Isolation of human cationic antimicrobial protein-18 from seminal plasma and its association with prostasomes

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BACKGROUND: Cathelicidins are a group of antibiotic peptides with broad antimicrobial activity. They are considered to be an essential part of the innate immune system. The only known human cathelicidin is the human cationic antimicrobial protein (hCAP-18), from which the antimicrobial peptide LL-37 is released. METHODS AND RESULTS: In the present study, we purified hCAP-18 from seminal plasma and confirmed its identity by N-terminal amino acid sequencing. Gel filtration of seminal plasma showed the presence of hCAP-18 in both a low and a high molecular weight peak. Fractions corresponding to the high molecular form of hCAP-18 also contained dipeptidyl peptidase IV (CD26), a prostasome marker. This finding suggested that hCAP-18 found in fractions corresponding to high molecular weight molecules, is prostasome-associated. Flow cytometry confirmed the association of hCAP-18 with prostasomes and indicated that the molecule is surface bound. Western blot showed the presence of intact hCAP-18 in sperm, prostasomes and ultracentrifuged seminal plasma. CONCLUSIONS: These findings suggest that hCAP-18 may have an important role in antimicrobial defence during human reproduction. The binding of hCAP-18 to prostasomes indicates that protasomes can serve as a reservoir of this precursor of the antibiotic peptide LL-37.

Key words: cathelicidin/hCAP-18/LL-37/prostasome/semen

Introduction

Innate immunity is important for the defence of the body against potentially invasive pathogenic micro-organisms. In contrast to adaptive immunity, it provides a rapid and non-specific response (Fearon and Locksley, 1996). In recent years, several antimicrobial peptides have been discovered to be effector molecules in innate immunity, therefore serving as the body’s first line of defence (Boman, 1995). One group of peptide antibiotics is the cathelicidin family. The human cationic antimicrobial protein (hCAP-18) is the only known member of this family of proteins in man (Larrick et al., 1994; Cowland et al., 1995). The antimicrobial peptide LL-37 becomes activated when released from the C-terminal end of the hCAP-18 holoprotein. LL-37 has a broad antimicrobial activity against both Gram-positive and Gram-negative bacteria, and also against many fungal species (Bals et al., 1998). It is synthesized in the bone marrow in progenitors of the neutrophil series of differentiation and is stored as an 18 kDa protein in specific, peroxidase-negative granules of mature neutrophils (Sorensen et al., 1997a). The gene coding for hCAP-18, CAMP (cathelicidin antimicrobial peptide), is also expressed in the epithelium of other tissues such as the airway epithelium (Bals et al., 1998), mouth, tongue, oesophagus, vagina, cervix (Frohm Nilsson et al., 1999) and epididymis (Malm et al., 2000). In addition to its antimicrobial activity, LL-37 has chemotactic activity for neutrophils, monocytes and T cells (Agerberth et al., 2000; De et al., 2000). LL-37 interacts with the formyl peptide receptor-like 1 receptor on the surface of these cells (De et al., 2000). LL-37 may thereby contribute to both innate and adaptive immunity, the latter by recruiting immunocompetent cells to sites of microbial invasion.

We have recently shown that hCAP-18 is expressed in the male reproductive system (Malm et al., 2000). High levels were found in seminal plasma and hCAP-18 was also associated with sperm. High expression of the hCAP-18 gene was demonstrated in the epithelium of epididymis.

In this study, hCAP-18 was purified from seminal plasma and its identity confirmed by N-terminal amino acid sequencing. We also show that hCAP-18 appears in two major peaks after gel filtration of seminal plasma. hCAP-18 in the high molecular peak represents hCAP-18 bound to prostasomes, small membrane-bound vesicles derived from prostate epithelial cells.

Materials and methods

Semen samples and preparation of prostasomes

Freshly ejaculated semen from healthy volunteers was collected at the Fertility Center, University Hospital MAS, Malmö, Sweden and
the Department of Growth and Reproduction, Copenhagen University Hospital, Denmark. After semen liquefaction for 1 h at room temperature, the ejaculates were centrifuged for 20 min at 1000 g at room temperature in order to separate sperm from seminal plasma. The pellet was suspended in 0.1 ml 30 mmol/l Tris–HCl buffer, pH 7.6, containing 0.13 mol/l NaCl. Cellular debris was pelleted by centrifugation at 10 000 g for 10 min at room temperature. The prostasomes were separated from seminal plasma by ultracentrifugation for 2 h at 109 000 g at room temperature. The pellet was resuspended in 0.1 ml 30 mmol/l Tris–HCl, 0.13 mol/l NaCl, pH 7.6. Sperm and prostasomes were washed three times in 1 ml of the same buffer.

