the human C1q protein and the stability of this complex at elevated ionic strength (0.5 M NaCl) was demonstrated in the present work.

RESULTS AND DISCUSSION

The affinity chromatography approach based on C1q interaction with immune complexes is widely used for preparative isolation of C1q. The immune complexes used in the conventional procedures for C1q isolation include immobilized human immunoglobulin G as the antigen and rabbit immunoglobulins G targeting the former as the antibody components [7,18, 19]. We have performed successful isolation of C1q in a system containing a different antigen (namely, myeloperoxidase). The C1q preparation retained functional activity manifested as the ability to restore the lytic activity of C1q-depleted serum towards *Escherichia coli* cells.

The C1q preparation isolated within the present study was analyzed using Western blotting with antibodies targeting three individual subunits of this protein (A, B, and C) (Fig. 2). Consistently with the previous reports [20], the electrophoretic bands of the A and B subunits overlapped, while the band of the C subunit ran a little lower. The interaction of antibodies targeting the C subunit with the B subunit can be due to the presence of common antigenic determinants in both subunits. The synthetic antigenic peptide used to generate antibodies targeting the C subunit contains a GKFTCKVPGLYYFVYHAS fragment found in the B subunit as well. The minor high-molecular weight components of the preparation that react with the antibodies and may consist of C1q oligomers are also visible on the electrophoregram.

Both enzyme-linked receptor sorbent assay and ELISA used in the present work for the analysis of the interaction of arenicin-1 and C1q have certain drawbacks. The receptor sorbent assay requires labeling of the receptor (C1q in the present work), and the label (horseradish peroxidase) can affect the properties of the receptor, including its ability to interact with the ligand. At the same time, the use of ELISA for the detection of protein–protein complexes can yield false negative results, since the antibodies can destroy the complex between the antigen and the interacting protein due to blocking of the epitopes involved in complex formation. Thus, we considered it important to use both methods to assess the capacity of arenicin-1 to form complexes with C1q. Protegrin-1, which was shown to interact with C1q in our previous study [8], was used as a reference peptide in these experiments.

Both receptor sorbent assay and ELISA revealed the interaction of arenicin-1 with the human C1q protein (Figs. 3 and 4). The complexes of the peptide with C1q remained stable as the ionic strength of the interaction medium was increased to 0.5 M, this being indicative of the nonexclusive role of ionic interactions for complex formation. Similar results were obtained for protegrin-1. The level of C1q binding in wells not containing adsorbed peptides was significantly lower ($p < 0.01$ in all cases). Thus, C1q shows strong specificity for interaction with arenicin and protegrin as compared to BSA used to block the well surface.

Ringed worms, including the lugworm, do not have a complement system, and therefore C1q is absent from their organisms. However, the capacity of arenicins for protein–protein interactions manifested in the experiments with C1q may be a functionally important property that emerged and was stabilized in the course of evolution. Each of the three C1q subunits contains a collagen-like *N*-terminal domain and a globular *C*-terminal domain. Both collagen possessing a range of unique structural features [21] and proteins containing domains homologous to the globular domains of C1q [22] were found in ringed worms, and some of these proteins can be assumed to interact with arenicin in the lugworm organism. The present study did not involve the identification of the site of the C1q molecule directly involved in the interactions with arenicins and protegrins. However, published reports of other AMPs point at the collagen-like domain of C1q as the site involved in the interaction with peptides.

