hCAP-18, a cathelin/pro-bactenecin-like protein of human neutrophil specific granules

Jack B. Cowland[a], Anders H. Johnsen[b], Niels Borregaard[a,*]

[a]*Granulocyte Research Laboratory, Department of Hematology, National University Hospital, Rigshospitalet L-4042, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark*

[b]*Department of Clinical Biochemistry, National University Hospital, Rigshospitalet L-4042, 9 Blegdamsvej, DK-2100 Copenhagen, Denmark*

Received 23 May 1995

Abstract A 19 kDa protein was identified in specific granules of human neutrophils. A full-length cDNA clone was isolated from a human CML cDNA library, based on amino-acid sequences of isolated tryptic fragments. This clone includes the recently identified cDNA for FALL-39/CAP-18. Aminoacid sequences of proteolytic fragments derived both from the conserved N-terminal cathelin-like region and the highly variable C-terminal region characteristic of this family of bactericidal, LPS binding proteins, were in complete agreement with the sequence deduced from the cDNA. Thus, the 19 kDa protein is hCAP-18, stored as a 'pro-peptide' in specific granules.

Key words: CAP-18; cDNA; Neutrophil; FALL-39; Cathelin

1. Introduction

The neutrophilic granulocyte is the main mobile phagocyte, essential in the primary defense against intruding microorganisms. Intracellular granules are important as stores of bactericidal substances that are liberated to the phagocytic vacuole during phagocytosis. In addition, the granule membrane contains proteins that serve important functions during adhesion, diapedesis and phagocytosis when these proteins are translocated to the plasma membrane and the phagosomal membrane [1]. Several subsets of granules have been identified in addition to the highly mobilizable secretory vesicles[2–4]. In order to systematically investigate the content of these different granule subsets, and in particular to identify novel proteins, human neutrophils were disrupted and fractionated on a 3-layer Percoll density gradient [5]. Isolated azurophil granules, specific granules, gelatinase granules, and light membranes (secretory vesicles and plasma membranes), were subjected to Triton X-114 phase separation and the protein profiles of the hydrophobic and hydrophilic phases investigated by SDS-PAGE. An unidentified 19 kDa band, that partitioned exclusively in the Triton X-114 rich phase of specific granules, was observed.

*Corresponding author. Fax: (45) (3) 545-4841.

Abbreviations: bp, base pair; CML, chronic myeloid leukemia; DFP, diisopropyl fluorophosphate; ELISA, enzyme linked immunosorbent assay; HLA, human leukocyte antigen; LPS, lipo-polysaccharide; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; PCR, polymerase chain-reaction; TFA, trifluoroacetic acid.

2. Materials and methods

2.1. Subcellular fractionation

Human neutrophils were isolated from freshly drawn blood and disrupted by nitrogen cavitation following treatment with 5 mM DFP (Aldrich) [6]. The post-nuclear supernatant (10 ml), was loaded on a 3-layer Percoll (Pharmacia) density gradient (gradient volume 27 ml) and fractionated [5]. Azurophil granules were identified by their content of myeloperoxidase [7], specific granules by lactoferrin [7], gelatinase granules by gelatinase [5,8], and plasma membranes by HLA, all assayed by ELISA [5,9,10]. Secretory vesicles were identified by their content of latent alkaline phosphatase [4], assayed as described [11] and by their content of albumin, assayed by ELISA [12].

