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Cell Attachment Peptide of C-Reactive Protein: Critical Amino Acids and Minimum Length

Michael C. Mullenix, Pravin T.P. Kaumaya, and Richard F. Mortensen

Departments of Microbiology (M.C.M., R.F.M.) and Medical Biochemistry (P.T.P.K.), The Ohio State University, Columbus, Ohio 43210

Abstract Human C-reactive protein (CRP) is an acute phase blood component that accumulates at sites of tissue damage and necrosis and is degraded by neutrophils to biologically active peptides. A dodecapeptide composed of amino acids 27–38 of CRP mediates cell attachment in vitro. This peptide was designated the cell-binding peptide (CB-Pep) of CRP. Characterization of the interaction between fibroblasts and modified synthetic peptides with sequential deletions from either the N-terminus or C-terminus revealed that the minimal sequence for cell attachment or inhibition of cell attachment to the CB-Pep was **Phe-Thr-Val-Cys-Leu,** which corresponds to residues 33–37 within each of the five 206 amino acid subunits of CRP. The pentapeptide by itself mediated cell attachment. Substitutions for each residue within the CB-Pep indicated that the critical residues for activity were Phe-33 and Thr-34. This cell-binding pentapeptide represents a recognition motif for cell adhesion not found in other proteins. © 1994 Wiley-Liss, Inc.

Key words: hepatocytes, pentraxins, phospholipids, neutrophils, tetrapeptides, wound repair

The acute phase of the systemic inflammatory response to tissue injury or infection is characterized by a rapid reorchestration in the pattern of blood proteins synthesized by liver hepatocytes in response to several cytokines [Kushner, 1988]. C-reactive protein (CRP) is the prototype acute phase reactant since its concentration can increase several thousandfold in humans and most vertebrates [Pepys and Baltz, 1983; Ballou and Kushner, 1992]. CRP is composed of five identical noncovalently associated subunits of 206 amino acids each [Osmand et al., 1977; Woo et al., 1985] and displays Ca^{++}-dependent binding to monophosphate esters, especially phosphorylcholine (PC) [Gotschlich and Edelman, 1967; Kilpatrick and Volanakis, 1991]. The homologous protein, serum amyloid P-component (SAP), and CRP are classified as pentraxins, proteins with a highly conserved secondary structure and gene organization [Liu et al., 1987]. Human CRP mediates several inflammatory biological activities, including leukocyte activation [Buchta et al., 1987; Tebo and Mortensen, 1990; Zeller and Sullivan, 1992] and initiation of the complement cascade [Volanakis and Narkates, 1981; Jiang et al., 1991]. One of CRP's unique properties is that it is selectively deposited at sites of tissue damage, suggesting a mechanism for focusing its biological activities [Kushner and Kaplan, 1961; Kushner et al., 1963; Gitlin et al., 1977; DuClos et al., 1981]. CRP also interacts via its PC-binding region with the extracellular matrix proteins fibronectin (Fn) [Salonen et al., 1984] and laminin [Swanson et al., 1989] and to phospholipids on damaged membranes [Volanakis and Narkates, 1981], substances that are likely to be more accessible in inflamed and damaged tissues. Neutrophil proteolysis of CRP generates peptides that also activate monocytes and neutrophils [Robey et al., 1987; Shephard et al., 1988, 1990]. The active peptides contain tuftsin tetrapeptide (Thr-Lys-Pro-Arg)-like sequences [Robey et al., 1987].

Abbreviations used: B, biotin or biotinylated; CB-Pep, cell-binding peptide; CRP, C-reactive protein; CRP-R, CRP-receptor; DTT, dithiothreitol; Fn, fibronectin; KLH, Keyhole limpet hemocyanin; mAb, monoclonal antibody (ies); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PC, phosphorylcholine; Pep, peptide; SAP, serum amyloid P-component; TMB, 3,3′5,5′-tetramethylbenzidine.

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Address reprint requests to Richard F. Mortensen, The Ohio State University, Department of Microbiology, 484 West 12th Avenue, Columbus, OH 43210.

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The analysis of one of the tuftsin-bearing peptides composed of residues 27–38 for its interaction with the monocyte CRP-receptor (CRP-R) [Tebo and Mortensen, 1990] resulted in the finding that the peptide supports cell attachment in vitro, and therefore the peptide is referred to as the cell-binding peptide or CB-Pep [Fernandez et al., 1992]. Characterization of the interaction between cells and the CB-Pep revealed that attachment occurs at physiological concentrations, is distinct from the attachment to the extracellular matrix proteins, Fn and laminin, and is not inhibited by the tuftsin tetrapeptide [Fernandez et al., 1992]. The interaction with the CB-Pep indicated that cell attachment was very different from that supported by RGDS-bearing peptides from Fn [Fernandez et al., 1992].

