The Human Antibacterial Cathelicidin, hCAP-18, Is Bound to Lipoproteins in Plasma*

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Cathelicidins are a family of antibacterial and lipopolysaccharide-binding proteins. hCAP-18, the only human cathelicidin, is a major protein of the specific granules of human neutrophils. The plasma level of hCAP-18 is >20-fold higher than that of other specific granule proteins relative to their levels within circulating neutrophils. The aim of this study was to elucidate the background for this high plasma level of hCAP-18. Plasma was subjected to molecular sieve chromatography, and hCAP-18 was found in distinct high molecular mass fractions that coeluted with apolipoproteins A-I and B, respectively. The association of hCAP-18 with lipoproteins was validated by the cofractionation of hCAP-18 with lipoproteins using two different methods for isolation of lipoproteins from plasma. Furthermore, the level of hCAP-18 in delipidated plasma was <1% of that in normal plasma. Immunoprecipitation of very low, low, and high density lipoprotein particles with anti-apolipoprotein antibodies resulted in coprecipitation of hCAP-18. The binding of hCAP-18 to lipoproteins was mediated by the antibacterial C-terminal part of the protein. The binding of hCAP-18 to lipoproteins suggests that lipoproteins may play an important role as a reservoir of this antimicrobial protein.

hCAP-18 belongs to the cathelicidins, a group of antimicrobial peptides found in mammalian neutrophils (1). The cathelicidins share a highly conserved N-terminal prosequence that is homologous to cathelin, a protein first isolated from porcine leukocytes (2). The active antimicrobial domains of the cathelicidins generally reside in their C termini. The antimicrobial activity is observed only when the C-terminal domain is cleaved from the holoprotein (3–5). The C termini of the cathelicidins show great variability in amino acid sequence, but they are all highly cationic and hydrophobic. The antimicrobial part of many cathelicidins, including the C terminus of hCAP-18 (also named LL-37), has been shown to bind lipopolysaccharide (6).

Porcine and bovine neutrophils contain a variety of cathelicidins, whereas hCAP-18 is the only cathelicidin identified in humans (6–8). hCAP-18 is a major protein of the specific granules of human neutrophils (9), but is also present in squamous epithelia (10) and in keratinocytes during inflammatory skin diseases (11). Transcripts for hCAP-18 have been found in lung tissue by in situ hybridization (12).

We have previously shown that the relative plasma levels of specific granule proteins from neutrophils are very low compared with the levels in circulating neutrophils (<1%) (13). In contrast, the concentration of hCAP-18 in plasma is ~1.2 μg/ml, which is >20% of the amount present in circulating neutrophils (14). In general, neutrophils are activated to release their granule proteins only when present outside the circulation. Thus, degranulation is unlikely to be the cause of this high plasma level of hCAP-18 since other granule proteins localized in the same granule subset would be expected to have equally high plasma levels as hCAP-18. This is not the case. Thus, a specific mechanism must exist to sequester hCAP-18 in the circulation and provide the relatively high concentration of this pro-bactericidal protein in plasma. Since hCAP-18 partitions mainly in the hydrophobic phase during Triton X-114 phase separation (8), we reasoned that hCAP-18 might partition into lipoprotein particles, possibly in the same way as the bactericidal C terminus of hCAP-18 is believed to insert into the phospholipid bacterial membrane and cause bacterial lysis. In the following, we demonstrate that hCAP-18 is found in plasma complexed with lipoproteins, and we suggest that lipoproteins may serve as a reservoir of a pro-bactericidal substance in plasma.

EXPERIMENTAL PROCEDURES

Materials

Blood was obtained from healthy volunteers and used to prepare human plasma anticoagulated with EDTA. Specific rabbit anti-hCAP-18 antibodies were generated by immunization of rabbits with recombinant hCAP-18 (14).

SDS-PAGE1 and Immunoblotting

SDS-PAGE (15) and immunoblotting (16) were performed according to the instructions given by the manufacturer (Bio-Rad). For immunoblotting, polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA) were blocked for 1 h with 5% skimmed milk in PBS after the transfer of proteins from 14% polyacrylamide gels. For visualization of hCAP-18, the polyvinylidene fluoride membranes were incubated overnight with protein A-purified rabbit anti-hCAP-18 antibodies. The following day, the membranes were incubated for 2 h with peroxidase-conjugated porcine antibodies to rabbit immunoglobulins (Dako, Glostrup, Denmark) and visualized by diaminobenzidine/metal concentration and Stable Substrate Buffer (Pierce).

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; HSA, human serum albumin.