2.2. Identification of hCAP-18

Fractions corresponding to peak values of azurophil granules (α-band), specific granules (β_1-band), gelatinase granules (β_2-band), and secretory vesicles and plasma membranes (γ-band), respectively [5], were pooled and Percoll removed by centrifugation [6]. The biological material was resuspended in relaxation buffer [6], and Triton X-114 was added to a concentration of 0.7%. Phase separation was then carried out as described by Bordier [13], and SDS-PAGE was performed on a 5–20% polyacrylamide gradient [14]. Proteins were electroblotted to ProBlott membranes (Applied Biosystems) in 10% (v/v) methanol, 10 mM CAPS buffer, pH 11, and stained with 0.1% Coomassie blue or Amido black. The 19 kDa band was cut out, and the piece of membrane cut into smaller pieces and washed once with 50% methanol followed by buffer. Then the protein was incubated overnight at 37°C with either 0.5 μg trypsin or 0.28 μg endoproteinase Asp-N (both sequence grade, Boehringer-Mannheim), including 0.1% reduced Triton X-100 and 10% acetonitrile in the buffer as suggested for trypsin [15], which also worked for endoproteinase Asp-N. Following incubation, the samples were sonicated for 5 min, centrifuged, and the supernatant collected. This was repeated with buffer and 0.1% and 10% TFA. The peptides in the combined supernatants were separated by reverse phase HPLC employing a Vydac C8 column (2.1 × 150 mm) eluted with a gradient from 0.1% TFA water to 0.1% TFA in acetonitrile. The fragments were identified by mass spectrometry and sequence analysis. Laser desorption mass spectrometry was performed in a Biflex instrument (Bruker-Franzen) using α-cyano-4-hydroxycinnamic acid as matrix. Sequence analyses were performed on a Protein Sequencer model 475 A, equipped with on-line HPLC detection or a Procise Protein Sequencer, both from Applied Biosystems). For cleavage with CNBr, the central part of the 19 kDa band was cut to fit the slot in the blot cartridge of the Procise sequencer. Ten μl of CNBr in 50% TFA (50 g/l) was added. The blot was incubated overnight at room temperature in the dark in a argon-atmosphere in a closed 50 ml tube containing 2 ml of 50% TFA in the bottom, dried and subjected to sequence analysis.

2.3. Cloning of hCAP-18

On the basis of the amino-acid sequence of tryptic fragments, a degenerate primer CAGCAGCCICCIGA(AG)GA(CT)TG(CT)GA-(CT)T was used along with a library specific primer for PCR amplification from a human CML cDNA library [16]. An approximately 450 bp PCR product was subcloned into the phagemid vector pBluescript II KS$^+$ (Stratagene). Two clones of the 3'-region were sequenced. The largest contained the last 279 bp of hCAP-18 and part of the poly(A) tail. The other clone terminated 19 bp upstream of the poly(A) site. Based on the nucleotide sequence, a specific primer CCCCTGGCCT-

SSDI 0014-5793(95)00634-6

GGTTGAGG was made and used against a library specific primer for PCR amplification from the cDNA library. A PCR product of approximately 750 bp was cloned in the plasmid vector pCR II using the TA cloning kit (Invitrogen). Two clones were sequenced and found to contain the 5'-end of hCAP-18. The largest clone included 35 bp of the 5'-non-translated region, the shortest clone terminated at the G of the start codon.

3. Results and discussion

The protein profiles of the isolated subcellular fractions show marked differences between azurophil granules, specific granules, gelatinase granules, and plasma membranes and secretory vesicles (Fig. 1). These differences relate both to proteins partitioning into the Triton-rich, hydrophobic phase, and to the Triton-poor, hydrophilic phase.

One of the dominating bands in specific granules is a 19 kDa protein, provisionally termed p19, that partitioned exclusively in the Triton X-114 rich phase. We sought to identify this protein by amino acid sequence analysis of intact electroblotted protein and of tryptic fragments generated by digestion of the protein after transfer to ProBlott membranes. We were unable to obtain the N-terminal sequence of the undigested protein, indicating that it is N-terminally blocked. However, the following internal sequences of tryptic peptides, given in one-letter codes, were identified: *Fragment 1:* AIDGINQR; *Fragment 2:* SSDANLYR; *Fragment 3:* LLDLDPRPXMD. A fourth fragment gave a double sequence in the first 6 residues followed by a single sequence for another 6 cycles. By alignment with the later cDNA deduced sequence, this fragment could be identified as two peptides: *4A* ETVXPR, and *4B* TTQQSPEDXDFK, linked by a disulfide bridge between C in the two X positions. The measured molecular mass is in accordance with this structure. A search in the EMBL database showed a high degree of homology to the pro-bactenecin family of bovine neutrophil proteins, but gave no hits on known human proteins.

