

Limunectin

A PHOSPHOCHOLINE-BINDING PROTEIN FROM *LIMULUS* AMEBOCYTES WITH ADHESION-PROMOTING PROPERTIES*

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A protein that binds to and precipitates with pneumococcal C-polysaccharide and a phosphocholine (PC) derivative of bovine serum albumin has been affinity purified from *Limulus* amebocytes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis reveals that the isolated protein consists of a single polypeptide chain of ~50 kDa. It is an intracellular protein localized in the secretory granules of amebocytes according to immunogold staining. Although it shares the PC-binding property with C-reactive protein isolated from *Limulus* and other animal species, it differs from C-reactive protein in that the latter binds to PC only in the presence of Ca^{2+} , whereas the newly isolated protein binds to PC in a Ca^{2+} -independent manner. In this respect, the newly isolated PC-binding protein resembles the antibodies to PC of mouse myelomas.

The gene coding for this protein has been isolated. The gene sequence predicts a protein of 54 kDa with an unusual structural feature: it consists almost entirely of 10 contiguous segments, 45 amino acids in length, with extensive homology. Some limited sequence homologies were found between the 54-kDa protein and segments of vitronectin, gelatinase, and collagenase. It binds to bacterial cells, fixed amebocytes, and a number of extracellular matrix molecules. Due to its structural and some functional similarities to other adhesion molecules, the *Limulus* 54-kDa protein was named "Limunectin."

For a number of years our laboratory has been engaged in the study of C-reactive proteins (CRPs),¹ Ca^{2+} -dependent phosphocholine binding proteins from human (Oliveira *et al.*, 1979; Lei *et al.*, 1985), rabbit (Syn *et al.*, 1986), and *Limulus*

(Robey and Liu, 1981; Nguyen *et al.*, 1986a, 1986b). Whereas in humans (Tillett and Francis, 1930) and rabbits (Anderson and McCarty, 1951), CRP synthesis is induced in response to inflammation or trauma, in invertebrates such as *Limulus*, CRP is constitutively produced and represents a major component of the horseshoe crab's hemolymph (Robey and Liu, 1981). Even though the exact physiological roles have not been assigned, CRPs are known to promote agglutination, complement fixation, bacterial capsular swelling, and phagocytosis (Gewurz *et al.*, 1982; Gal and Miltenyi, 1955; Kaplan and Volanakis, 1974; Löfstrom, 1944; Hokama, 1962). CRP has also been shown to bind to chromatin (Robey *et al.*, 1984) and to histones (Du Clos *et al.*, 1988) *in vitro*. We question if CRP or CRP-like molecules exist intracellularly and if they do what would be their physiological functions.

Adhesion proteins are a group of complex glycoproteins which play critical roles in the regulation of the immune response, in wound healing, cellular development, morphogenesis, and metastasis (Suzuki *et al.*, 1985; Clark and Colvin, 1985; Terhorst *et al.*, 1988). A substantial body of literature dealing with tissue injury and repair indicates that adhesion molecules, soluble or membrane bound, are involved. These proteins serve as mediators for cell to cell adhesion and cell to extracellular matrix interaction through different mechanisms (Filippsen *et al.*, 1990). Fibronectin and von Willebrand factor contain several types of internal repeats, whereas vitronectin contains no internal repeat (Verweij *et al.*, 1986; Petersen *et al.*, 1983; Suzuki *et al.*, 1985). Adhesion proteins in general share little sequence homology but often contain several separate domains each of which exhibits specific physiological functions (Suzuki *et al.*, 1984; Clark and Colvin, 1985).

By using PC affinity column chromatography similar to that used in the purification of CRP (Oliveira *et al.*, 1980), we have isolated a 54-kDa intracellular protein from *Limulus* amebocytes that binds to PC independent of Ca^{2+} . Its possible role as an adhesion protein will be discussed.

MATERIALS AND METHODS

Reagents—CH-Sepharose 4B was purchased from Pharmacia LKB Biotechnology Inc. The calcium salt of phosphocholine chloride was from Sigma. *Limulus* amebocytes were purchased from the Associate of Cape Cod, Woods Hole, MA. The molecular weight standards used for SDS-PAGE were from Bio-Rad. The ionophore, 4-bromo-calcionophore A23187 was from Sigma. All other chemicals used in this study were reagent grade and were purchased from Fisher.

Purification of Limunectin—The phosphocholine affinity matrix used for the protein isolation was made essentially as described previously (Oliveira *et al.*, 1980). Thirty ml of frozen amebocytes were thawed and washed three times with 3% saline containing 0.05 M PMSF. The cells were then homogenized in 100 ml of 50 mM Tris, pH 7.5, 0.15 M NaCl, 0.01 M CaCl_2 , 0.1% NaN_3 (Buffer I) containing

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¹ The abbreviations used are: CRP, C-reactive proteins; PC, phosphocholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

0.05 mM PMSF. The homogenate was centrifuged at $10,000 \times g$ for 20 min at 4 °C. To the supernatant ammonium sulfate was added to 40% saturation followed by centrifugation at $10,000 \times g$ for 20 min. After centrifugation, ammonium sulfate was added to the supernatant to 70% saturation. The protein precipitate was collected by centrifugation at $10,000 \times g$ for 20 min and dissolved in 10 ml of Buffer I and loaded onto a phosphocholine-Sepharose 4B column (1 $\times$ 10 cm) (Oliveira *et al.*, 1980) previously equilibrated with the Buffer I. The column was washed extensively with the Buffer I containing 2 M NaCl until the absorbance of the eluent at 280 nm was constant. The proteins were eluted by including 0.1 M phosphocholine chloride in Buffer I.