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Purification of hCAP-18 from Neutrophils

Isolation of Neutrophils—Human neutrophils were isolated from freshly prepared buffy coats as described (17). Briefly, after sedimentation with 2% dextran T-500 (Amersham Pharmacia Biotech, Uppsala, Sweden) in isotonic NaCl, the leukocyte-rich supernatant was pelleted and resuspended in saline for subsequent centrifugation on Lymphoprep (Nycomed Pharma A/S, Oslo, Norway) at 400 x g for 30 min for removal of lymphocytes and monocytes. The remaining erythrocytes were lysed in ice-cold water for 30 s. Toxicity was restored by the addition of 1% NaCl. The cells were washed once and resuspended in the desired buffer. All steps were carried out at 4 °C, except sedimentation in dextran, which was carried out at room temperature.

Subcellular Fractionation—Neutrophils were disrupted by nitrogen cavitation after the addition of 5 mM disopropyl fluorophosphosphate (Sigma). The post-nuclear supernatant was loaded on a three-layer Percoll gradient (1.05/1.09/1.12 g/ml; Amersham Pharmacia Biotech) (18) and centrifuged for 30 min at 37,000 x g. This resulted in four visible bands. Starting at the bottom, the bands were designated the α-band, which contains the azurophil granules; the β1-band, which contains the specific granules; the β2-band, which contains the gelatinase granules; and the γ-band, which contains the plasma membranes and the secretory vesicles.

The β1-band containing specific granules was harvested, and the Percoll removed by ultracentrifugation. The granules were lysed in PBS containing 1% Triton X-100 (Roche Molecular Biochemicals, Heidelberg, Germany), 1 mM phenylmethylsulfonyl fluoride (Sigma), 100 μg/ml kalikrein inhibitory units/ml aprotinin (Bayer, Leverkusen, Germany), 100 μg/ml leupeptin (Sigma), and 1 mM EDTA (Sigma). The lysate was centrifuged, and the supernatant was applied to an affinity chromatography column with anti-hCAP-18 antibodies immobilized on CNBr-activated Sepharose (Amersham Pharmacia Biotech) as described by the manufacturer. The column was washed extensively, and the bound protein was eluted with 0.2 M glycine HCl (pH 2.5). The purity of the eluted protein was assessed by SDS-PAGE.

Preparation of Exocytosed Material from Neutrophils

Isolated neutrophils from freshly prepared buffy coats were resuspended in Krebs-Ringer phosphate solution (10 mM NaH2PO4/Na2HPO4, 130 mM NaCl, 5 mM KCl, 0.95 mM CaCl2, and 5 mM glucose) at a concentration of 3 x 107 cells/ml. Cells were preincubated at 37 °C for 5 min and then stimulated with 1 μM ionomycin (Calbiochem) for 20 min at 37 °C. The stimulation was stopped by placing the cells on ice. The cells were pelleted by centrifugation, and the supernatant containing the exocytosed material was harvested and stored at −20 °C until further use.

Purification of Recombinant Cathelin and hCAP-18

A recombinant form of cathelin, the N-terminal part of hCAP-18, was produced using the baculovirus expression vector system. The cDNA for the cathelin part of hCAP-18 was polymerase chain reaction-amplified from a human bone marrow cDNA library (CLONTECH, Palo Alto, CA) using specific primers and was cloned into the pAcGP67(b) vector (Pharmingen, San Diego, CA). The sequence of the construct was checked by DNA sequencing. Recombinant protein was produced by Sf9 cells (Pharmingen) after cotransfection of the cells with recombinant pAcGP67(b) and BaculoGold DNA (Pharmingen). The recombinant protein was harvested from the supernatant of the infected Sf9 cells and purified by affinity chromatography as described above for native hCAP-18. SDS-PAGE and subsequent staining with Coomassie Blue of the purified protein showed a single band of the expected molecular mass. This band reacted with anti-hCAP-18 antibodies in immunoblotting. Recombinant hCAP-18 was produced and purified as described (14).

Purification and Identification of Cathelin

Fragments of hCAP-18, exocytosed from neutrophils after stimulation with ionomycin, were affinity-purified on an anti-hCAP-18 antibody column. The eluted material was subjected to anion-exchange chromatography on a MonoQ column (Pharmacia Biotech) using a linear gradient of 0-1 M NaCl in 50 mM Tris (pH 8.0). One peak containing a 14-kDa protein was eluted at 0.2 M NaCl. The sample was concentrated on a Centricron 10 microcentricon (Amicon, Inc., Beverly, MA) and subjected to gel filtration on a Superose 12 column (Amersham Pharmacia Biotech). The protein was re-purified and desalted by reverse-phase HPLC employing a Vydac C4 column (2.1 x 150 mm) equilibrated with 10% solvent B (0.1% trifluoroacetic acid in acetonitrile) and eluted with a 1%/min gradient from solvent A (0.1% trifluoroacetic acid) to solvent B. An aliquot was analyzed by mass spectrometry (see below) using horse myoglobin as an internal standard, whereas the remainder was reduced and derivatized with iodoacetamide as described by Manoharan (19), followed by HPLC purification as described above. The derivatized cathelin was digested with endoproteinase Asp-N (Roche Molecular Biochemicals) as described by the manufacturer. The resulting fragments were separated by HPLC on a Vydac C8 column (2.1 x 150 mm) using the solvents described above. Peak fractions were collected manually. Intact cathelin and proteolytic fragments were subjected to matrix-assisted laser desorption mass spectrometry in a Biflex instrument (Bruker-Franzen) using a-cyano-4-hydroxycinnamic acid as the matrix. Sequence analysis was performed on a 494 A Procise Protein Sequencer (Perkin-Elmer).