Bactenecins were originally identified as small highly bactericidal peptides known as Bac 7 (7 kDa) and Bac 5 (5 kDa)[17], and cyclic dodecapeptide [18], all with little amino acid sequence similarity. These peptides were later shown to be stored as 15–20 kDa 'pro-peptides' in a specialized granule subset of bovine neutrophils called large granules [19]. The proteins are all highly homologous in their 'pro-peptide' region to cathelin, a protein initially identified in porcine neutrophils as an inhibitor of cathepsin L [20]. This activity was later shown to reside in a contaminating protein [21]. PCR cloning of mRNA from porcine bone marrow has revealed that cathelin most likely is a processing product of a 'pro-peptide' of similar composition as the 15–20 kDa proteins of bovine neutrophils [22–24]. In rabbits, 3 members of the same family of proteins have been identified, CAP-18 [25], and two p15's [26]. While the bactericidal effects of these proteins have only been demonstrated for the C-terminal peptides generated through proteolytic cleavage (as effectuated by elastase, a protein of azurophil granules) [27], the holo-peptides are able to bind and inactivate LPS, as demonstrated by CAP-18 [28].

To further characterize p19, cDNA amplification of a human myeloid (CML) cDNA library was performed by PCR, using library specific primers and degenerate primers designed on the basis of the amino acid sequence of the tryptic fragments identified. The cDNA sequence is given in Fig. 2 along with the deduced amino acid sequence of the protein. The cDNA sequence includes two sequences recently cloned from human bone marrow cDNA libraries on the basis of similarity with cDNA of porcine PR-39 and rabbit CAP-18, respectively [29,30]. One has been termed FALL-39 based on the assumption that this cDNA codes for a pre-pro-peptide that would result in a 39 amino acid peptide starting with the aminoacids FALL after full processing. However, no translation or processing product corresponding to this cDNA was demonstrated. Larrick et al. [30] showed that polyclonal antibodies generated against recombinant human CAP-18 protein reacted with an 18 kDa and a 14 kDa protein of human neutrophils, the latter presumed to be the N-terminal part of the 18 kDa protein, and representing the cathelin-like sequence that is highly conserved