To determine the relationship between the receptor for CB-Pep to other known cell attachment receptors, it became important to determine both the critical residues involved and the minimal length of the peptide that supports cell attachment. Our approach was to synthesize substituted peptides for determining the critical residues involved and truncated peptides to determine the minimal length required. The experiments described herein using both direct cell attachment and inhibition of cell attachment to the CB-Pep by the various synthetic peptides show that the critical residues are present within a five residue minimal sequence in the C-terminal half of the dodecapeptide. The findings suggest that CRP, and especially a peptide derived from CRP, may contribute to the wound repair process by supporting cell attachment via receptors distinct from the integrin receptors for extracellular matrix proteins.

MATERIALS AND METHODS

Peptide Synthesis

The list below shows the sequence of the CB-Pep of CRP, as well as the substituted and truncated peptides that were synthesized. The numbering of the residues is based on the published sequence by Woo et al. [1985]. The substituted amino acid is shown in bold, and its position is part of the designation for each of their peptides. The truncated peptides with amino acids deleted from the C- and N-terminus are designated by the remaining residues.

CB27-38 T-K-P-L-K-A-F-T-V-C-L-H
CB30I T-K-P-**I**-K-A-F-T-V-C-L-H
CB31R T-K-P-L-**R**-A-F-T-V-C-L-H
CB32L T-K-P-L-K-**L**-F-T-V-C-L-H
CB33Y T-K-P-L-K-A-**Y**-T-V-C-L-H
CB34S T-K-P-L-K-A-F-**S**-V-C-L-H
CB35L T-K-P-L-K-A-F-T-**L**-C-L-H
CB37I T-K-P-L-K-A-F-T-V-C-**I**-H
CB38K T-K-P-L-K-A-F-T-V-C-L-**K**

CB28-38 K-P-L-K-A-F-T-V-C-L-H
CB29-38 P-L-K-A-F-T-V-C-L-H
CB30-38 L-K-A-F-T-V-C-L-H
CB31-38 K-A-F-T-V-C-L-H
CB32-38 A-F-T-V-C-L-H
CB33-38 F-T-V-C-L-H
CB33-37 F-T-V-C-L
CB27-37 T-K-P-L-K-A-F-T-V-C-L
CB27-36 T-K-P-L-K-A-F-T-V-C
CB27-35 T-K-P-L-K-A-F-T-V
CB27-34 T-K-P-L-K-A-F-T

The substituted and truncated peptides were synthesized by FMoc-*tert*-butyl solid phase synthesis strategy on an advanced chemtech model 350 multiple peptide synthesizer. The solid support consisted of 4-methylbenzhydrylamine resin (0.54 mmol of Cl/g) using an acid labile linkage agent 4-(hydroxymethyl) phenoxyacetic acid [Kaumaya et al., 1990, 1992]. Side chain protection was as follows: S-trityl for cysteine, *tert*-butyl ethers for serine, threonine, and tyrosine, N-*im*-trityl for histidine, *tert*-butyloxycarbonyl (Boc) for lysine, and N^G-PMC for arginine. The resin support was neutralized with 10% diisopropylethylamine (DIEA) in dichloromethane (DCM). The linkage agent was activated as its pentafluorophenyl (pfp) ester (fivefold) and attached to the resin by double coupling (30 min each). During the last 15 min, three equivalents of catalyst [1-hydroxybenzotriazole (HOBt)] were added. Ninhydrin Kaiser test indicated that no amino groups were left unreacted. The C-terminal amino acids were esterified to the linker-activated support by double coupling of the preformed pfp ester (sixfold excess) for 1 h in the presence of dimethylaminopyridine (0.1 equivalent) as the catalyst.