Immunodiffusion

Immunodiffusion of affinity-purified hCAP-18 from plasma was carried out as described by Ouchterlony and Nilsson (20).

Precipitation of LDL and VLDL

LDL was precipitated from plasma as described (21) with minor modifications. Plasma (0.5 ml) was mixed with 0.05 ml (10 g/liter) of dextran sulfate (Amersham Pharmacia Biotech) in 0.5 M MgCl2 and incubated for 10 min at room temperature, followed by centrifugation at 12,000 x g for 5 min. The supernatant was collected manually, and the pellet was resuspended in 0.5 ml of PBS.

Separation of Lipoproteins in Plasma by Ultracentrifugation

Plasma was adjusted to densities of 1.060 and 1.215 by the addition of solid KBr. The 350-μl sample was centrifuged in an Airfuge at 100,000 x g for 2.5 h as described (22). Fractions of 100 μl were collected manually from the top and the bottom, respectively, for analysis of plasma proteins as described below.

Preparation of Lipoprotein-deficient Plasma

The density of plasma was adjusted to 1.215 by the addition of solid KBr. Samples of 400 μl were centrifuged for 28 h in an Airfuge at 100,000 x g. Delipidated plasma (200 μl) was collected from the bottom of the tube with a syringe to prevent contamination with lipoproteins from the top phase. Delipidated plasma was dialyzed against PBS to remove the KBr before further use.

Quantitation of Proteins

IgG, IgM, IgA, apolipoprotein A-I (a marker of HDL), apolipoprotein B (a marker of VLDL and LDL), and albumin were quantitated by a semiquantitative enzyme-linked immunosorbent assay. The samples were diluted in 50 mM Na2CO3/NaHCO3 buffer (pH 9.6) and incubated in 96-well flat-bottom immunoplates (Nunc, Roskilde, Denmark) overnight at 4 °C. Unspecific binding was blocked by incubation with 200 μl/well dilution buffer (0.5 M NaCl, 3 mM KCl, 8 mM Na2HPO4/KH2PO4, 1% bovine serum albumin (Sigma), and 1% Triton X-100 (pH 7.2) for 1 h. Rabbit antibodies (Dako) against the above-mentioned antigens were diluted 2000-fold in dilution buffer and incubated for 2 h. Horse-radish peroxidase-labeled goat anti-rabbit antibodies (Dako) were diluted 1000-fold in dilution buffer and incubated for 1 h. The plates were washed three times in wash buffer (0.5 M NaCl, 3 mM KCl, 8 mM Na2HPO4/KH2PO4, and 1% Triton X-100 (pH 7.2)) after each incubation using a Microwash-II (Skatron, Roskilde). The plates were washed once in substrate buffer (0.1 M sodium phosphate and 0.1 mM citric acid (pH 5.0)) prior to color development and then incubated with substrate buffer containing 0.04% ortho-phenylendiamine (Kem-En-Tec, Copenhagen, Denmark) and 0.03% H2O2 and incubated for each incubation step unless otherwise stated. The color development was stopped by the addition of 100 μl of 1 N H2SO4; absorbance was measured at 492 nm in a Multiscan Plus ELISA Reader (Labsystems, Helsinki, Finland); and the concentrations are expressed as absorbance units (read at 492 nm). An arbitrary standard of diluted plasma was used in the experiments with immunoprecipitations and gel filtration of plasma. hCAP-18 was measured by enzyme-linked immunosorbent assay as described previously (14).

