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COMPLEMENTARY DNA SEQUENCE OF RABBIT CAP18--A UNIQUE LIPOPOLYSACCHARIDE BINDING PROTEIN

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CAP18 is a novel 18 kDa cationic protein [pI ≈ 10] originally purified from rabbit granulocytes using as an assay the agglutination of lipopolysaccharide (LPS) coated erythrocytes. cDNA clones encoding CAP18 were isolated from a rabbit bone marrow cDNA library using a PCR generated oligonucleotide probe derived from the N-terminal amino acid sequence. The deduced amino acid sequence reveals a putative signal sequence of 29 amino acids and a mature protein of 142 amino acid residues. The predicted size of the encoded protein is 16.6 kDa with a pI of 10. There are no N-linked glycosylation sites. The CAP18 sequence bears no homology with other known LPS-binding proteins including human bacterial permeability increasing protein (BPI)(1) and rabbit LPS binding protein (LBP)(2).

A rational approach to the control of Gram-negative sepsis is to neutralize the toxic effects of endotoxin or LPS(3). Cationic antibiotics such as polymyxin B bind to and neutralize some types of LPS, however clinical use is limited by toxicity(4). LPS neutralizing monoclonal antibodies recognizing the most common types of LPS is another approach, but the numerous species of Gram-negative bacteria limit this approach. Antibodies made to the cross-species conserved regions of LPS, such as the lipid A core, appear to be ineffective against smooth LPS, possibly due to steric effects by the type-specific polysaccharides(5). Recently, several proteins have been identified that bind to and appear to neutralize the toxic effects of LPS. These include mammalian BPI derived from granulocytes and LBP an acute phase protein produced primarily by the liver. In addition the LPS binding protein of the horseshoe crab, Limulus has been purified(6). Hirata et al.(7) identified an LPS binding protein from rabbit granulocytes. This protein, rabbit CAP18, is known to attenuate the activity of LPS in a

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Abbreviations: CAP18/CAP37/CAP57-cationic antimicrobial proteins of 18 kDa, 37kDa, 57 kDa; BPI-bactericidal permeability increasing protein; LPS-lipopolysaccharide; LBP-LPS binding protein; PCR-polymerase chain reaction.

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number of assays. CAP18 binds to core mutant and smooth LPS coated onto erythrocytes. Purified CAP18 attenuates LPS induced generation of tissue factor in vivo and blocks lethality of LPS in galactosamine sensitized mice. Here, we describe the cloning and characterization of the CAP18 cDNA. Analysis of the LPS binding domains of the various LPS binding proteins may lead to novel therapeutic strategies to neutralize LPS.

MATERIALS AND METHODS

Protein sequence of CAP18 - Rabbit CAP18 was purified from rabbit peritoneal exudate cells elicited by IP injection of 500 ml of 0.25% sodium caseinate. Cells were washed and extracted with 0.1M citric acid. The acid soluble fraction was precipitated with 80% ethanol and applied to heparin-sepharose CL-6B column. The tightly bound cationic proteins eluting with 2M NaCl were used for further study. C8 reverse phase HPLC of the heparin bound material yielded two major peaks. The first peak (@26.1 min.) actively inhibited LPS induced tissue factor generation from murine macrophages (Hirata M, et al., unpublished data). The active peak was sequenced using an Applied Biosystems Model 477A protein/peptide sequencer with an on-line Applied Biosystems 120A PTH-amino acid analyzer. The sequence of the first 30 amino acids of the putative N-terminus of CAP18 was: GLY-LEU-ARG-LYS-ARG-LEU-ARG-LYS-PHE-ARG-ASN-LYS-ILE-LYS-GLU-LYS-LEU-LYS-LYS-ILE-GLY-GLN-(ASP/LYS)-ILE-GLN-(GLN-ILE)-(GLY-GLN)-LEU-LEU. Searches of the GenBank and the National Protein databases revealed that this sequence is unique.
Construction cDNA library - Rabbit bone marrow cells were harvested and lysed with guanidinium thiocyanate, and spun on a cesium chloride gradient as per Maniatis(8). Poly(A)+ mRNA was selected on an oligi dT-cellulose column (Pharmacia). 5 μg mRNA was used to construct a cDNA library, using the cDNA Synthesis System Plus kit (Amersham). EcoRI restriction sites were methylated, and EcoR1 linkers were ligated overnight at room temperature using T4 DNA Ligase (New England Biolabs). Excess linker was digested with EcoRI. The cDNA was size fractionated over a Sepharose S-400 column, ligated into lambda gt10 vector (Lambda Vector Kit, Stratagene). C600Hfl bacteria were infected with the packaged lambda and grown on NZYDT plates. Random plaques were amplified via polymerase chain reaction (PCR) (Perkin Elmer Cetus) using primers made to the lambda arms flanking the insert site.
Isolation of cDNA clones - A 20 nucleotide, degenerate probe was designed from amino acids #11-17 of CAP18: 5'-AA(CT)AA(GA)AT(CTA)AA(GA)GA(GA)AA(GA)CT. The probe was paired with vector derived primers flanking the insert site. Amplified PCR fragments were subcloned into m13mp18 phage and pcDNAI plasmids for sequencing by the dideoxy chain termination method(9),(10). A 200 bp PCR fragment was found to encode the known CAP18 amino acid sequence, some additional 3' information, and a poly A tail. This fragment was used to generate random primed probes as per manufacturer's instructions (Boehringer-Mannheim), and used to screen the library for the full-length cDNA.
cDNA library screening - Confluent plates were transferred to nitrocellulose filters. The filters were UV-crosslinked (Stratagene, UV Stratalinker) and incubated for 2 hours in 50% formamide, 50% PAM. Filters were hybridized at 42°C overnight with fresh solution containing the random primed probe. Filters were washed once at room temperature for 15 minutes in 2 X SSC, 0.1% SDS; then in 1 X SSC, 0.1% SDS; and a third time in 0.5 X SSC, 0.1% SDS. Positives were subcloned into m13mp18 and/or pcDNAI(Invitrogen) and sequenced.
Computer Analysis of DNA and Protein Sequences - Generation of the predicted amino acid sequence was performed using the DNA Matrix program. Analysis of the primary