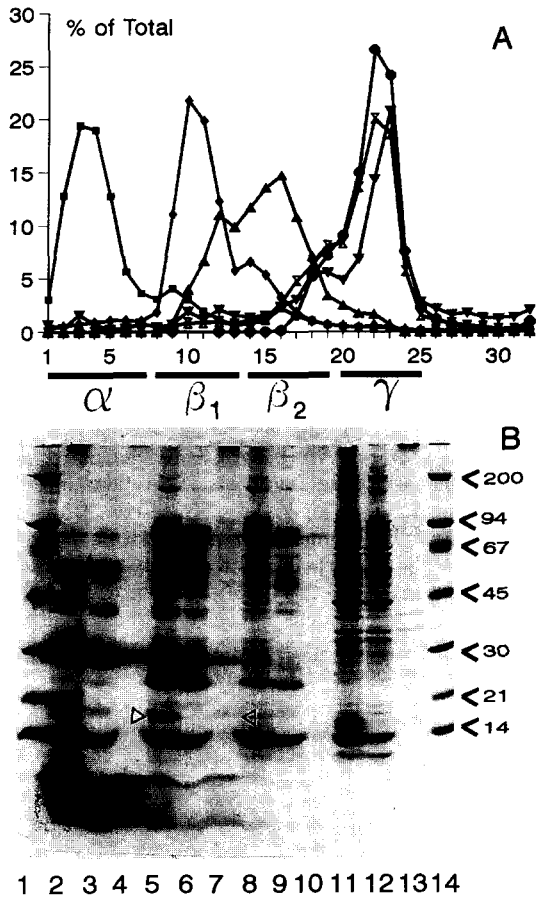

Fig. 1. Subcellular fractionation and SDS-PAGE profile of human neutrophil granules. (A) 7×10^8 neutrophils were fractionated on a 3-layer Percoll density gradient and fractions of 1.0 ml collected and analyzed. Distribution of myeloperoxidase (■), lactoferrin (◆), gelatinase (▲), latent alkaline phosphatase (⧖), Albumin (●), and HLA (▼) is given and expressed as % of total. Fractions were pooled as indicated by α, β_1, β_2, and γ. (B) The α (azurophil granules), β_1 (specific granules), β_2 (gelatinase granules) and γ (secretory vesicles and plasma membranes) bands were each resuspended to 1.1 ml and subjected to Triton X-114 phase separation. The Triton micelles collected were resuspended to the original volume. Seventy-five μl of the material before phase separation (total), of the Triton X-114 supernatant (hydrophilic), and of the Triton X-114 pellets (hydrophobic) were run on a 5–20% polyacrylamide gel under reducing conditions: Lanes: 1 = MW-std; 2 = α(total); 3 = α(hydrophilic); 4 = α(hydrophobic); 5 = β_1(total); 6 = β_1(hydrophilic); 7 = β_1(hydrophobic); 8 = β_2 (total); 9 = β_2(hydrophilic); 10 = β_2(hydrophobic); 11 = γ(total); 12 = γ(hydrophilic); 13 = γ(hydrophobic); 14 = MW-std. p19 is indicated by triangle.

```
CGAATTCGGCACGAGGTGGGCTAGAGGGAGGCAGACATGGGAACC                    (-1)

ATG AAG ACC CAA AGG GAT GGC CAC TCC CTG GGG CGG TGG TCA CTG      (45)
 M  K  T  Q  R  D  G  H  S  L  G  R  W  S  L                    (15)

GTG CTC CTG CTG CTG GGC CTG GTG ATG CCT CTG GCC ATC ATT GCC      (90)
 V  L  L  L  L  G  L  V  M  P  L  A  I  I  A                    (30)

CAG GTC CTC AGC TAC AAG GAA GCT GTG CTT CGT GCT ATA GAT GGC      (135)
[Q  V  L  S  Y  K  E  A  V  L  R [A  I  D  G                    (45)

ATC AAC CAG CGG TCC TCG GAT GCT AAC CTC TAC CGC CTC CTG GAC      (180)
 I  N  Q  R] [S  S  D  A  N  L  Y  R] [L  L  D                  (60)

CTG GAC CCC AGG CCC ACG ATG GAT GGG GAC CCA GAC ACG CCA AAG      (225)
 L  D  P  R  P  T  M {D] G  D  P  D  T  P  K                    (75)

CCT GTG AGC TTC ACA GTG AAG GAG ACA GTG TGC CCC AGG ACG ACA      (270)
 P  V  S  F  T  V  K [E  T  V  C  P  R] [T  T                   (90)

CAG CAG TCA CCA GAG GAT TGT GAC TTC AAG AAG GAC GGG CTG GTG      (315)
 Q  Q  S  P  E} D  C  D  F  K] K  D  G  L  V                    (105)

AAG CGG TGT ATG GGG ACA GTG ACC CTC AAC CAG GCC AGG GGC TCC      (360)
 K  R  C  M {G  T  V  T  L  N  Q  A  R  G  S                    (120)

TTT GAC ATC AGT TGT GAT AAG GAT AAC AAG AGA TTT GCC CTG CTG      (405)
 F  D  I  S  C  D  K  D  N  K  R  F  A  L  L                    (135)

GGT GAT TTC TTC CGG AAA TCT AAA GAG AAG ATT GGC AAA GAG TTT      (450)
 G  D  F  F  R  K  S  K  E  K  I  G  K  E  F                    (150)

AAA AGA ATT GTC CAG AGA ATC AAG GAT TTT TTG CGG AAT CTT GTA      (495)
 K  R} I  V  Q  R  I  K  D  F  L  R  N  L  V                    (165)

CCC AGG ACA GAG TCC TAG TGTGTGCCCTACCCTGGCTCAGGCTTCTGGGCTCTG
 P  R  T  E  S      (170) (MW 19301)

AGAAATAAACTATGAGAGCAATTTCAAAAAAAAAAAAAAAAAAAAAAAAA(599)
```