Gel Filtration of Plasma

Plasma was diluted with 1 volume of PBS. A 200-μl sample was applied to a Superose 12 column. Fractions of 0.5 ml were collected and analyzed for their content of hCAP-18, apolipoproteins B and A-I, and IgA. Molecular mass standards (Amersham Pharmacia Biotech) and
Concentrations of apolipoproteins are given as absorbance units at 492 nm and subsequently incubated with purified hCAP-18 before gel filtration. The density was increased to 1.215. Delipidated plasma was dialyzed against PBS 0.5% HSA was similarly subjected to gel filtration. Plasma was subjected to gel filtration, and the content of apolipoprotein A-I, apolipoprotein B, and hCAP-18 in plasma was indicated. Purified hCAP-18 in PBS with 0.5% HSA was similarly subjected to gel filtration. Plasma was subjected to gel filtration, and the content of apolipoprotein A-I, apolipoprotein B, and hCAP-18 in the fractions was measured. Concentrations of apolipoproteins are given as absorbance units at 492 nm. Gel filtration of delipidated plasma incubated with purified hCAP-18. Plasma was delipidated by ultracentrifugation after adjusting the density to 1.215. Delipidated plasma was diazylized against PBS and subsequently incubated with purified hCAP-18 before gel filtration.

endogenous IgA were used to estimate the molecular sizes of the plasma proteins investigated.

**Immunoprecipitation**

Antibodies against apolipoproteins A-I and B, normal rabbit immunoglobulins, and hCAP-18 were immobilized on CNBr-activated Sepharose. Plasma was diluted 200-fold in PBS, and 400 μl were incubated with 40 μl of antibodies coupled to Sepharose. The Sepharose particles were pelleted by centrifugation after 4 h of incubation at room temperature. The supernatants were aspirated, and the pellets were washed three times in PBS before elution with glycine HCl (0.2 M, pH 2.5). The Sepharose beads were pelleted again, and the eluted material was aspirated and neutralized by the addition of 2 μl Tris-HCl (pH 8). Protein concentrations were measured in the supernatant and pellet after immunoprecipitation. The fractions containing the highest concentration of hCAP-18 after gel filtration of plasma (fractions 17 and 23) were diluted 5-fold in PBS with 1% bovine serum albumin, and immunoprecipitation was performed as described above.

**RESULTS**

**Gel Filtration**—When hCAP-18 isolated from the specific granules of neutrophils was applied to molecular sieve chromatography on a Superose 12 column, a major peak of hCAP-18 was found in fraction 29 (15–30 kDa) as expected for monomeric hCAP-18. When isolated hCAP-18 was subjected to chromatography in the presence of HSA, a minor high molecular mass peak of hCAP-18 was observed in fraction 22 (Fig. 1A). When human plasma was subjected to gel filtration, two major high molecular mass peaks of hCAP-18 were observed at fraction 17 (void volume) and fraction 22 (150 kDa), and no hCAP-18 was found at 15–30 kDa (Fig. 1A). This indicates that the high molecular mass complexes of hCAP-18 found in plasma were not caused by self-aggregation of hCAP-18. When plasma was separated by SDS-PAGE under reducing and non-reducing conditions, followed by immunoblotting with anti-hCAP-18 antibodies, the only band observed was at the expected molecular mass of 18 kDa (Fig. 1A). This indicates that hCAP-18 is bound noncovalently to a high molecular mass component present in plasma. To identify the nature of these high molecular mass complexes, hCAP-18 was isolated from plasma by affinity chromatography using anti-hCAP-18 antibodies. The resulting eluate contained several proteins, including hCAP-18 and apolipoproteins, as judged by immunodiffusion and SDS-PAGE (data not shown).

It has previously been found that hCAP-18 from neutrophil granules is very hydrophobic and partitions into the Triton-rich (hydrophobic) phase during phase separation of neutrophil granules with Triton X-114 (8). We therefore reasoned that hCAP-18 might be associated with lipoproteins.

Analysis of the fractions obtained by gel filtration of plasma showed that the low molecular mass peak of hCAP-18 co-localized with apolipoprotein A-I and that the high molecular mass peak co-localized with apolipoprotein B (Fig. 1B). To investigate this further, lipoproteins were isolated from plasma, and their content of hCAP-18 was determined.

**LDL/VLDL Precipitation**—80% of hCAP-18 and almost 100% of apolipoprotein B, but no IgG, IgM, albumin, or apolipoprotein A-I present in plasma, coprecipitated with LDL/VLDL (by the dextran sulfate method) (Fig. 2A). To ensure that hCAP-18 itself is not precipitated by this method, a sample of purified hCAP-18 was precipitated under the same conditions. No significant precipitation of hCAP-18 was found (Fig. 2B).

**Separation of Lipoproteins by Ultracentrifugation**—Ultracentrifugation was used to further examine the association of plasma hCAP-18 with lipoproteins. When plasma was subjected to ultracentrifugation at a density of 1.060, VLDL and LDL were found in the top fraction, and HDL was found in the bottom fraction; hCAP-18 was found in both the HDL- and LDL/VLDL-enriched fractions (Fig. 3). Common plasma proteins like IgG and albumin were more evenly distributed in the fractions at all densities examined. Thus, hCAP-18 in plasma was found to partition with lipoproteins obtained by two different separation procedures.