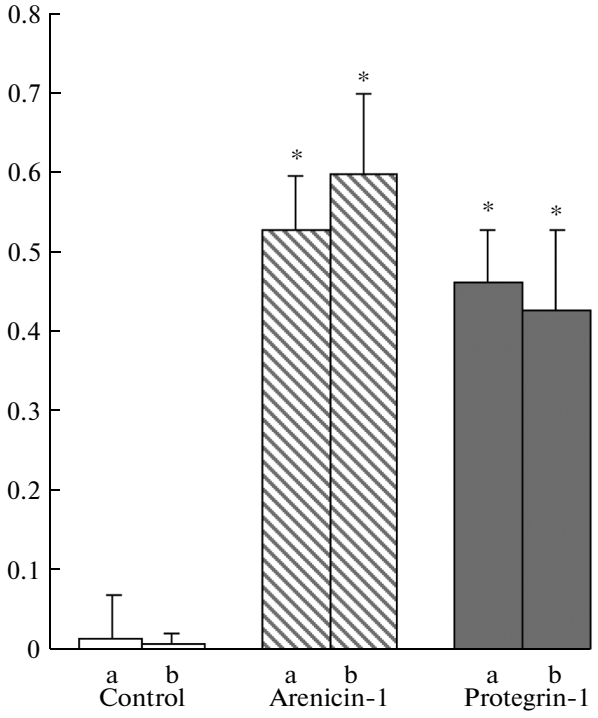

Fig. 3. Receptor sorbent assay-based assessment of the interaction of arenicin-1 and protegrin-1 with C1q in the presence of 0.15 (a) and 0.5 (b) M NaCl. Control samples did not contain peptides, and the values for these samples characterize nonspecific interaction with BSA used to block the well surface. Absorbance of the probes at λ_{492} is plotted on the ordinate axis.

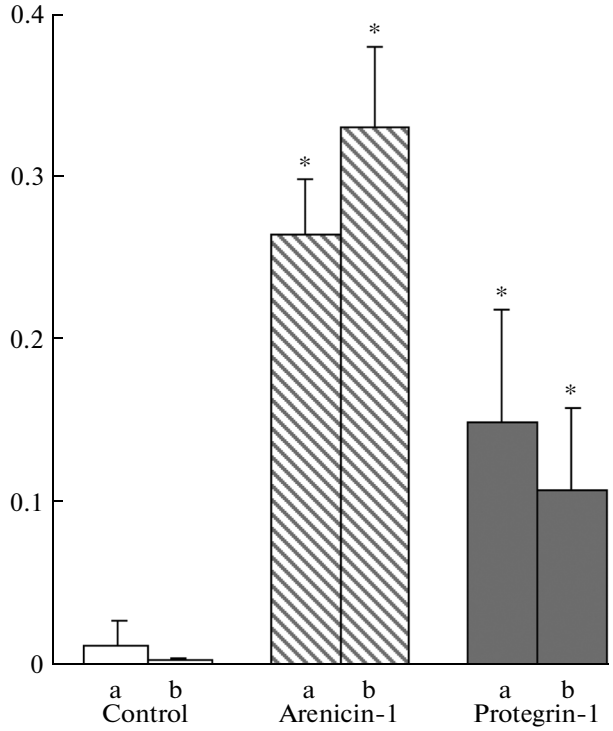

Fig. 4. ELISA assessment of the interaction of arenicin-1 and protegrin-1 with C1q in the presence of 0.15 (a) and 0.5 (b) M NaCl. Control samples did not contain peptides, and the values for these samples characterize nonspecific interaction with BSA used to block the well surface. Absorbance of the probes at λ_{492} is plotted on the ordinate axis.

Such results were obtained for tachyplesin [10] and defensins [6, 7]. Notably, α- and β-defensins were shown to interact with another recognition molecule of the complement system, the mannan-binding lectin, which contains a collagen-like domain as well [7]. Recent studies demonstrated constitutive expression of arenicins in the body wall, extravasal tissue, and intestine of the lugworm, in addition to the expression in celomocytes [23]. These peptides may be assumed to fulfill other functions than defense; the ability of these peptides to interact with different proteins (probably including collagen) can be of importance for various physiological processes, such as morphogenesis.

Notably, all experimental data on the interaction of AMPs with C1q, both obtained in the present work and reported previously, concern peptides with β-structure stabilized by disulfide bonds predominating in the molecule (α- and β-defensins, protegrin, tachyplesin, arenicin). The latter three peptides form β-hairpin structures. Positive charge and amphipathic properties of the molecule are characteristic of these peptides, as well as for most other AMPs. These structural properties may favor the formation of complexes between the peptides and C1q. Human cathelicidin LL-37, which was the object of our previous studies, does not contain cysteine residues, is prone to α-helix formation, and devoid of the ability to interact with C1q [8]. Despite the absence of sequence homology between protegrins, on one hand, and arenicins and tachyplesins, on the other hand, a common amino acid motif including a cationic amino acid residue, a hydrophobic amino acid residue, and a cysteine residue involved in the formation of a disulfide bond, is present in the *N*-terminal part of these molecules: the sequence of this fragment is RWC in arenicin-1 (amino acid residues 1–3), KWC in tachyplesin-1 (residues 1–3), and RLC in protegrin-1 (residues 4–6). Further experiments are required to assess the role of this motif in the interaction of peptides with C1q.