**Isolation of hCAP-18 from seminal plasma and N-terminal amino acid sequencing**

An immunoglobulin fraction of rabbit anti-hCAP-18 antiserum (Sorensen et al., 1997b) was coupled to Affi 10 gel (BioRad, Hercules, CA, USA), 7 mg antibody/ml gel. A fresh sample of seminal plasma (4 ml) diluted 1:3 in equilibration buffer [50 mmol/l Tris–HCl, 0.5 mol/l NaCl, 0.1% Triton-X 100 (v/v), pH 7.5] was applied to the affinity chromatography column. The bound protein was eluted by lowering the pH [0.1 mol/l glycine, 0.5 mol/l NaCl, 0.1% Triton-X 100 (v/v), pH 2.2]. The fractions were analysed by Western blotting using rabbit anti-hCAP-18 antibody. Fractions containing hCAP-18 were pooled and concentrated using a Centricron 3 (Millipore, Bedford, MS, USA) centrifugal filter. The protein present in the pooled and concentrated fractions was cleaved by pyrogglutamate aminopeptidase (EC 3.4.19.3; Sigma, St Louis, MO, USA) to remove pyroglutamate before Edman degradation. Thirty units of the enzyme were incubated with 2.7 µg hCAP-18 in 100 mmol/l Na2HPO4, pH 8.0, 10 mmol/l EDTA, 5 mmol/l dithiothreitol (ICN Biomedicals Inc., Aurora, OH, USA) and 5% (v/v) glycerol for 16 h at 50°C. After cleavage, the sample was run on sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) (see below) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The 18 kDa doublet band was cut out and N-terminal amino acid sequencing performed (Procie 494 Protein Sequencer; Applied Biosystems).

**Analysis for detection of glycosylation of hCAP-18**

The purified hCAP-18 was investigated by using the DIG Glycan/Protein Double Labeling Kit (Biocheimca Boehringer Mannheim, Mannheim, Germany). In brief, the purified hCAP-18 was subjected to electrophoresis on a SDS–PAGE (Laemmli, 1970) and the protein was then transferred onto a nitrocellulose membrane (Towbin et al., 1979). Staining of the blotted protein was performed according to the manufacturer’s instructions.

**Flow cytometry**

Prostasomes were investigated for the presence of surface-associated hCAP-18 by flow cytometry (FACScan; Becton Dickinson, San José, CA, USA). The freshly prepared prostasomes were washed extensively with PBS (50 mmol/l, pH 7.5) containing 0.1% bovine serum albumin and thereafter incubated with the primary antibody. Rabbit anti-hCAP-18 antibody (0.2 µg) was used against an amount of prostasomes corresponding to ~90 µl human semen. After repeated washings, bound hCAP-18 antibodies were detected by a secondary fluorescein isothiocyanate-labelled anti-rabbit IgG antibody (Dakopatts). The prostasomes were neither fixed nor permeabilized before analysis in the flow cytometer. As a negative control, the primary antibody was replaced with an irrelevant isotype-matched factor V antibody (kindly provided by Professor Björn Dahlbäck, Malmö, Sweden).

**SDS–PAGE and Western blot**

Prostasomes were isolated from seminal plasma and N-terminal amino acid sequencing performed (Procie 494 Protein Sequencer; Applied Biosystems). Affinity chromatography column. The bound protein was eluted by lowering the pH 2.2. The fractions were analysed by Western blotting using rabbit anti-hCAP-18 antibody. Fractions containing hCAP-18 were pooled and concentrated using a Centricron 3 (Millipore, Bedford, MS, USA) centrifugal filter. The protein present in the pooled and concentrated fractions was cleaved by pyrogglutamate aminopeptidase (EC 3.4.19.3; Sigma, St Louis, MO, USA) to remove pyroglutamate before Edman degradation. Thirty units of the enzyme were incubated with 2.7 µg hCAP-18 in 100 mmol/l Na2HPO4, pH 8.0, 10 mmol/l EDTA, 5 mmol/l dithiothreitol (ICN Biomedicals Inc., Aurora, OH, USA) and 5% (v/v) glycerol for 16 h at 50°C. After cleavage, the sample was run on sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) (see below) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The 18 kDa doublet band was cut out and N-terminal amino acid sequencing performed (Procie 494 Protein Sequencer; Applied Biosystems).