As an additional control, plasma was delipidated through a 28-h ultracentrifugation after adjustment of the density to 1.215 with solid KBr. Less than 1% of the original concentration of hCAP-18 was found in plasma after delipidation, whereas the concentrations of the common plasma proteins like albumin, IgG, and IgM were increased (Table I). A small amount of hCAP-18 was incubated with delipidated plasma for 2 h at 37°C. hCAP-18 in delipidated plasma was then subjected either to gel filtration or to ultracentrifugation (after adjustment of the density to 1.215). In the gel filtration experiment, exogenous hCAP-18 was found in the low molecular mass fractions (Fig. 1C). Following ultracentrifugation of the delipidated plasma, the added hCAP-18 was present at the same concentrations in the top and bottom fractions (Table I), as expected when no formation of complexes takes place.

**Immunoprecipitation from Plasma**—To further substantiate the association of hCAP-18 with lipoproteins in plasma, immunoprecipitation was performed with antibodies against hCAP-18, apolipoprotein A-I (HDL), and apolipoprotein B (VLDL and LDL).
addition of solid KBr before ultracentrifugation. After centrifugation, the density of the plasma sample was adjusted to 1.215 by the adjustment of the density and ultracentrifugation. hCAP-18 was incubated with delipidated plasma (dialyzed against PBS), followed by centrifugation, 100 μl were collected from the bottom (bottom fraction) and 100 μl from the top (top fraction); the remaining 100 μl were the middle fraction. The concentration in the sample before the precipitation. All concentrations are given as percent of the initial concentration in the supernatant. The concentrations are presented as percent of the total concentration (bottom plus top) in the samples. Black bars represent the bottom fraction, and gray bars represent the top fraction. Alb., albumin.

**TABLE I**

| Fraction          | hCAP-18 | ApoA-I | ApoB | Albumin | IgG | IgM | hCAP-18 in delipidated plasma |
|-------------------|---------|--------|------|---------|-----|-----|-----------------------------|
| Bottom fraction   | 0.3     | 7.2    | 1.6  | 83.1    | 68.5| 54.2| 49.9                        |
| Middle fraction   | 35.9    | 19.6   | 22.9 | 13      | 29.5| 43.5| 25                          |
| Top fraction      | 43.8    | 73.2   | 75.5 | 3.9     | 2   | 2.3 | 25.2                        |

**FIG. 2. LDL/VLDL precipitations.** A. LDL/VLDL precipitation of plasma. LDL/VLDL from plasma was precipitated by the dextran sulfate method. Gray bars represent the concentration in the supernatant after precipitation, and black bars represent the concentration in the precipitate after resuspension of the precipitate to a volume equal to that of the supernatant. The concentrations are given as percent of the initial concentration in the sample before the precipitation. Alb., albumin. B. LDL/VLDL precipitation of a sample of purified hCAP-18 and 0.5 mg/ml HSA. A sample of purified hCAP-18 with 0.5 mg/ml HSA was precipitated by the dextran sulfate method. The precipitate was resuspended to a volume equal to that of the supernatant. Gray bars represent the concentration in the supernatant, and black bars represent the concentration in the precipitate.

**FIG. 3. Separation of lipoprotein from plasma by ultracentrifugation.** The density of the plasma sample was adjusted to 1.215 by the addition of solid KBr before ultracentrifugation. After centrifugation, 100 μl were collected from the top and bottom, respectively. The data are presented as percent of the total concentration (bottom plus top) in the samples. Black bars represent the bottom fraction, and gray bars represent the top fraction. Alb., albumin.

LDL). 46% of hCAP-18 was found in the apolipoprotein B precipitate (n = 4, S.D. = 10.5), and 17.3% was found in the apolipoprotein A-I precipitate (n = 4, S.D. = 12.2). It was not possible, however, to immunoprecipitate all of the lipoproteins from plasma without increasing the antibody concentration to an extent that resulted in high unspecific precipitation. No hCAP-18 was found after precipitation with preimmune rabbit immunoglobulins, whereas >96% of hCAP-18 was precipitated with anti-hCAP-18 antibodies. Immunoprecipitation of hCAP-18 present in plasma resulted in coprecipitation of 30% of LDL/VLDL (n = 4, S.D. = 15.2) and 12.4% of HDL (n = 4, S.D. = 10.8), indicating that not all lipoproteins in plasma are associated with hCAP-18.

**Immunoprecipitation from Fractions after Gel Filtration of Plasma**—The association of hCAP-18 with apolipoproteins during gel filtration of plasma was investigated by immunoprecipitation of the fractions that contained peak concentrations of hCAP-18. No hCAP-18 was precipitated from fraction 17 (void volume), but 48.3% (n = 4, S.D. = 10.3) of hCAP-18 was precipitated with anti-apolipoprotein A-I antibodies from fraction 23 (150 kDa). 72.9% of hCAP-18 in fraction 17 was precipitated with anti-apolipoprotein B antibodies (n = 4, S.D. = 19.8), and 40.5% was precipitated in fraction 23 (n = 3, S.D. = 11.5). All of apolipoproteins A-I and B were precipitated, respectively.