and secondary structures of the protein based on the DNA sequence was performed using the PCGENE program. Genebank searches at the nucleotide and amino acid levels were conducted using the programs FASTA and TFASTA(11).

Northern Blot analysis - Poly(A)+ rabbit bone marrow mRNA was electrophoresed in 1% agarose under denaturing conditions and transferred to nylon filters. Filters were UV-crosslinked and hybridized under conditions as described above using the ^{32}P-labelled 200 bp PCR derived from the CAP18 cDNA. Filters were washed twice in 0.5 X SSC, 0.1% SDS, for fifteen minutes at RT prior to autoradiography.

RESULTS

Two pairs of oligonucleotide probes were designed corresponding to amino acids #11-17 and #15-21 respectively. Initial attempts using conventional screening failed to identify a positive clone. An alternative approach using PCR was employed. CAP18 oligonucleotide #11-17 was matched with primers designed from the lambda phage arms.

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1     ATG GAG ACC CAT AAG CAC GGA CCT TCC CTG GCC TGG TGG TCA CTG    45
      Met Glu Thr His Lys His Gly Pro Ser Leu Ala Trp Trp Ser Leu
      -29                                                    -15

46    TTG CTG CTG CTG CTG GGC CTG CTG ATG CCC CCA GCC ATC GCC CAG    90
      Leu Leu Leu Leu Leu Gly Leu Leu Met Pro Pro Ala Ile Ala Gln
                                                              1

91    GAC CTC ACC TAC CGG GAG GCT GTG CTC CGC GCT GTG GAT GCC TTC   135
      Asp Leu Thr Tyr Arg Glu Ala Val Leu Arg Ala Val Asp Ala Phe
                                                             16

136   AAC CAG CAG TCC TCA GAG GCC AAC CTC TAC CGC CTC CTG AGC ATG   180
      Asn Gln Gln Ser Ser Glu Ala Asn Leu Tyr Arg Leu Leu Ser Met
                                                             31

181   GAC CCC CAG CAG CTG GAG GAT GCG AAG CCA TAC ACC CCG CAG CCT   225
      Asp Pro Gln Gln Leu Glu Asp Ala Lys Pro Tyr Thr Pro Gln Pro
                                                             46

226   GTG AGC TTT ACG GTG AAG GAG ACG GAG TGC CCC CGG ACA ACA TGG   270
      Val Ser Phe Thr Val Lys Glu Thr Glu Cys Pro Arg Thr Thr Trp
                                                             61

271   AAG CTA CCA GAG CAG TGT GAC TTC AAG GAA GAT GGG CTG GTG AAG   315
      Lys Leu Pro Glu Gln Cys Asp Phe Lys Glu Asp Gly Leu Val Lys
                                                             76

316   CGG TGT GTG GGG ACT GTG ACA CGG TAC CAG GCC TGG GAC TCC TTT   360
      Arg Cys Val Gly Thr Val Thr Arg Tyr Gln Ala Trp Asp Ser Phe
                                                             91

361   GAC ATC CGC TGC AAC AGG GCC CAA GAG TCC CCA GAA CCT ACT GGG   405
      Asp Ile Arg Cys Asn Arg Ala Gln Glu Ser Pro Glu Pro Thr Gly
                                                            106