**Analysis for detection of glycosylation of hCAP-18**

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**Gel filtration of seminal plasma and immunoblots**

Seminal plasma was diluted in 1 volume of phosphate-buffered saline (PBS). A 200 µl sample was applied to a Superose 12 column (Pharmacia, Uppsal, Sweden). The gel filtration was run under non-denaturing conditions. Fractions of 0.5 ml were collected and analysed with an hCAP-18 ELISA (Sorensen et al., 1997b). To investigate a possible co-localization of prostasomes and hCAP18, an antibody against the prostasome marker dipeptidyl peptidase IV (CD26) (Schrimpf et al., 1999) was used for immunoblotting (Dakopatts, Glostrup, Denmark). A total of 6 µl of each fraction was dotted onto PVDF membranes (Millipore). After blocking in skimmed milk [3% (w/v) in 10 mmol/l Tris–HCl, pH 8.0, 150 mmol/l NaCl, 0.05% Tween 20, (TBST)] the membranes were washed and CD26 visualized using monoclonal mouse anti-human CD26 antibody (2 µg/ml; Dakopatts) and porcine anti-mouse IgG conjugated with alkaline phosphatase (Promega Corp., Madison, WI, USA) as a secondary antibody. Bound antibodies were visualized by nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Sigma) in developing buffer (100 mmol/l Tris–HCl, 100 mmol/l NaCl, 5 mmol/l MgCl2, pH 9.5).

**ELISA**

A previously described (Sorensen et al., 1997b) sandwich ELISA was used to quantify hCAP-18 in the fractions after gel filtration.

**Results**

**Isolation and N-terminal amino acid sequencing of hCAP-18 from seminal plasma**

Affinity chromatography was performed using a polyclonal rabbit anti-hCAP-18 antibody. Fresh seminal plasma was applied to the column and, after washing, hCAP-18 was eluted. Fractions containing hCAP-18 were pooled and concentrated. As judged from SDS–PAGE, the eluted hCAP-18 was >95% pure. From a single semen sample ~25 µg hCAP-18 was purified, giving a total yield of ~7%. N-terminal amino acid sequencing of the doublet band was performed after removal of pyrogglutamate. Thirteen steps were run, and 10 residues characterized corresponded to the earlier published sequence of hCAP-18 obtained from neutrophil granulocytes (Agerberth et al., 1995; Cowland et al., 1995; Larrick et al., 1995) (Figure 1).
The human cathelicidin hCAP-18 in semen

Figure 1. Isolation of hCAP-18 from seminal plasma by affinity chromatography. The sequence obtained (A) was compared with the previously published sequence of hCAP-18 from neutrophil granulocytes (B). The affinity chromatography purified protein displayed sequence identity with hCAP-18. The arrow indicates removal of pyroglutamate (<Q) by pyroglutamate aminopeptidase, before performing N-terminal amino acid sequencing.

Figure 2. hCAP-18 in seminal plasma is found both as free monomeric protein and associated with prostasomes. A fresh sample of seminal plasma was applied on a Superose 12 column. Fractions were collected and the concentration of hCAP-18 measured by ELISA. All fractions were also analysed for immunoreactivity against the prostasome marker dipeptidyl peptidase IV (CD26) (circles below graph). Presence of dipeptidyl peptidase IV (CD26) was detected in fractions 14–22, corresponding to the high molecular peak of hCAP-18 and thus indicating that hCAP-18 is associated with prostasomes.