The results of the specific lipoprotein isolation and the immunoprecipitation indicate that 80% of hCAP-18 is bound to LDL or VLDL. The 20% of hCAP-18 in plasma that is not isolated with LDL/VLDL-specific precipitation is bound to HDL and represents the amount of hCAP-18 that was precipitated with anti-apolipoprotein A-I antibodies in fractions with a molecular mass of ~150 kDa after gel filtration of plasma.

**Capacity of Lipoproteins to Bind hCAP-18**—Different amounts of hCAP-18 purified from neutrophils were added to EDTA-treated plasma and incubated for 2 h at 37°C, followed by gel filtration. The fractions were subsequently analyzed for their content of hCAP-18. More than 85% of hCAP-18 was still found in the high molecular mass complexes even when >20 times the endogenous amount of hCAP-18 was added to the plasma (Fig. 5).

**Identification of the Lipoprotein-binding Domain of hCAP-18**—The antimicrobial active domain of hCAP-18 (LL-37) is cleaved off during exocytosis from human neutrophils (23). Immunoblotting (with anti-hCAP-18 antibodies) of the exocytosed material revealed three bands with apparent molecular masses of 18, 14, and 4 kDa (Fig. 6, lane A). The molecular mass of hCAP-18, calculated from the amino acid sequence, is 16 kDa, 11.5 kDa for cathelin and 4.5 kDa for LL-37. To confirm that the 14-kDa band seen by immunoblotting was the cathelin part of hCAP-18, the exocytosed material from neutrophils was

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The detailed figures (FIG. 2, FIG. 3, FIG. 5, FIG. 6) are not transcribed here but are available in the original document.
The legend to Fig. 1). The concentration of apolipoproteins are given as absorbance units at 492 nm (the same fraction volume and column as described in the legend to Fig. 1).

All the fragments generated were from the cathelin part of hCAP-18, and the last fragment detected (DNKRFA) was consistent with the C terminus of cathelin (Fig. 7). Furthermore, 66. Fragment 42–67 was also detected.

The molecular mass of the purified intact 14-kDa fragment was determined by mass spectrometry. The protein was heterogeneous, showing three peaks at 11,910, 12,178, and 12,462 Da, respectively, compared with 11,520 Da calculated for the cathelin part of hCAP-18 (Fig. 4). The heterogeneity of the protein and the discrepancy from the calculated value remain unexplained, but do suggest either a post-translational modification located in one or more of the non-recovered fragments or a non-consensus cleavage by Asp-N after the C-terminal alanine (Fig. 7). Thus, the data obtained by mass spectrometry and the sequences from the Asp-N-derived fragments identified the 14-kDa fragment as the cathelin part of hCAP-18. To substantiate that the 4-kDa fragments seen by immunoblotting represented the antibacterial C terminus of hCAP-18, the excised material from neutrophils was subjected to SDS-PAGE and immunoblotting with anti-hCAP-18 antibodies. The addition of recombinant hCAP-18 in excess abolished the binding of the antibodies to all three bands, thus demonstrating that all three bands represent hCAP-18 or fragments of the protein (Fig. 6, lane B). When an excess of recombinant cathelin was added, binding to the 14-kDa band was abolished, whereas the holoprotein of 18 kDa and the 4-kDa band were still labeled by the antibody (Fig. 6, lane C). This identified the 4-kDa band as the non-cathelin C terminus of hCAP-18.

When the excised material from neutrophils was subjected to gel filtration, all detectable hCAP-18 was of low molecular mass (monomeric form) (data not shown). The excised material was incubated with plasma and subjected to gel filtration, and the 14-kDa fragment of hCAP-18 was reduced, and the Cys residues were derivatized with iodoacetamide. The protein was then digested with endoproteinase Asp-N, and the resulting fragments were separated by HPLC. The theoretical fragments (according to the specificity of the enzyme) are listed as well as their calculated molecular masses (see also Fig. 7). The molecular masses of the isolated fragments were measured by mass spectrometry. A number of fragments were not detected (ND). All of these were calculated to have lower retention times than any of the recovered fragments; thus, they most likely eluted in the void volume.

| hCAP-18 fragment | Calculated | Measured |
|-----------------|------------|----------|
| 1–13            | 1472.8     | 1472.9   |
| 14–21           | 875.9      | ND       |
| 22–29           | 977.1      | 977.2    |
| 30–31           | 246.3      | ND       |
| 32–37           | 715.8      | ND       |
| 38–39           | 190.2      | ND       |
| 40–41           | 230.2      | ND       |
| 42–67           | 3008.3     | 3008.5   |
| 68–71           | 536.6      | ND       |
| 72–91           | 2210.6     | 2210.1   |
| 92–95           | 493.5      | ND       |
| 96–97           | 261.3      | ND       |
| 98–103          | 749.4      | 749.4    |