A typical coupling cycle was as follows: the N-protected peptide resin was washed once and treated for 12 min with 50% piperidine in DMF followed by 10 DMF washes. Duplicate 30 min couplings for each amino acid were performed using a sixfold excess of FMoc amino acid, HOBt, and 1,3-diisopropyl-carbodiimide (DIC). After the chain was elongated the N-terminal FMoc group was removed and the resin washed, transferred,

and dried in a desiccator. The side chain protecting groups and the peptide resin anchoring bond were cleaved by treatment with trifluoroacetic acid (TFA) containing 5% anisole, 3% thioanisole, and 2% ethanedithiol. The resins were washed several times with neat TFA and the combined washings were rotary-evaporated to an oily residue. The crude peptides were precipitated with diethyl ether and taken up in 0.1 M acetic acid. Several ether extractions were carried out to remove scavengers, and the aqueous acidic phase was lyophilized to a dry powder. One of the full-length substituted peptides was N-terminally sequenced on a Milligen prosequencer model 6600, and the analysis confirmed the sequence accuracy. The substituted and truncated peptides were $> 70\%$ pure on the basis of reverse phase HPLC profiles.

The first CB-Pep was synthesized by the Ohio State University Biochemical Instrument Center using t-boc synthesis on a model 9500 peptide synthesizer (Millegen/Millipore, Burlinton, MA) and was estimated to be $\sim 90\%$ pure on the basis of amino acid composition.

Cell Attachment Assays

Polystyrene microplates (Immulon-2; Dynatech, Chantilly, VA) were coated with 10 nmoles of CB-Pep, 2 nmoles of intact CRP, or 0.5 pmoles of fibronectin (Fn) in carbonate-bicarbonate buffer (pH 9.6) as described previously [Swanson et al., 1989; Fernandez et al., 1992]. The wells were washed 3× in phosphate-buffered saline (PBS) and blocked for 1 h with 1% BSA in PBS. Normal rat kidney fibroblasts (NRK-49F) from American Type Culture Collection between passages 2 and 10 were added at 10^5/well and allowed to adhere for 30 min in RPMI medium containing 5% calf serum. For inhibition of cell attachment soluble CB-Pep at 100 nmoles/well was added at the same time as the cells. The plates were washed 3× with PBS to remove unattached cells and the percentage of attached cells determined by the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay [Mosmann, 1983]. Briefly, 100 μl of medium and 20 μl of a 5 mg/ml MTT in PBS were added to the attached cells in each well and incubated at 37°C in 5% CO_2 for 2 h. The reduced MTT crystals formed by viable cells were dissolved in 0.01 M HCl in isopropanol, and the amount of MTT dye incorporated was determined by reading its absorbance at 550 nm. The absorbance of the converted dye was proportional to the number of viable cells remaining in the wells. The percentage of cell attachment was calculated by dividing the average absorbance of three wells by the average absorbance of three wells coated with Fn.

Reduction of Cys in CB-Pep

Dithiothreitol (DTT) reduction of the CB-Pep was done by dissolving 5 mg of the peptide in 0.1 M acetic acid and 0.05 M DTT. DTT was removed from the peptide solution by gel filtration through a sephadex G15 column by collecting fractions at the void volume. DTT removal was assayed by the addition of Ellman's reagent. The peptide was then dialyzed against PBS (pH 7.4) to remove the acetic acid. All buffers were purged with nitrogen gas prior to use in any steps involving the reduced peptide.

Direct Cell Attachment to Substituted and Truncated Peptides

Serial twofold dilutions of substituted peptides and peptides with deleted residues were diluted in carbonate buffer (pH 9.6) and coated onto Immulon-2 microplate wells. The dilution range of the coated peptides was from 20 to 0.16 nmoles/well. Cell attachment was determined as described above.

Inhibition of Cell Attachment to CB Peptide

Microplates were coated with 10 nmoles/well of CB-Pep washed and blocked as described above. Serial twofold dilutions of either substituted or deleted peptides at 100 to 12.5 nmoles/well were added with 10^5 NRK-49F cells and allowed adhere for 30 min in RPMI medium plus 5% calf serum. The plates were washed 3× with PBS to remove the unattached cells, and the percentage of attached cells was determined by the MTT viability assay as described above.

RESULTS

Cell Attachment to Intact CRP, Fn, and the CB-Pep

To compare the relative efficiency of cell attachment to both the CB-Pep of CRP and the intact protein, we compared cell attachment to equimolar amounts of CRP and CB-Pep vs. plasma Fn. Similar levels of cell attachment were observed when comparing CB-Pep or intact CRP at 10 nmoles/well (Fig. 1). The molar concentration for the intact CRP was calculated