Fig. 2. cDNA and amino acid sequence of hCAP-18. The cDNA sequence is given as determined from clones with overlapping sequences. The initial 10 bases in italics are library specific. The localization of sense and antisense primers for PCR amplification is underlined. The deduced amino acid sequence is given. The amino acids identified by protein sequence analysis are given in boldface and the fragments indicated by [] for trypsin, and {} for CNBr. r indicates N-terminal of mature protein

among the members of this family. It is therefore possible that the antibody may have recognized other members of the cathelin-family than the protein whose cDNA was cloned and termed CAP-18.

To verify that the cloned cDNA corresponds to the p19 protein, and to further characterize this, the protein was digested with endoproteinase Asp-N and CNBr. Digestion with endoproteinase Asp-N gave rise to several fragments, two of which gave no signal by sequence analysis. However, their molecular masses (1472.4 Da and 2331.6 Da) fit those calculated for fragment 31–43 (1472.8 Da) and 31–51 (2330.7 Da) of the deduced protein sequence, assuming the N-terminal residue to be pyroglutamate. Both residue 44 and 52 are Asp, in accordance with the specificity of the enzyme. This finding strongly indicates that residues 1–30 constitute the signal peptide, as predicted by the 'von Heijne rules'[31], and as also suggested by [30]. Sequence analysis of the CNBr-treated ProBlott showed two sequences which fit residues 68–95 and 110–152, respectively. From amino acid no. 28 of this analysis, only one sequence could be recognized, thus unequivocally identifying residues 138–152 of the cDNA deduced sequence to be present in p19. Hence, the cDNA cloned by all 3 groups reflects a mRNA that is transcribed to a protein stored as an 18–19 kDa protein in specific granules of human neutrophils, and that this is the major if not the only member of the cathelin family that is stored intact in specific granules. We propose that p19 should be named hCAP-18.

The subcellular localization of the proteins belonging to the cathelin/pro-bactenecin family has only been determined in bovine neutrophils. No human correlate of the peroxidase negative large granules, the dominating granule subset of bovine neutrophils[32], has been described. This granule subset is the store of cationic, bactericidal proteins, like pro-bactenecins [19], and contains lactoferrin but lacks lysozyme and vitamin B_{12}-binding protein [32]. In the human neutrophil, most bactericidal cationic proteins are found in azurophil granules [33]. The demonstration of hCAP-18 in specific granules of mature human neutrophils raises the important questions: how is the protein targeted to these granules in the human neutrophil? Is this protein further processed to generate bactericidal C-terminal fragments such as FALL-39 during degranulation? What is the interplay between azurophil granules and specific granules of human neutrophils in this process?

Acknowledgements: The expert technical assistance of Charlotte Horn and Allan Kastrup is greatly appreciated. This work was supported by grants from The Danish Cancer Society (94 100 37), The Danish Medical Research Council, The Novo Nordic Fund, The Danish Arthritis Foundation. N.B. is the recipient of a Neye-Research Professorship. The cDNA library from human CML cells was a generous gift from dr. Nancy Berliner, Department of Hematology, Yale University Medical School.