*This is the result of incomplete cleavage between Glu-65 and Asp-66. Fragment 42–65 was also detected.*
Our results demonstrate that it is the antibacterial C terminus of hCAP-18 (LL-37) that is bound to lipoproteins. LL-37 is antibacterial and cytotoxic, depending on the degree of α-helical conformation of the peptide (25). The antibacterial and cytotoxic effect of LL-37 is inhibited by serum, presumably by binding to a serum protein (25). Two synthetic α-helical peptides have been found to have their cytotoxic effect attenuated by binding to LDL in plasma (26). These observations indicate that lipoproteins may play a role in protection against the harmful effects of LL-37 and other α-helical amphiphilic peptides by scavenging these peptides.

The LDL/VLDL particles have a different protein composition than HDL, and as the binding of hCAP-18 to lipoproteins was due to hydrophobic interactions, it seems likely that hCAP-18 (through the C terminus) interacted with the lipid bilayer of the lipoproteins. This hypothesis is supported by the fact that synthetic LL-37 has been shown to bind to liposomes (27).

Even though cathelicidins and other antibacterial peptides differ greatly in amino acid sequence, they are all hydrophobic and cationic (1). These properties enable the peptides to bind and to be inserted into the surface membrane of target cells, and it is possible that the same mechanism mediates the binding of hCAP-18 to lipoproteins. The C terminus of another cathelicidin, indolicidin, which is a tridecapeptide rich in tryptophan and structurally very different from LL-37, has also been shown to bind to liposomes. The liposome-bound indolicidin retained its antifungal activity, but the cytotoxic activity was diminished (28). Thus, lipoproteins in general may bind antimicrobial peptides (or their pro-proteins) to preserve high plasma levels or to protect against the cytotoxic effects, or both.

Recently, the protein responsible for the binding of LL-37 in serum was identified as apolipoprotein A-I (29). The interaction was proposed to result from ionic interaction, as the binding of LL-37 to apolipoprotein A-I was dissociated by lowering the pH to 5. Our results show that most of the hCAP-18 in plasma is bound to LDL/VLDL particles. When LL-37 (in the exocytosed material from neutrophils) was added to plasma, most of the peptide was found together with LDL/VLDL. Furthermore, we could not dissociate the hCAP-18-lipoprotein complex by high ionic concentrations or by lowering the pH to 4.5. Although our data show that ~20% of hCAP-18 in plasma is associated with LDL particles containing apolipoprotein A-I, the data do not support a specific ionic interaction between apolipoprotein A-I and the lipoprotein-binding domain of hCAP-18.

Defensins are the major antibacterial peptides of human neutrophils and constitute ~5% of the total proteins of the cells. Defensins have been shown to bind to α₂-macroglobulin (30), serpins (31), and C1q (32). Despite the formation of these complexes, the plasma concentration of defensins is only 50 ng/ml in healthy subjects (33) and thus very low in comparison with the levels of defensins in neutrophils.

Defensins have been shown experimentally to bind to lipoprotein(a), a subclass of HDL, and to facilitate the binding of...
lipoprotein(a) to endothelial and smooth muscle cells (34). Defensins are found in both normal and atherosclerotic coronary vessels (35), and the deposition of defensins is proposed to contribute to the development of atherosclerosis. By immunohistochemistry, no positive staining for hCAP-18 was found, although we could confirm the positive staining for defensins in atherosclerotic coronary vessels.2 Thus, the association between lipoproteins and hCAP-18 does not result in deposition of the latter in blood vessels.

In summary, we found that the human cathelicidin, hCAP-18, is associated with VLDL, LDL, and HDL in plasma. The binding to lipoproteins results in a very high plasma concentration of hCAP-18 compared with other granule proteins of neutrophils. The finding that a member of a family of lipopolysaccharide-binding and antibacterial proteins binds to lipoproteins in plasma may add to our understanding of the complex role lipoproteins play in the defense against bacterial infections.