Alternatively, the protein was prepared by using the same procedure as above except that calcium was eliminated in all the buffers. A phosphocholine-Sepharose 4B column was stripped of calcium by passing through two column volumes of 0.1 M EDTA, pH 8.0, then equilibrated with Buffer I without CaCl_2 before loading a protein sample. For binding studies, PC was removed from the purified limunectin by concentrating 1 ml of protein to 0.1 ml and passing through a PD-10 Sephadex column (Pharmacia).

SDS-PAGE—Gel electrophoresis was carried out in 10% polyacrylamide and 0.1% SDS according to Laemmli (1970). Protein samples were denatured in 0.1% SDS in the presence of 0.1% β -mercaptoethanol. The molecular weight of the protein was calculated on the basis of a linear relationship of the log of the molecular weight to the distance migrated in the SDS gel.

Ion Spray Mass Spectroscopy—To accurately determine the molecular weight of limunectin, the purified protein was subjected to ion spray mass spectrometry using a Sciex Mass Spec., API III system (Covey, *et al.*, 1988). The reliability of this method and the accuracy of the instrument were affirmed by determining the molecular weight of a recombinant human growth hormone. The value obtained was consistently within two mass units of its expected mass of 22,123.²

Amino Acid and Carbohydrate Analyses—Protein was hydrolyzed in 4 M methanesulfonic acid for 90 min at 150 °C in a hermetically sealed capillary tube (Liu and Boykins, 1989). Amino acid composition was obtained by analyzing the sample on a Beckman 6300 amino acid analyzer (Spackman, 1967). Carbohydrate content was determined on a sugar analyzer (Boykins and Liu, 1980).

Carboxymethylation of Proteins—To determine if limunectin has any free SH-groups, the native protein (1 nmol) was exposed to 1 μCi [¹⁴C]iodoacetate (from Du Pont-New England Nuclear) and recrystallized unlabeled iodoacetate (50 nmol) in 0.1 ml of 0.1 M Tris, pH 8.5, 6 M guanidine hydrochloride, 1 mM EDTA, at 25 °C for 1 h under nitrogen. The protein was subsequently separated from the reagents by gel filtration on a Sephadex G-25 column (1 $\times$ 60 cm) in 10% acetic acid, and aliquots from the effluent were counted for radioactivity.

Generation of Internal Protein Sequences—Native protein (7–10 nmol) was subjected to CNBr cleavage (100-fold excess over the total methionine, 88% HCOOH , 25 °C, 20 h) (Gross and Witkop, 1962) and limited tryptic digestion (1% trypsin, 0.1 M NH_4HCO_3 , pH 8, 25 °C, 12 h) independently to generate peptides for sequencing. A cyanogen bromide-cleaved peptide and a tryptic peptide were purified by a combination of gel-permeation chromatography on Sephadex G-50 (1 $\times$ 60 cm) in 10% acetic acid followed by reverse-phase chromatography on a C-18 column (Bio-Rad 4.6 $\times$ 200 mm) as previously described (Nguyen *et al.*, 1986b). The purified peptides were subjected to automated Edman degradation.

Protein Sequence Analysis—Automated Edman degradation was carried out with a 477A gas-phase microsequencer (Applied Biosystems Inc.), connected to an on-line model 120A phenylthiohydantoin analyzer. The amount of protein or peptide applied to the sequencer was determined from amino acid analysis of an aliquot of the protein or peptide solution. This value was used to compute the absolute yield of phenylthiohydantoin derivatives in the automated Edman degradation. The absolute and repeated yields were in the range of 95–97 and 85–88%, respectively, for the automated Edman degradation.

Double Diffusion—Double diffusion was performed on glass plates coated with 1% agarose in 50 mM Tris, pH 7.4, 0.15 M NaCl in the presence and in the absence of 0.01 M CaCl_2 .

Polymerase Chain Reaction—Three mixed oligonucleotides based on the amino acids determined by protein sequencing were synthesized with an Applied Biosystem DNA Synthesizer: 3C, $\text{CA}^{\text{G}}/\text{GAC}$ -

$\text{NGA}^{\text{T}}/\text{CAA}^{\text{A}}/\text{GACNGA}$; 1a/b, $\text{GG}^{\text{C}}/\text{TTC}^{\text{G}}/\text{ATC}^{\text{T}}/\text{CTC}^{\text{G}}/\text{ATA}^{\text{T}}/\text{CTT}$, and 2a/b, $\text{TTNCC}^{\text{T}}/\text{CTTC}^{\text{G}}/\text{AA}^{\text{G}}/\text{AA}^{\text{G}}/\text{TA}$. The mixed oligonucleotides were used in pairs (3C and 1a/b, 3C and 2a/b) for the polymerase extension and amplification. Single-stranded cDNA made from amebocyte mRNA was used as a template. The reaction mixtures were analyzed on 0.8% agarose gels. Ethidium bromide-stained bands were excised from the gel, and the isolated DNA was cloned into pUC vectors. DNA sequences were determined by the dideoxy-chain termination method (Sanger *et al.*, 1977). One of the clones, 3a-4 (1.2 kilobases in length made from primers 3C and 1a/b) contained most of the coding sequence for the limunectin, including three regions of the amino acid sequence determined by protein sequencing.

Screening for Full-length cDNA Coding for Limunectin—A cDNA library made from amebocyte mRNA and a genomic DNA library from *Limulus* sperm DNA were prepared by Clontech using a $\lambda\text{gt}10$ vector (Huynh *et al.*, 1985). Several overlapping cDNA clones which contained most of the coding sequence, excluding both the three amino acids at the NH_2 terminus and the 5'-noncoding sequence, were identified and sequenced. Several attempts to screen for cDNA clones containing the NH_2 -terminal sequence failed. A 300-base pair *EcoRI* and *ClaI* fragment was isolated from the 5'-end of the cDNA clone and was used to screen the genomic DNA library. One of the clones was shown to contain the 5'-noncoding sequence, coding sequence for a putative signal peptide, and the entire coding sequence of limunectin.