References

[1] Borregaard, N., Lollike, K., Kjeldsen, L., Sengeløv, H., Bastholm, L., Nielsen, M.H. and Bainton, D.F. (1993) Eur. J. Haematol. 51, 187–198.
[2] Rice, W.G., Kinkade, J.M. and Parmley, R.T. (1986) Blood 68, 541–555.
[3] Kjeldsen, L., Bainton, D.F., Sengeløv, H. and Borregaard, N. (1993) Blood 82, 3183–3191.
[4] Borregaard, N., Miller, L. and Springer, T.A. (1987) Science 237, 1204–1206.
[5] Kjeldsen, L., Sengeløv, H., Lollike, K., Nielsen, M.H. and Borregaard, N. (1994) Blood 83, 1640–1649.
[6] Borregaard, N., Heiple, J.M., Simons, E.R. and Clark, R.A. (1983) J. Cell Biol. 97, 52–61.
[7] Cramer, E., Pryzwansky, K.B., Villeval, J.-C., Testa, U. and Breton-Gorius, J. (1985) Blood 65, 423–432.
[8] Dewald, B., Bretz, U. and Baggiolini, M. (1982) J. Clin. Invest. 70, 518–525.
[9] Hibbs, M.S. and Bainton, D.F. (1989) J. Clin. Invest. 84, 1395–1402.
[10] Bjerrum, O.W. and Borregaard, N. (1990) Scand. J. Immunol. 31, 305–313.
[11] DeChatelet, L.R. and Cooper, M.R. (1970) Biochem. Med. 4, 61–68.
[12] Borregaard, N., Kjeldsen, L., Rygaard, K., Bastholm, L., Nielsen, M.H., Sengeløv, H., Bjerrum, O.W. and Johnsen, A.H. (1992) J. Clin. Invest. 90, 86–96.
[13] Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Fernandez, J., Andrews, L. and Mische, S.M. (1994) Anal. Biochem. 218, 112–117.
[16] Johnston, J., Bollekens, J., Allen, R.H. and Berliner, N. (1989) J. Biol. Chem. 264, 15754–15757.
[17] Gennaro, R., Skerlavaj, B. and Romeo. D. (1989) Infect. Immun. 57, 3142–3146.
[18] Romeo, D., Skerlavaj, B., Bolognesi, M. and Gennaro, R. (1988) J. Biol. Chem. 263, 9573–9575.
[19] Zanetti, M., Litteri, L., Gennaro, R., Horstmann, H. and Romeo, D. (1990) J. Cell Biol. 111, 1363–1371.

[20] Ritonja, A., Kopitar, M., Jerala, R. and Turk, V. (1989) FEBS Lett. 255, 211–214.
[21] Lenarcic, B., Ritonja, A., Dolenc, I., Stoka, V., Berbic, S., Pungercar, J., Strukelj, B. and Turk, V. (1993) FEBS Lett. 336, 289–292.
[22] Zhao, C., Liu, L. and Lehrer, R.I. (1994) FEBS Lett. 346, 285–288.
[23] Pungercar, J., Strukelj, B., Kopitar, G., Renko, M., Lenarcic, B., Gubensek, F. and Turk, V. (1993) FEBS Lett. 336, 284–288.
[24] Zanetti, M., Storici, P., Tossi, A., Scocchi, M. and Gennaro, R. (1994) J. Biol. Chem. 269, 7855–7858.
[25] Larrick, J.W., Morgan, J.G., Palings, I., Hirata, M. and Yen, M.H. (1991) Biochem. Biophys. Res. Commun. 179, 170–175.
[26] Levy, O., Weiss, J., Zarember, K., Ooi, C.E. and Elsbach, P. (1993) J. Biol. Chem. 268, 6058–6063.
[27] Scocchi, M., Skerlavaj, B., Romeo, D. and Gennaro, R. (1992) Eur. J. Biochem. 209, 589–595.
[28] Hirata, M., Shimomura, Y., Yoshida, M., Morgan, J.G., Palings, I., Wilson, D., Yen, M.H., Wright, S.C. and Larrick, J.W. (1994) Infect. Immun. 62, 1421–1426.
[29] Agerberth, B., Gunne, H., Odeberg, J., Kogner, P., Boman, H.G. and Gudmundsson, G.H. (1995) Proc. Natl. Acad. Sci. USA 92, 195–199.
[30] Larrick, J.W., Hirata, M., Balint, R.F., Lee, J., Zhong, J. and Wright, S.C. (1995) Infect. Immun. 63, 1291–1297.
[31] von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690.
[32] Gennaro, R., Dewald, B., Horisberger, U., Gubler, H.U. and Baggiolini, M. (1983) J. Cell Biol. 96, 1651–1661.
[33] Gabay, J.E., Heiple, J.M., Cohn, Z.A. and Nathan, C.F. (1986) J. Exp. Med. 164, 1407–1421.