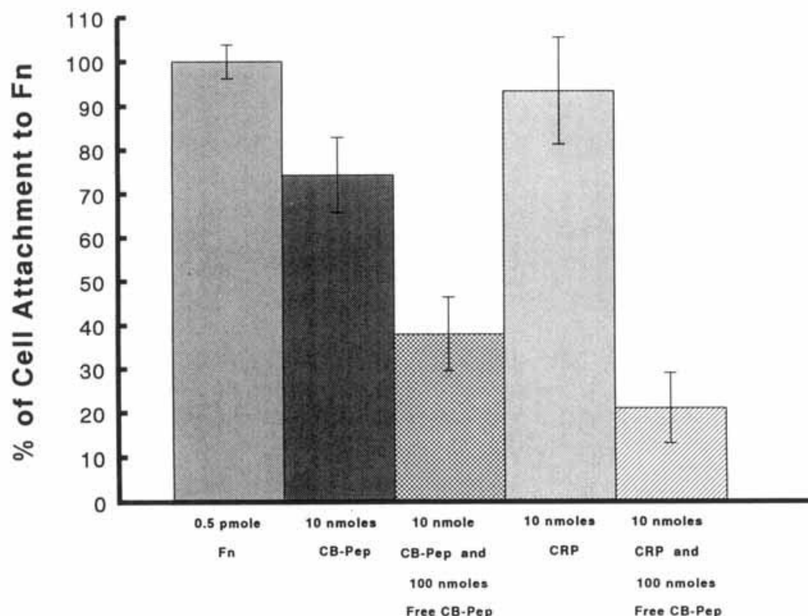

Fig. 1. Efficiency of cell attachment to Fn, CRP, and the CB-Pep of CRP. Soluble CB-Pep at 100 nmoles/0.1 ml was added to the cells before allowing them to attach to either CB-Pep or CRP coated directly onto the plate. The amount of CRP is expressed as nmoles of CRP subunits. Data are the mean (+/− SD) values from a representative experiment in which cell attachment was measured in triplicate.

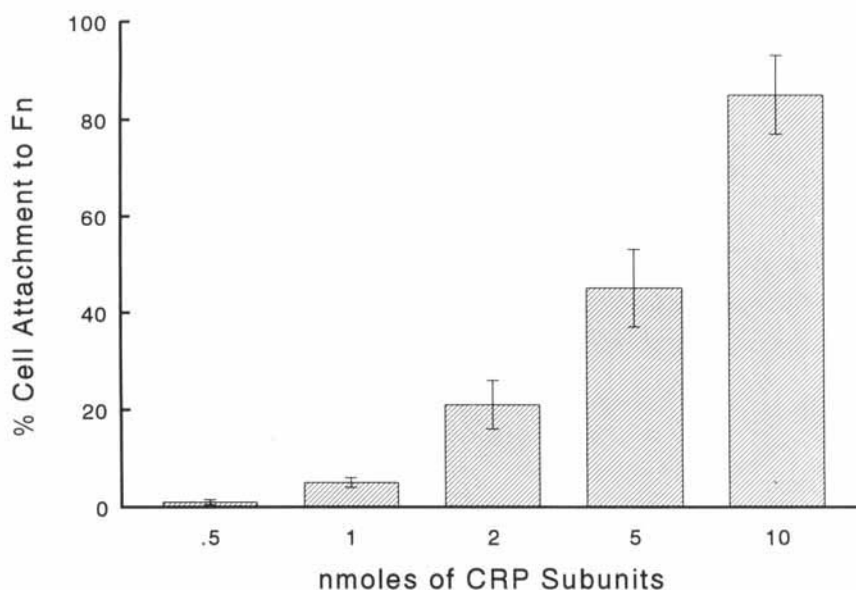

Fig. 2. Cell attachment to different amounts of immobilized CRP. The amount of CRP is expressed in terms of nmoles of CRP subunits. The data are the mean values (+/− SD) from three experiments.

using a m.w. of 23 kDa/subunit, and therefore 10 nmoles of CRP subunits is equivalent to 2 nmoles of the intact pentraxin. The 2 nmoles of CRP are equal to 230 μg. A variety of moderate systemic inflammatory responses are associated with CRP concentrations >230 μg/ml [Ballou and Kushner, 1992]. CRP would be further concentrated as it deposits at sites of tissue damage. A dose-response relationship was observed between cell attachment activity and the amount of intact, immobilized CRP expressed in terms of nmoles of CRP subunits (Fig. 2). The results suggest that a threshold concentration of CRP is required for efficient cell attachment.