Computer Analysis for Sequence Homology—The FASTA program was used to determine protein sequence homology between limunectin and other proteins of known sequences.

Binding of Limunectin with Phosphocholine—Purified limunectin (0.15–0.2 nmol) was incubated at room temperature for 1 h with 2–120 μM of [¹⁴C]phosphocholine (5 mCi/mM) in 50 mM Tris buffer, pH 7.5, 0.15 M NaCl, 0.1% NaN_3 in a total volume of 120 μl . For nonspecific binding, 20.8 mM of unlabeled PC (>200-fold excess) was included in the incubation mixture. The values of specific binding were computed by subtracting the nonspecific binding from the total binding. A Pharmacia PD-10 Sephadex column (1 $\times$ 5 cm) was used to separate bound from free PC. The PD-10 column was first coated with 1 ml of a 2% BSA solution followed by equilibration with 25 ml of 50 mM Tris buffer, pH 7.5, 0.15 M NaCl, 0.1% NaN_3 . The reaction mixture was then loaded on to the column and eluted with the same buffer. The first 2.5 ml was discarded and the next 2.0 ml were collected for the radioactivity counting.

Preparation and Purification of Polyclonal Anti-limunectin Antibody—Rabbits were initially immunized with a 1:1 mixture of purified limunectin (300 $\mu\text{g}/\text{ml}$) and complete Freund's adjuvant. For subsequent booster injections incomplete Freund's adjuvant was used. Blood was collected 2 weeks after the second booster injection. Serum from the last bleed was used for Western blot analysis without purification. For immunocytochemistry studies, the antibody was purified with a DEAE-Affi-Gel Blue column (Bio-Rad) according to the manufacturer's protocol.

Electron Microscopy and Immunocytochemistry—For electron microscopy studies the *Limulus* blood was collected in an equal volume of HEPES 0.05 M, pH 7.4, containing 0.1 M *N*-ethylmaleimide, 0.165 M NaCl, 0.01 M EDTA, and 0.1% glutaraldehyde. Amebocytes were pelleted by centrifugation and resuspended in the same buffer where they remained for 1 h for fixation. After washing several times in PBS, the cells were dehydrated, embedded in "LR White" resin (Ted Pella, Inc.), and sectioned (Newman *et al.*, 1982). The nickel grids containing the amebocyte sections were placed in PBS solution containing 1% BSA for 30 min followed by a solution of rabbit anti-limunectin (1:50 dilution) in PBS containing 20% of normal goat serum. After 1 h the grids were washed three times in PBS, 5 min each, and were placed in PBS-normal goat serum containing goat anti-rabbit IgG serum gold conjugate (1:10 dilution) (Auro Probe, 10 nM gold labeled, Amersham Corp.) for 1 h after which they were washed in distilled water and stained by conventional methods with uranyl acetate (De May *et al.*, 1981). The sections were analyzed in a JEOL-100CX electron microscope with an $\times 8000$ magnification.

Iodination of Limunectin—Limunectin (210 μg) was radiolabeled with 1 mCi of carrier-free Na^{125}I (Du Pont-New England Nuclear) using Iodo-Beads (Pierce) (Markwell, 1982). After 20 min at room temperature the free Na^{125}I was removed by passing the reaction mixture through a DG-10 column (Pharmacia) in 0.02 M Tris-HCl, pH 7.4, containing 2% BSA and collected in tubes containing 0.1% BSA. The specific activity of the radiolabeled limunectin was 3 $\mu\text{Ci}/\mu\text{g}$, and when it was analyzed on a SDS-PAGE a single band corresponding to ~50 kDa was obtained.

² Ion spray mass spectrometry of limunectin was performed in the laboratory of Drs. W. S. Hancocks and A. Guzzetta of Genentech, Inc.

Binding of ^{125}I -Limunectin to Bacteria and Amebocytes—For the binding studies, the overnight cultures of *Staphylococcus aureus* and *Escherichia coli* were harvested and washed twice in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, and 10 mM CaCl_2 (binding buffer) and resuspended in the binding buffer to a final concentration of 2×10^8 cell/ml (Filipssen *et al.*, 1990; Paulsson and Wadström, 1990). For formalin-fixed *S. aureus* and *Limulus* amebocytes, cells were resuspended in the binding buffer to an $\text{OD}_{600} = 1.2$. Fifty μl of cell suspension containing 0.1% of BSA were incubated with 30 μl (20,000 cpm) of ^{125}I -limunectin and 20 μl of unlabeled limunectin in different concentrations (0–2 μM) for 2 h at room temperature. For inhibition studies, inhibitors were added 30 min prior to the addition of ^{125}I -limunectin. The reaction mixtures were quenched by the addition of 200 μl of ice-cold binding buffer and centrifuged for 10 min in an Eppendorf microfuge. The pellets were washed three times in 500 μl each of PBS, and amounts of the bound radioactive limunectin were measured in an LKB gamma counter.