Thus, we have demonstrated specific interaction of arenicin-1 with the human protein C1q that implies the possibility of using arenicin as a base for development of pharmaceuticals targeting the complement system.

EXPERIMENTAL

Peptide isolation. Arenicin-1 and protegrin-1 were isolated from lugworm celomocytes and porcine leukocytes, respectively, according to the procedures described previously [11, 13].

C1q isolation. Blood serum from healthy donors was used to obtain C1q. The procedure of C1q isolation was modified from that reported previously [18]. The proteins were precipitated by 7% PEG 3350 and redissolved in 0.01 M sodium phosphate buffer, pH 7.4 (buffer A) containing 0.075 M NaCl and 2 mM EDTA. The solution was centrifuged (10000 g, 40 min), filtered through a membrane with pore diameter 45 μm, and fractionated using cation exchange chromatography on carboxymethyl cellulose. The column was washed with buffer A containing 0.075 M NaCl and 2 mM EDTA immediately after application of the sample, and thus the complex between C1q and the proteinases C1r and C1s was destroyed. The proteins were eluted in a linear gradient of NaCl concentration (0.075–1 M) in buffer A supplemented with 2 mM EDTA. The fractions containing C1q (as shown by electrophoresis) were pooled, diluted to the final NaCl concentration of not more than 0.15 M, and used for the further affinity chromatography-based purification of C1q. A column with immobilized human myeloperoxidase was used for the affinity chromatography. A PBS solution of rabbit immunoglobulins precipitated by ammonium sulfate from the specific antiserum was loaded onto the column for the immune complex to be formed. The column with immobilized myeloperoxidase and the rabbit antiserum specific for this protein were obtained by the authors previously for use in research not related to the present study. Elution of C1q bound to the column was performed using buffer A supplemented with 1 M NaCl and 2 mM EDTA. The C1q solution obtained was dialyzed against PBS.

Western blotting. The protein components of the sample were separated by SDS electrophoresis [24] and transferred to a nitrocellulose membrane [25]. Immunochemical detection was performed according to the standard procedure. A, B, and C subunits of the protein C1q were detected using polyclonal antibodies from Sigma-Aldrich (HPA 002350, HPA052116, and HPA001471, respectively) produced against synthetic peptides identical to fragments of polypeptide chains of the C1q subunits. The bound antibodies were detected using peroxidase-conjugated antibodies targeting rabbit immunoglobulin G. Peroxidase bound to the membrane was detected using the reaction of luminol oxidation.

Interaction of the peptides with C1q. The peptides under investigation were adsorbed to the wells of a polystyrene plate (at 0.5 μg per well) in 0.05 M sodium carbonate buffer, pH 9.5, during 2 h at +37°C. The well surface was subsequently blocked by 1% BSA in PBS containing 0.05% Tween-20. Enzyme-linked receptor sorbent assay of the ability of peptides to interact with C1q involved the addition of 20 μg of horseradish peroxidase-conjugated C1q into each well; the conjugate was obtained using the periodate method [26]. The plates used for ELISA experiments were sequentially incubated with 20 μg C1q, polyclonal antibodies to the C subunit of C1q (Sigma-Aldrich, AV-35418), and peroxidase-conjugated antibodies targeting rabbit immunoglobulins G. All incubations were performed for 1 h at +37°C, and all components were diluted in PBS supplemented with 0.05% Tween-20. The experiments addressing the effects of ionic strength on the stability of the complexes involved the addition of 0.5 M NaCl to the buffer used for the incubation of the samples with the C1q-peroxidase conjugate (in receptor sorbent assay) or with the free C1q (in ELISA). The peroxidase reaction used in both procedures was performed with *ortho*-phenylendiamine as the substrate of oxidation, the time of incubation with the substrate was 10 minutes, and the results were quantitated using photometry at the wavelength of 492 nm. Mean values ± standard deviation are shown on the graphs. The significance of the differences was assessed using pairwise Student's t-test.

ACKNOWLEDGMENTS

The present work was supported by a grant from the Federal Target Program "Research and Development on Priority Directions of Scientific Technological Complex of Russia for 2014–2020" (agreement no. 14.604.21.0104); unique identifier for Applied Scientific Research (project) is RFMEFI60414X0104.

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Translated by S. Semenova