Dual distribution of hCAP-18 in seminal plasma

Fresh seminal plasma was subjected to gel filtration and the concentration of hCAP-18 in each fraction was measured (Figure 2). There was a large variation in the total concentration of hCAP-18 in seminal plasma between donors (mean 48 µg/ml, range 12–121; n = 6). After gel filtration, two peaks appeared, one of high and one of low molecular weight (Figure 2). Irrespective of the total amount of hCAP-18, approximately the same percentage of hCAP-18 was found in each molecular form in all samples. Approximately 70% (range 64–78; n = 6) of the total hCAP-18 in the sample was found in the high molecular peak and ~30% (range 22–36; n = 6) in the low molecular weight peak. The gel filtration fractions were also analysed for the prostasome marker dipeptidyl peptidase IV (CD26). Fractions in the first peak contained CD26 whereas fractions in the low molecular weight peak did not (circles below graph in Figure 2). This indicated that the high molecular weight peak contained prostasomes. The experiment was repeated with seminal plasma from five different individuals and essentially the same pattern was seen.

hCAP-18 in sperm, prostasomes and ultracentrifuged seminal plasma

Pelleted sperm, prostasomes and ultracentrifuged seminal plasma were subjected to SDS–PAGE and Western blotting (Figure 3). Western blot analysis of a neutrophil homogenate, used as a positive control, showed a single band at 18 kDa corresponding to the hCAP-18 holoprotein. In contrast, a doublet band at 18 kDa was seen in all of the different semen components: sperm, prostasomes and ultracentrifuged seminal plasma (Figure 3). We were not able to detect any glycosylated forms of hCAP-18, as shown by using a staining protocol specific for glycoproteins (Biochemica Boehringer Mannheim). Therefore, the doublet band seen in Figure 3 is unlikely to be due to glycosylation of the protein. Neither the cathelin fragment (14 kDa) nor the LL-37 peptide (4 kDa) was seen in the semen samples.

Detection of hCAP-18 associated with the surface of prostasomes

Freshly prepared prostasomes from five different donors were analysed using a FACSscan flow cytometer. The cytogram suggested that hCAP-18 was present on the surface of prostasomes. The positive signal shown when prostasomes were exposed to hCAP-18-specific primary antibody (Figure 4A) was not seen when the hCAP-18 antibody was replaced with an isotype-matched irrelevant antibody (Figure 4B).

Discussion

In this study we have isolated hCAP-18 from seminal plasma and the identity of the protein was confirmed with N-terminal amino acid sequencing. Furthermore, we have shown that hCAP-18 has a dual distribution in seminal plasma. Gel
and essentially the same cytograms were seen. Separate experiments with prostasomes from different donors resulted in a much lower signal, representing background labelling. The data obtained is from one out of five different donors and essentially the same cytograms were used.

The presence of hCAP-18 is attached to the surface of prostasomes. By using flow cytometry, prostasomes were gated using their characteristics in forward and side scatter. (A) Bound hCAP-18 antibody, on the surface of prostasomes, was detected by a secondary fluorescein isothiocyanate-conjugated antibody indicating the presence of hCAP-18. (B) Replacement of the specific primary antibody with an irrelevant isotype-matched antibody at the same concentration resulted in a much lower signal, representing background labelling. The data obtained is from one out of five separate experiments with prostasomes from five different donors and essentially the same cytograms were seen.

Figure 4. hCAP-18 is attached to the surface of prostasomes. By using flow cytometry, prostasomes were gated using their characteristics in forward and side scatter. (A) Bound hCAP-18 antibody, on the surface of prostasomes, was detected by a secondary fluorescein isothiocyanate-conjugated antibody indicating the presence of hCAP-18. (B) Replacement of the specific primary antibody with an irrelevant isotype-matched antibody at the same concentration resulted in a much lower signal, representing background labelling. The data obtained is from one out of five separate experiments with prostasomes from five different donors and essentially the same cytograms were seen.