Binding of ^{125}I -Limunectin to Immobilized Proteins and Heparin—Binding assays were performed according to Gebb *et al.* (1986) with the exception that a radiolabeled ligand instead of an antibody to the ligand was used. Wells of a polyvinyl microtiter plate were coated overnight at room temperature with 100 μl of solutions containing 10 $\mu\text{g}/\text{ml}$ of collagens, gelatin, fibronectin, or heparin (Sigma) in 0.1 M NaHCO_3 , pH 9.5. The wells were then washed three times with 0.02 M Tris buffer, pH 7.4, and this buffer was used in all subsequent steps. Radiolabeled limunectin (10,000 cpm) was added to each well and incubated overnight at room temperature. For competition studies 100-fold excess of unlabeled limunectin was added to the wells 30 min prior to the addition of radiolabeled limunectin. The unbound materials were removed by three washes with the same buffer. The wells were counted individually in an LKB gamma counter.

RESULTS³

Purification of Limunectin—When *Limulus* amebocyte lysates were passed through a phosphocholine affinity column in the presence of Ca^{2+} , a protein of ~50 kDa and two proteins of approximately 30 kDa, were selectively adsorbed. In the absence of divalent ions, only the 54-kDa protein was selectively retained by the affinity column (Fig. 1). The purified 54-kDa protein precipitated with pneumococcal C-polysaccharide and phosphocholine derivative of bovine serum albumin (BSA-PC) in a Ca^{2+} -independent manner (Fig. 2).

SDS-PAGE and NH_2 -terminal residue analysis revealed that the newly isolated protein consisted of a single polypeptide chain. Ion spray mass spectroscopy analysis indicated that the molecular mass for limunectin is $55,323 \pm 4$ daltons. The carbohydrate analysis indicated that the protein is probably not glycosylated. The protein as a whole is dominated by hydrophilic regions and consists of 35.0% non-polar residues, 42.4% polar residues, 9.8% acidic residues, and 12.8% basic residues with a pI of 9.1 as calculated from the pI values of individual amino acids.

Nucleotide and Deduced Amino Acid Sequence of Limunectin—The nucleotide and the deduced amino acid sequence of limunectin are shown in Fig. 3. The DNA sequence is derived from a genomic clone (full-length) and several cDNA clones (base 217 to the 3'-end). The NH_2 -terminal residues and two internal segments of the sequence of limunectin were verified by amino acid sequence analyses of the purified protein, a CNBr fragment and a tryptic peptide of the molecule, as indicated by *solid underlines* in Fig. 3. In all cases there is excellent agreement between the amino acid sequence determined by protein sequence and the deduced amino acids from the DNA sequence. The DNA sequence defines a polypeptide of 54,146 daltons which agrees closely with the molecular

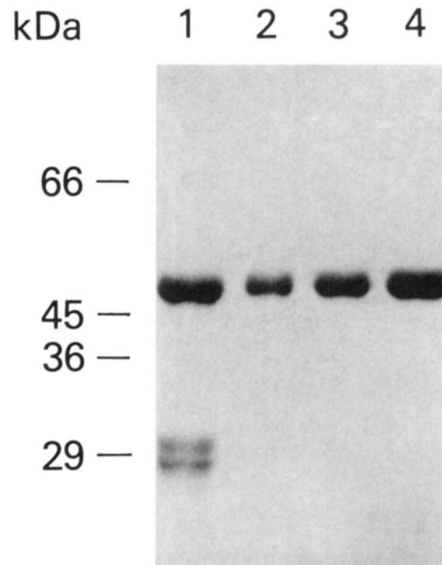


FIG. 1. **Purification of limunectin.** A 10% SDS-PAGE analysis of proteins eluted from PC-column. *Lane 1*, limunectin coeluted with 30-kDa proteins when the PC affinity column was run in the presence of Ca^{2+} and the adsorbed proteins eluted with PC. *Lanes 2–4*, limunectin (10 μg , *lane 2*; 25 μg , *lane 3*; 50 μg , *lane 4*) eluted from the PC affinity column with PC in the absence of Ca^{2+} .

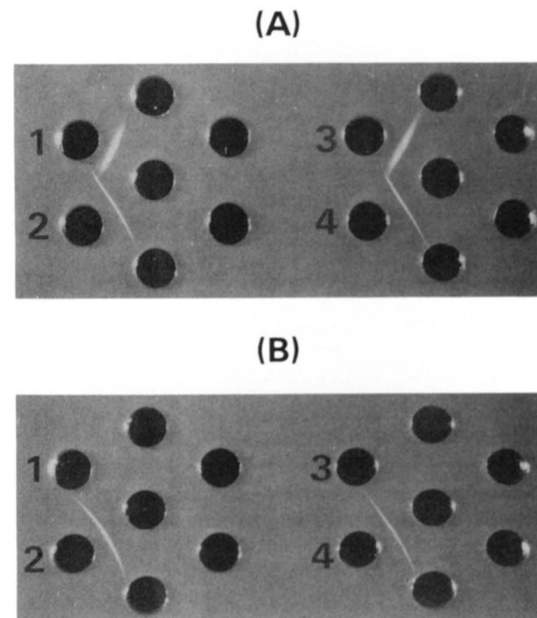


FIG. 2. **Double diffusion of limunectin and C-reactive protein.** Double diffusions were carried out in 1% agarose plates containing 50 mM Tris, pH 7.5, 0.15 M NaCl. *Panel A*, the agarose plate contains 0.01 M CaCl_2 . Center well at left contains C-polysaccharide, 0.2 mg/ml, 20 μl . Center well at right contains BSA-PC, 1 mg/ml, 20 μl . *Panel B*, identical arrangement with *panel A* except that the agarose plate did not contain CaCl_2 . *Samples 1 and 3*, human CRP, 20 μl ($\text{OD}_{280}/\text{ml} = 2$). *Samples 2 and 4*, limunectin, 20 μl ($\text{OD}_{280}/\text{ml} = 2$).

mass determined by ion spray mass spectroscopy, 55,333 daltons. The gene sequence predicts a leader sequence of 15 amino acid residues which is absent in the mature protein. Further evidence that the deduced amino acid sequence ac-