406   CTG CGC AAG CGC TTA CGA AAA TTT AGA AAC AAG ATT AAA GAA AAG   450
      Leu Arg Lys Arg Leu Arg Lys Phe Arg Asn Lys Ile Lys Glu Lys
                                                            121

451   CTT AAA AAA ATT GGT CAG AAA ATC CAG GGT TTG CTG CCG AAA CTT   495
      Leu Lys Lys Ile GLy Gln Lys Ile Gln Gly Leu Leu Pro Lys Leu
                                                            136

496   GCA CCC AGG ACA GAT TAC TAG GGTCTGCCCTGCCCTGGACTCTGAAAAATAA   547
      Ala Pro Arg Thr Asp Tyr END
                        142

548   ACTGTGTGAAAGCAACAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Nucleotide and deduced amino acid sequence of rabbit CAP18. The nucleotide sequence is numbered from the ATG. The amino acid sequence is numbered from the N-terminus of the mature protein.

A 200 bp fragment corresponding to CAP18 was amplified, purified and used to screen the cDNA library. In the library of 400,000 plaques, greater than 100 clones hybridized to the probe. Twenty of these primary positives were replated and 4 were subcloned into plasmids for sequence analysis.

The cDNA and predicted amino acid sequences of CAP18 is shown in figure 1. The CAP18 cDNA encodes a 29 amino acid signal peptide followed by a mature protein of 142 amino acids. The protein is predicted to be 16.6 kDa and to have a pI of 10. No N-linked glycosylation sites are predicted. There are four cysteines. The sequence derived from Edman degradation is found at the carboxy-terminus of the protein beginning at amino acid position 116. It is possible that the sequenced peptide was generated by proteolysis of CAP18 during preparation or storage.

Northern blot analysis was performed to look for alternative forms of the CAP18 mRNA. The 200 bp fragment derived by PCR was subcloned, isolated and used to generate random-primed probes. Probing of poly(A)+ mRNA derived from rabbit bone marrow revealed a major band at 0.8-0.9 kb corresponding to full length CAP18 mRNA (see figure 2).

DISCUSSION

The characteristics of the cDNA clone we have isolated for CAP18 correspond to the purified protein. The predicted size of CAP18 is 16.6 kDa with a pI of 10. The sequence obtained from Edman degradation matches the predicted carboxy-terminus of the rabbit cDNA. Granulocytes contain large amounts of proteolytic enzymes and we postulate that the protein underwent cleavage during isolation or storage. At the present time we have no evidence that the protein undergoes cleavage in vivo.

There is very limited homology of the CAP18 cDNA with known sequences in the Genbank database. Several other cationic antimicrobial proteins have been found in

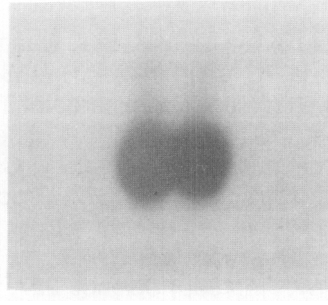

Figure 2. Northern blot hybridization analysis of rabbit CAP18 mRNA. Duplicate samples of rabbit bone marrow poly(A)+ mRNA were probed with a 200 bp fragment of the CAP18 cDNA. The major band is at 0.8 kb.

granulocytes. None of these share sequence with CAP18, including CAP37(12), BPI (see below) or the defensins(13).

Recently several reports of LPS binding enzymes(14) or receptors(15) have been made. Perhaps best characterized is LBP, a serum protein produced by hepatocytes during the acute phase(16). LBP is a 60 kDa glycoprotein of pI of 6.8 that binds the lipid A portion of many types of LPS. LBP is believed to be composed of two regions: an amino-terminal domain that binds to LPS and a carboxy-terminal domain that may mediate binding of the LBP-LPS complex to the CD14 receptor on leukocytes(17). LBP shares significant homology with BPI, a human neutrophil granule protein of 57 kDa and pI of 10 (originally named CAP 57). BPI has a domain structure similar to LBP and is known to block LPS stimulated complement receptor activation on granulocytes(18). The genes for both of these molecules are closely linked on chromosome 21 and they share 44% homology. Neither BPI nor LBP shares significant sequence with CAP18 at the protein or nucleotide level.

Expression of recombinant CAP18 will aid future studies aimed to characterize CAP18 binding to LPS and the capacity of CAP18 to neutralize the activity of LPS in various in vitro and in vivo models of endotoxinemia. A human homolog of CAP18 may have use as a therapy to neutralize LPS in conditions associated with excessive endotoxinemia.

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