Prostasomes are complex membrane-bound corpuscular organelles produced by prostate epithelial cells. These organelles are expelled with the prostate secretions at ejaculation and can be purified from seminal plasma (Ronquist et al., 1978a,b). Prostasomes have a diameter of ~150 nm (Ronquist et al., 1990) and are claimed to have several functions. For example, prostasomes possess immunosuppressive capacity (Kelly et al., 1991; Skibinski et al., 1992) and after fusion with sperm (Arienti et al., 1997), they enhance sperm motility (Stegmayr and Ronquist, 1982). The biochemical background behind these effects is still unknown. Apart from hCAP-18, some other seminal plasma proteins have been found in association with prostasomes, e.g. tissue factor (Fernandez et al., 1997), the complement-regulatory protein membrane cofactor protein (CD46) (Simpson and Holmes, 1994; Kitamura et al., 1995) and membrane attack complex inhibitory protein CD59 (Rooney et al., 1993). The finding that hCAP-18 is associated with prostasome membranes is analogous to the finding that hCAP-18 is bound to the lipid part of lipoproteins in blood plasma (Sorensen et al., 1999). Essentially all hCAP-18 in blood plasma is bound to lipoproteins. Prostasome-associated hCAP-18 accounts for ~70% of all hCAP-18 in seminal plasma. The association of hCAP-18 with lipoproteins has been characterized as a non-covalent, hydrophobic interaction, with the cationic C-terminal end of LL-37 associated with the lipoprotein (Sorensen et al., 1999). It is therefore reasonable to believe that hCAP-18 in seminal plasma is associated with the prostasome membrane in the same way. This is also supported by the fact that LL-37 interacts with lipid bilayers (Turner et al., 1998).

The antimicrobial activity seen in prostasomes (Carlsson et al., 2000) could be due to hCAP-18, possibly in interaction with other antimicrobial peptides in seminal plasma such as the C terminal fragment of chromogranin B, secretelytin (Strub et al., 1995; Stridsberg et al., 1996). Expression of the CAMP gene and the large amount of hCAP-18 found in the epididymis (Malm et al., 2000) indicate its importance, probably in local defence against potentially invasive pathogenic microorganisms. hCAP-18 has also been described intracellularly in the acrosome of human sperm (Hammani-Hamza et al., 2001), and this hCAP-18 is assumed to originate from the epithelial cells of testis (Hammani-Hamza et al., 2001). It is, however, possible that hCAP-18 attached to sperm (Malm et al., 2000) originates from prostasomes which have fused with sperm.

The binding of hCAP-18 to prostasomes instead of directly to sperm could be a way of protecting sperm cells from the cytotoxic effects of LL-37. LL-37 could be cytotoxic to prokaryotes and not eukaryotic cells due to the absence of cholesterol in the plasma membrane of prokaryotes. The plasma membrane of sperm possesses only a low cholesterol content (Mack et al., 1986) compared with prostasome membranes (Arvidson et al., 1989). When prostasomes, with associated hCAP-18, are fused with sperm, the sperm membranes will be enriched in cholesterol and the sperm could subsequently become resistant to the toxicity of LL-37.

Several studies have shown that human semen has antimicrobial activity. The antimicrobial capacity has been ascribed to different components of semen, for example lysozyme (Mardh and Colleen, 1974), spermine (Williams-Ashman and Lockwood, 1970) and the high zinc concentration (Mardh and Colleen, 1975). A possible reason for the relatively weak antimicrobial effect of seminal fluid is that hCAP-18 is present predominantly in its inactive proform with the C-terminal part, LL-37, still bound to the cathelin region. Activation may take place in the uterus. About 45 min after the sperm have entered the uterus, there is an extensive invasion of granulocytes from the female. These granulocytes release high concentrations of proteases, such as elastase and proteinase 3, which kill most of the sperm cells (Phillips and Mahler, 1977). Both elastase (data not shown) and proteinase 3 (Sorensen et al., 2001) have been shown to activate hCAP-18. The neutrophil granulocytes also contribute LL-37, since hCAP-18 is released from its specific granules in parallel with the release of proteinase 3.
from the azurophilic granules (Sorensen et al., 2001). The physiological importance of semen-mediated and prostasome-mediated antimicrobial effects is easily envisaged. Both Gram-positive and Gram-negative bacteria and also some fungal species are found in the environment outside the cervix. We suggest that seminal plasma hCAP-18 is important for the protection of sperm cells in this unfriendly milieu. Free, uncomplexed hCAP-18 does not pass the cervix barrier and consequently does not enter the uterus. In contrast, hCAP-18 bound to sperm, probably transferred from prostasomes, may have its main function in the uterus or at conception in the oviduct. hCAP-18 may thus contribute to the sterile environment during fertilization.

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