³ Portions of this paper (including Figs. S-1–S-3 and Tables S-1–S-3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

activity of vitronectin or fibronectin and von Willebrand factor (Ruoslahti, 1988)

Binding Properties of Limunectin with Phosphocholine—The binding of radiolabeled PC to limunectin was determined by a Scatchard analysis (Fig. 6). The results indicate that the binding is specific and saturable. The binding of limunectin to radiolabeled PC was effectively displaced by a 200-fold excess of the unlabeled PC. The stoichiometry of limunectin-PC interaction at pH 7.4 was estimated to be approximately 2 PC bound/protein at four different protein concentrations with an average $K_d = 4 \times 10^{-6}$ M. The binding of limunectin to pneumococcal C-polysaccharide, a PC containing cell wall polysaccharide of bacteria and BSA-PC are illustrated by double diffusion analysis (Fig. 2).

Cellular Localization—Polyclonal antibody used in this study is reactive only to the limunectin as shown by Western blot analysis of both purified limunectin and *Limulus* amoebocyte lysate. Immunocytochemical analysis revealed that limunectin is localized in the secretory granules of the amoebocytes and is absent from the nucleus and other cytoplasmic structures (Fig. 7).

Adhesion-promoting Properties of Limunectin—Since limited sequence homologies were found to exist between limunectin, vitronectin, gelatinase, and collagenase (Fig. 5), a number of binding studies were carried out to test the adhesion properties of limunectin. Bacterial cells, gelatin, collagen, and heparin which are known to bind specifically to vitronectin were used for binding to radiolabeled limunectin. Fibronectin was also used to test the binding specificity of limunectin.

Adhesion to Bacteria and Amoebocytes—Fig. 8 shows the binding of ^{125} I-limunectin to *S. aureus*, *E. coli*, and formalin-fixed *S. aureus* and amoebocytes. The binding was displaced by the addition of unlabeled limunectin. The ID_{50} for the bindings as calculated from these data is approximately 10^{-7}

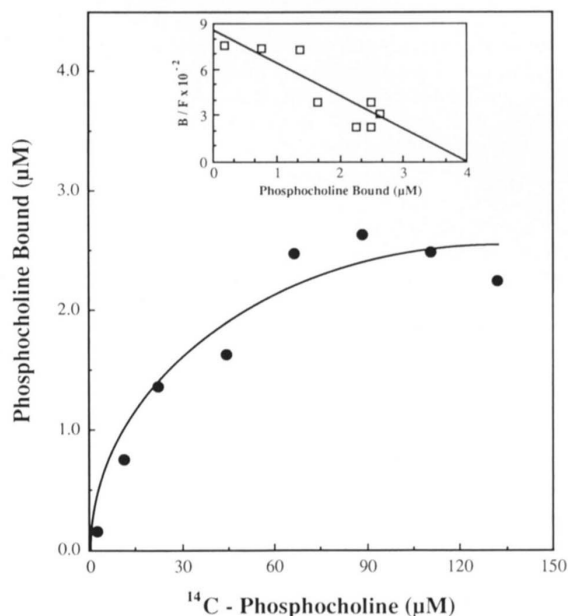


FIG. 6. Specific binding of ^{14} C-radiolabeled phosphocholine to limunectin. The binding reactions were carried out in the presence of >200-fold excess of unlabeled PC (nonspecific binding) and in the absence of unlabeled PC (total binding). Subtracting the nonspecific binding from the total binding, a saturation curve of specific binding of PC to limunectin was obtained. The insert is a Scatchard plot which gives a K_d of 4×10^{-6} M.

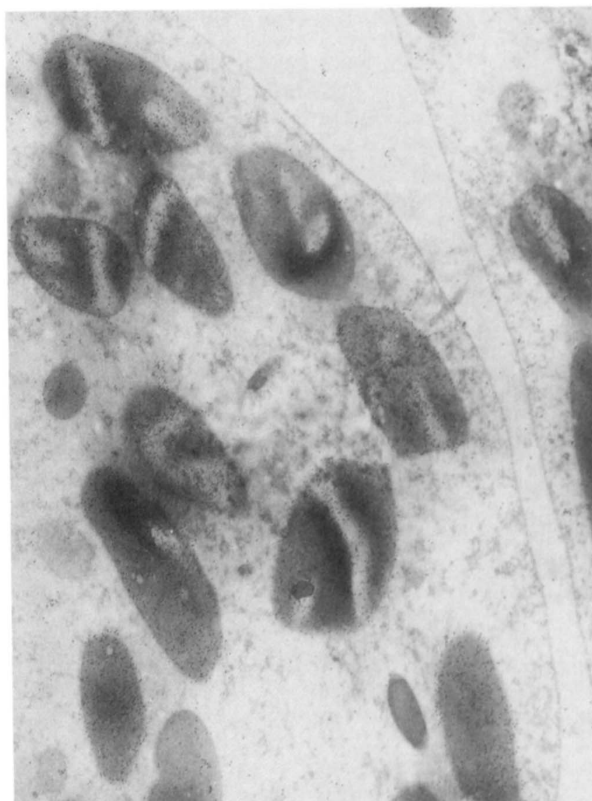


FIG. 7. Electronmicrograph of *Limulus* amoebocytes with immunogold staining. The first antibody used was an affinity purified rabbit anti-limunectin, and the second antibody was a gold-labeled rabbit anti-IgG. A large number of granules with gold particle staining are visible. Magnification, $\times 8000$.