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REFERENCES

1. Zanetti, M., Gennaro, R., and Romeo, D. (1995) FEBS Lett. 374, 1–5
2. Bitseja, A., Kopitar, M., Jeral, R., and Turk, V. (1998) FEBS Lett. 255, 211–214
3. Scoechi, M., Skerlavaj, B., Romeo, D., and Gennaro, R. (1992) Eur. J. Biochem. 209, 589–595
4. Zanetti, M., Litteri, L., Griffiths, G., Gennaro, R., and Romeo, D. (1991) J. Immunol. 146, 4295–4300
5. Panyutich, A., Shi, J., Bouza, P. L., Zhao, C., and Ganz, T. (1997) Infect. Immun. 65, 979–985
6. Larrick, J. W., Michimasu, H., Balint, R. F., Lee, J., Zhong, J., and Wright, S. C. (1995) Infect. Immun. 63, 1291–1297
7. Agerberth, B., Ganne, H., Odeberg, J., Roquer, P., Romao, H. G., and Gudmundsson, G. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 195–199
8. Cowland, J. B., Johnsen, A. H., and Borregaard, N. (1995) FEBS Lett. 368, 173–176
9. Sørensen, O., Arnjots, K., Cowland, J. B., Bain ton, D. F., and Borregaard, N. (1997) Blood 90, 2796–2803

2 O. Sørensen, M. Sehested, and N. Borregaard, unpublished data.

10. Frohn Nilsson, M., Sandstedt, B., Sørensen, O., Weber, G., Borregaard, N., and Stähle-Backdahl, M. (1999) Infect. Immun. 67, 2561–2566
11. Frohn, M., Agerberth, B., Ahangari, G., Stähle-Backdahl, M., Lidén, S., Wigtell, H., and Gudmundsson, G. H. (1997) J. Biol. Chem. 272, 15258–15263
12. Bals, R., Wang, X., Zasloff, M., and Wilson, J. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9541–9546
13. Lollide, K., Kjeldsen, L., Sørensen, O., and Borregaard, N. (1995) Leukemia (Baltimore) 9, 159–164
14. Sørensen, O., Cowland, J. B., Askaa, J., and Borregaard, N. (1997) J. Immunol. Methods 206, 53–59
15. Laemmli, U. K. (1970) Nature 227, 680–685
16. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
17. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21, 77–90
18. Kjeldsen, L., Sørensen, O., and Borregaard, N. (1994) Blood 6, 1640–1649
19. Matsudaira, P. T. (1989) A Practical Guide to Protein and Peptide Purification for Microsequencing, pp. 20–23, Academic Press, New York
20. Ouchterlony, O., and Nilsson, L.-A. (1986) Handbook of Experimental Immunology, Vol. 6, pp. 32.1–32.50, Blackwell Publishers, Oxford
21. Finley, P. R., Schiffman, R. B., Williams, R. J., and Lichti, D. A. (1978) Clin. Chem. 24, 931–933
22. Bronzert, T. J., and Brewer, H. B., Jr. (1977) Clin. Chem. 23, 2089–2098
23. Gudmundsson, G. H., Agerberth, B., Odeberg, J., Bergman, T., Olsson, B., and Salcedo, R. (1996) Eur. J. Biochem. 238, 325–332
24. Arnjots, K., Sørensen, O., and Borregaard, N. (1998) Leukemia (Baltimore) 12, 1789–1785
25. Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D., and Agerberth, B. (1998) J. Biol. Chem. 273, 3718–3724
26. Peck-Miller, K. A., Darveau, R. P., and Fell, H. P. (1993) Cancer Chemother. Pharmacol. 32, 109–115
27. Turner, J., Cho, Y., Dinh, N.-N., Waring, A., and Lehrer, R. I. (1998) J. Antimicrob. Chemother. 42, 2206–2214
28. Ahmad, I., Perkins, W. R., Lupan, D. M., Selsted, M. E., and Janoff, A. S. (1995) Biochim. Biophys. Acta 1257, 109–114
29. Wang, Y., Agerberth, B., Löthgren, A., Almstedt, A., and Johansson, J. (1998) J. Biol. Chem. 273, 33115–33118
30. Panyutich, A. V., and Ganz, T. (1991) Am. J. Respir. Cell Mol. Biol. 12, 351–357
31. Panyutich, A. V., Hiemstra, P. S., van Wetering, S., and Ganz, T. (1995) Am. J. Respir. Cell Mol. Biol. 12, 351–357
32. Panyutich, A. V., Stold, O., Poon, P. H., Toeng, Y., and Ganz, T. (1994) FEBS Lett. 356, 169–173
33. Panyutich, A. V., Panyutich, E. A., Krapivin, V. A., Baturevich, E. A., and Ganz, T. (1993) J. Lab. Clin. Med. 122, 202–207
34. Higazi, A. A.-R., Lavi, E., Edeir, K., Uhrich, A. M., Jamieson, D. G., Rader, D. J., Usher, D. C., Kane, W., Ganz, T., and Cines, D. B. (1997) Blood 90, 4290–4298
35. Barnathan, E. S., Raghunath, P. N., Tomaszewski, J. E., Ganz, T., Cines, D. B., and Higazi, A. A.-R. (1997) Am. J. Pathol. 150, 1009–1020