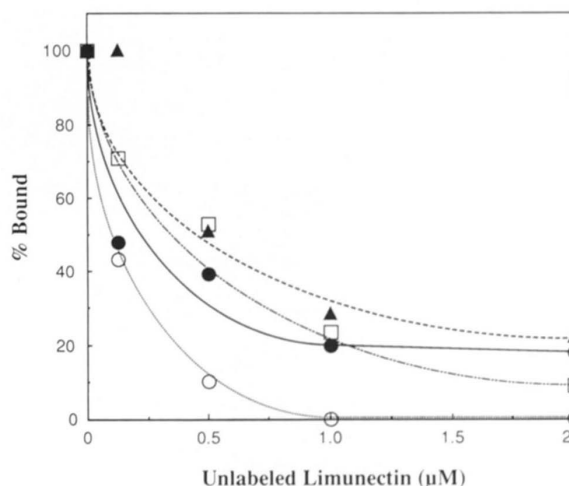


FIG. 8. Competition studies of limunectin to live (□), formalin-fixed (●) *S. aureus*, *E. coli* (○), and formalin-fixed amoebocytes (▲). ^{125} I-Limunectin (20,000 cpm) was added to 50 μ l of 10^6 cells/ml with increasing amounts of unlabeled limunectin in the range of 0–2 μ M.

M. Binding was not altered by formalin fixation when comparing live or formalin-fixed *S. aureus* cells. When PC was used to compete for the binding of limunectin to *S. aureus*, a high concentration was needed with an ID_{50} of 18 mM. Other

substances tested (heparin, glucosamine, and lipopolysaccharide) were ineffective as inhibitors.

Binding of ^{125}I -Limunectin to Immobilized Proteins and Heparin—Table II shows the maximal binding as well as specific binding of limunectin to several types of collagen (native and heat denatured), gelatin, heparin, and fibronectin. Limunectin bound to all types of collagen tested; heat denaturation did not affect the binding significantly. The binding of ^{125}I -limunectin to collagen, gelatin, fibronectin, and heparin was displaced by the addition of 100-fold excess unlabeled limunectin. The result indicates that the binding activity of limunectin is not restricted to gelatin and collagens, and it also binds specifically to other matrix adhesion proteins like fibronectin.

DISCUSSION

A 54-kDa protein has been purified from a soluble extract of *Limulus* amebocytes by phosphocholine affinity chromatography. The protein is found mainly in the secretory granules of the amebocytes as revealed by immunocytochemistry (Fig. 7).

Based on the amino acid sequence deduced from the gene sequence, the protein is probably synthesized in a precursor form with a leader sequence of 15 amino acids that are essentially hydrophobic in nature (Fig. 3). The deduced protein sequence consists of 469 amino acid residues corresponding to a molecular mass of 54,146 Da which is 1,187 Da less than that determined by ion spray mass spectroscopy. This small discrepancy could derive from an as yet unidentified post-translational modification. Even though there is one possible *N*-glycosylation site in the amino acid sequence (residue 273), sugars were not detected in the carbohydrate analysis. Due to the sensitivity of our carbohydrate determination, the possibility for a small carbohydrate chain attached to limunectin cannot be excluded.

It is important to point out that a genomic clone isolated from the genomic DNA library contains the coding sequence for the entire limunectin (10 repeats) without any intervening sequence. One of the cDNA clones initially isolated, 3A4, contains, however, only nine repeats. Five additional cDNA

clones subsequently isolated all contain 10 repeats. The extra repeat was located between segment 8 and 9 of clone 3A4 and contains coding sequence for 45 amino acids identical to segment 8 with only two silent base substitutions in the DNA sequence (Fig. 4). Due to the homologous repeating segments in limunectin, distinguishing a protein with nine or 10 repeats is difficult based on either total amino acid analysis or partial sequence analysis. An accurate molecular weight determination could, however, provide the distinction. A sample of purified limunectin was therefore, subjected to ion spray mass spectrometric analysis (Covey *et al.*, 1988) and resulted in a molecular weight of $55,333 \pm 4$. The result strongly favors the suggestion that limunectin consists of 10 rather than nine repeats. The origin of clone 3A4 is not clear. It might have come from an unusual splicing mechanism or more likely from an artifact in the polymerase chain reaction used to generate the cDNA clones.

In gel filtration studies (Superose 12, Pharmacia, 1×30 cm) carried out in high salt (0.1 M Tris, pH 7.6, containing 1.0 M KCl), the protein elutes in a position corresponding to an apparent molecular mass of 12,000 daltons (data not shown) which is considerably smaller than its actual size. The reason for this anomalous behavior is unknown but may be related to the compact structure imposed by the 10 disulfide linkages or to lectin-like properties, *e.g.* affinity for the agarose gel matrix.

The most striking feature of the molecule, as revealed by sequence analysis, is the repeated homologous segments. With the exception of 25 amino acids at the NH_2 terminus, the rest of the molecule consists of 10 contiguous segments of about 45 amino acids that share extensive homology. Within each repeat, there is a pair of cysteines that are equally spaced and 11 amino acid residues that are absolutely conserved. The homology between each of the 10 domains and the consensus domain is shown in Fig. 4. In summary, six domains share over 90% homology, two exhibit over 80% homology, and the remaining two over 70 and 50% homology, respectively. The alignment of repeated internally homologous segments is reminiscent of the Ig superfamily of proteins including the N-cell adhesion molecule polypeptides (Cunningham *et al.*, 1987) where five internally homologous segments are found to be aligned contiguously in the molecule. These proteins serve, among other functions, as mediators of cell-cell interaction. Another group of adhesion proteins, regulators of complement activation, are also known to contain a variable number (4–30) of internal repeats corresponding to the consensus sequence of complement regulatory proteins (Hourcade *et al.*, 1989). A granular membrane protein isolated from platelets and endothelial cells belongs to this family and contains nine homologous repeats and six functional domains (Johnston *et al.*, 1989).

A limited sequence homology was observed between the consensus sequence (Fig. 4) of limunectin and a region of vitronectin (residues 207–233, 52% identity, 80% homology, Fig. 5) (Suzuki *et al.*, 1985). Such homology becomes more significant when one considers the fact that these sequences are repeated 10 times in the *Limulus* protein. This finding was the basis of our investigation into the possible functional similarities between the two proteins regarding their binding specificities to the cell surface, collagen and to heparin, which are common properties among several adhesion molecules. An R-G-D sequence present in a number of vertebrate and invertebrate proteins has been shown, at least in some cases, to confer the ability to interact with specific receptors on the cell surface designated as integrins (Ruoslahti, 1988). Such a sequence was not found in limunectin. Even though limunec-

TABLE II
Binding of ^{125}I -limunectin to collagen, gelatin, heparin, and fibronectin

The binding was carried out with 100 μl of the test compound (10 $\mu\text{g}/\text{ml}$) and 10,000 cpm of ^{125}I -limunectin in the presence and in the absence of unlabeled limunectin.

Compound	^{125}I -limunectin bound	
	Total	Specific ^a
	cpm	
Collagen		
Type I (native)	5506	1959
Type I (denatured)	4120	2708
Type III (native)	9310	5749
Type III (denatured)	8160	3721
Type IV (native)	7505	3506
Type IV (denatured)	6855	3783
Type V (native)	5719	2915
Type V (denatured)	6736	3129
Gelatin	4949	3652
Heparin	3503	3367
Fibronectin	4196	2289
Control ^b	1595	0

^a Specific binding was calculated by subtracting the nonspecific binding (with 100-fold molar excess of unlabeled limunectin) from the total binding. The values were the average of three determinations.

^b As a control, the wells received only the binding buffer.

tin and vitronectin both bind to *S. aureus*, collagen, and heparin, the specific mechanisms may be different. In fact, the region of sequence similarity between both molecules comprises a segment in vitronectin that does not correspond to the reported binding sites for heparin (Suzuki *et al.*, 1984) and collagen (Izumi *et al.*, 1988). Furthermore, in competition studies of binding to both *S. aureus* and amoebocytes, we found that neither heparin nor sugars inhibited the limunectin binding activity, while PC was effective but only at high concentrations ($ID_{50} = 18 \text{ mM}$).

Additional information from the sequence homology search in the protein sequence bank demonstrated that the consensus sequence of limunectin that is homologous with vitronectin also shares homology with gelatinase (residues 539–582) and with collagenase (residues 427–464) (Fig. 5). The natural substrate for collagenase is collagen and for gelatinase is gelatin (collagen IV). Vitronectin is also known to bind to collagen (Gebb *et al.*, 1986). To date, no enzymatic activity has been attributed to vitronectin, but recent studies reveal that fibronectin contains a potential gelatinase activity (Plancheault *et al.*, 1990). The finding that limunectin shares a common sequence with vitronectin, gelatinase, and collagenase suggests that this common sequence might be responsible for the binding of limunectin to gelatin and collagen.

Limunectin binds to PC specifically with a K_d of $4 \times 10^{-6} \text{ M}$ which is similar to that of CRP and PC (Robey *et al.*, 1983). This binding was the basis for its purification using PC affinity chromatography as described under "Materials and Methods." While CRP-PC binding requires Ca^{2+} , limunectin binding is independent of Ca^{2+} . In this respect limunectin resembles the PC binding of mouse myeloma proteins (Segal *et al.*, 1974). There is no sequence homology between limunectin and CRP or the myeloma protein. However, binding of PC involves relatively few amino acids at the binding site. X-ray crystallographic analysis of myeloma PC-binding protein reveals that the binding of PC involves the side chains of Arg-52 and Tyr-33 for phosphate and Glu-35 for the choline group with additional van der Waals interactions between the choline and main chain atoms of residues 102–103 of H-chain residues and residues 91–94 of the L-chain (Segal *et al.*, 1974). Limunectin binds two molecules of PC/molecule of protein suggesting that the binding loci for PC are most likely the result of the folding of the molecule rather than the consequence of a linear arrangement of certain amino acid residues. Since PC is an integral component of a variety of polysaccharides and phospholipids from cellular and organelle membranes and from bacterial and fungal cell walls (Baldo, 1979), part of the biological function of limunectin could be related to its PC-binding property as was proposed for CRP (Narkates and Volanakis, 1982).

In considering the possible biological function of limunectin, one must keep in mind that it is an intracellular protein. However, at the time of cell lysis or exocytosis, induced for instance by endotoxin from Gram-negative bacteria, amoebocytes release limunectin together with other cellular components including the proteins of the coagulation cascade system. In this context, limunectin could conceivably function as an adhesion molecule to assist in both recognition and neutralization of the invading microorganisms. Since immunoglobulins are absent in horseshoe crabs, molecules such as agglutinins, lectins, and complement-like proteins could work in concert to serve as part of the defense mechanism. In this regard, our finding of a protein which belongs to the adhesion protein family in the amoebocytes of horseshoe crabs lends some support to this hypothesis. To further substantiate this notion we have also demonstrated that limunectin can be

detected as a component of extracellular fluid when amoebocytes are treated with an ionophore, 4-bromo-calcio-ionophore A23187 (Armstrong and Quigley, 1985) to promote the exocytosis (not shown).

Most adhesion proteins contain transmembrane segments with the amino-terminal domains facing the extracellular space. However, soluble adhesion proteins have also been reported (Johnston, 1989). Data from the cDNA sequence and the immunocytochemistry indicate that limunectin is most likely a soluble protein located within the secretory granules. It is however possible that membrane-bound species also exist in the amoebocyte as in the case of the platelet granular membrane protein (Johnston *et al.*, 1989).

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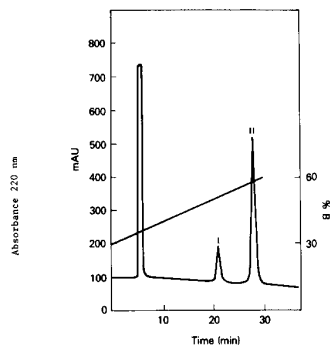
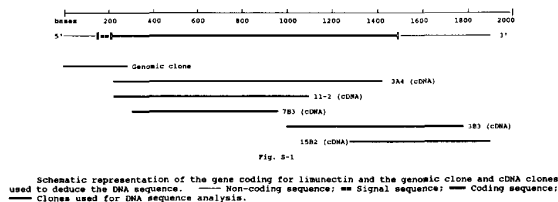
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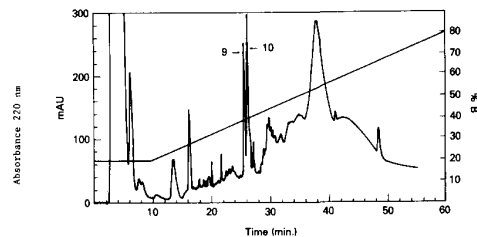
Supplementary Material

Limunectin: A Phosphocholine Binding Protein from *Limulus* Amebocytes with Adhesion Promoting Properties

Teresa Liu, Yuan Lin, Teresa Cisto, Conceicao A. S. A. Minetti, Julia M. K. Babe and Teh-Yung Liu



Purification of tryptic peptides. A sample from Sephadex G-50 column chromatography was rechromatographed on a C-18 reverse-phase column. (Bio-Rad 4.6 x 200mm); mobile phase: 0.1% TFA in H₂O; B=0.1% TFA in CH₃CN; flow rate=0.5ml/min; gradient=30% - 60% B, 30 min. Peak I was submitted to automated Edman degradation and the results are recorded in Table S-2 and Figure 3 (Text).



Purification of a CNBr fragment. Sample from Sephadex G-50 Column Chromatography was re-chromatographed on a C-18 reverse-phase column (Bio-Rad 4.6 x 200 mm); mobile phase: 0.1% TFA in H₂O; B=0.1% TFA in CH₃CN; flow rate = 0.5 ml/min; gradient = 30% - 60% B, 1 hr. Peak 9 gave the results shown in Table S-1 and Figure 3 (Text), upon automated Edman degradation. Peak 10 gave identical amino acid sequence as Peak 9 and is the lactone-form of the CNBr fragment.

Table S-1

SEQUENCE OF NATIVE LIMUNECTIN
(5.5 n moles of native protein were sequenced)

CYCLE NUMBER	AMINO ACID	YIELD (n mole)
1	Glutamic acid	4.60
2	Valine	3.3
3	Serine	0.45
4	Glutamine	2.8
5	Threonine	0.8
6	Aspartic acid	1.5
7	Lysine	2.0
8	Threonine	0.9
9	Glutamic acid	1.4
10	Leucine	2.4
11	Histidine	0.6
12	Serine	0.2
13	Threonine	0.7
14	Glycine	0.7
15	Methionine	1.1
16	Glutamic acid	0.74
17	Isoleucine	0.67
18	Leucine	1.15
19	Glutamine	0.61
20	Serine	-
21	Isoleucine	-
22	Phenylalanine	0.66
23	Proline	0.23
24	Serine	-
25	Isoleucine	-

- Detected but not quantitated

Table S-2

SEQUENCE OF A TRYPTIC PEPTIDE DERIVED FROM LIMUNECTIN
(2 n moles of peptide were sequenced)

CYCLE NUMBER	AMINO ACID	YIELD (n mole)
1	Lysine	1.32
2	Isoleucine	1.69
3	Arginine	0.92
4	Valine	1.31
5	Valine	1.37
6	Aspartic acid	1.53
7	Alaine	1.21
8	Valine	0.87
9	Phenylalanine	1.02
10	Arginine	1.09
11	Tryptophan	0.69
12	Serine	-
13	Asparagine	0.50
14	Glycine	0.64
15	Isoleucine	0.52
16	Threonine	0.37
17	Tyrosine	0.61
18	Phenylalanine	0.83
19	Phenylalanine	0.90
20	Lysine	0.60
21	Glycine	0.47
22	Asparagine	0.33
23	Cysteine	x
24	Tyrosine	0.59
25	Tyrosine	0.82
26	Arginine	0.48
27	Tyrosine	0.57
28	Glutamic acid	0.27
29	Aspartic acid	0.26
30	Lysine	0.29
31	Threonine	0.11
32	Asparagine	0.34
33	Lysine	0.27
34	Leucine	0.17
35	Serine	-
36	Glutamine	0.11

x Not detected
- Detected but not quantitated

Table S-3

SEQUENCE OF A CYNOGEN BROMIDE FRAGMENT OF LIMUNECTIN
(7.0 n moles of peptides were sequenced)

CYCLE NUMBER	AMINO ACID	YIELD (n mole)
1	Lysine	6.22
2	Tyrosine	4.00
3	Glutamic acid	3.92
4	Aspartic acid	1.60
5	Lysine	2.72
6	Proline	2.54
7	Glutamine	2.48
8	Lysine	1.84
9	Leucine	1.84
10	Serine	0.82
11	Glycine	1.71
12	Glutamic acid	0.80
13	Asparagine	0.52
14	Proline	0.92
15	Isoleucine	0.60