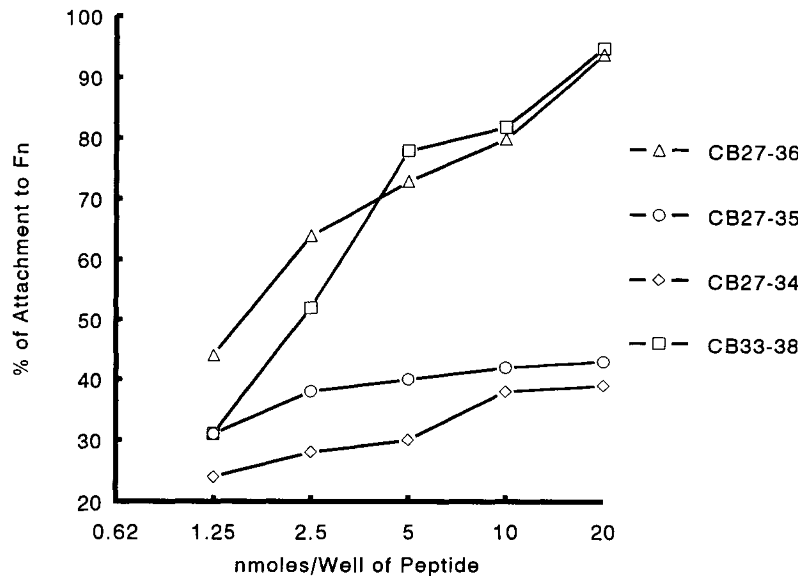

Fig. 3. Direct cell attachment to modified peptides based on the CB-Pep with N-terminal and C-terminal amino acid deletions. Serial twofold dilutions of the peptides were coated onto plates, and fibroblasts were added at 10^5 cells/well. The percentage of cell attachment vs. the attachment to 0.5 pmoles of Fn was measured. Results are mean values of three experiments.

Fn at only 0.5 pmoles/well supported a similar level of cell attachment as that of 10 nmoles of CB-Pep, indicating that attachment to Fn is much more efficient. The addition of soluble CB-Pep at 100 nmoles/ml as a competing peptide to the fibroblasts significantly inhibited cell attachment to both the CB-Pep and intact CRP, suggesting that the cell interaction with CRP occurs at this region. The CB-Pep was shown previously by us not to inhibit cell attachment to Fn [Fernandez et al., 1992]. The specificity of the cell-binding interaction with the CB-Pep was explored further.

Cell Attachment to Reduced CB-Pep

Since dimer formation through the Cys-SH at position 36 in the CB-Pep readily occurs in solution, the possibility that only dimers support cell attachment was considered. A comparison of reduced vs. nonreduced CB-Pep failed to detect any difference over a range of concentrations (1–20 nmoles/well) in the level of cell attachment (data not shown). These results clearly indicated that dimer formation does not affect the cell attachment activity of the peptide.

Cell Attachment to Truncated Peptides

To determine the minimal amino acid sequence required for cell attachment to the CB-Pep, a series of synthetic peptides with sequential N-terminal and C-terminal deletions were used in direct cell attachment assays. Two of the ten truncated peptides, CB27-34 and CB27-35, failed to support cell attachment, indicating that residues 33–36 are required for cell attachment (Table I; Fig. 3). A comparison of the relative cell attachment activity of different amounts of four truncated peptides shown in Figure 3 suggests that the critical residues for activity are 33–36.

TABLE I. Cell Attachment to Truncated Synthetic Peptides vs. the CB-Pep of CRP*

Peptide	Sequence	% of attachment to fibronectin[a]
CB-Pep	T-K-P-L-K-A-F-T-V-C-L-H	100
CB27-37	T-K-P-L-K-A-F-T-V-C-L	95
CB27-36	T-K-P-L-K-A-F-T-V-C	80
CB27-35	T-K-P-L-K-A-F-T-V	42
CB27-34	T-K-P-L-K-A-F-T	38
CB28-38	K-P-L-K-A-F-T-V-C-L-H	94
CB29-38	P-L-K-A-F-T-V-C-L-H	NS[b]
CB30-38	L-K-A-F-T-V-C-L-H	90
CB31-38	K-A-F-T-V-C-L-H	96
CB32-38	A-F-T-V-C-L-H	82
CB33-38	F-T-V-C-L-H	85

*The peptides were coated at 10 nmoles/well. Normal rat kidney fibroblasts were added to the wells at 10^5 cells/well and the percentage of cell attachment calculated vs. the attachment to 0.5 pmoles Fn.
[a]Results are mean values of three experiments.
[b]Peptides that were not soluble (NS) under the conditions of the experiment.

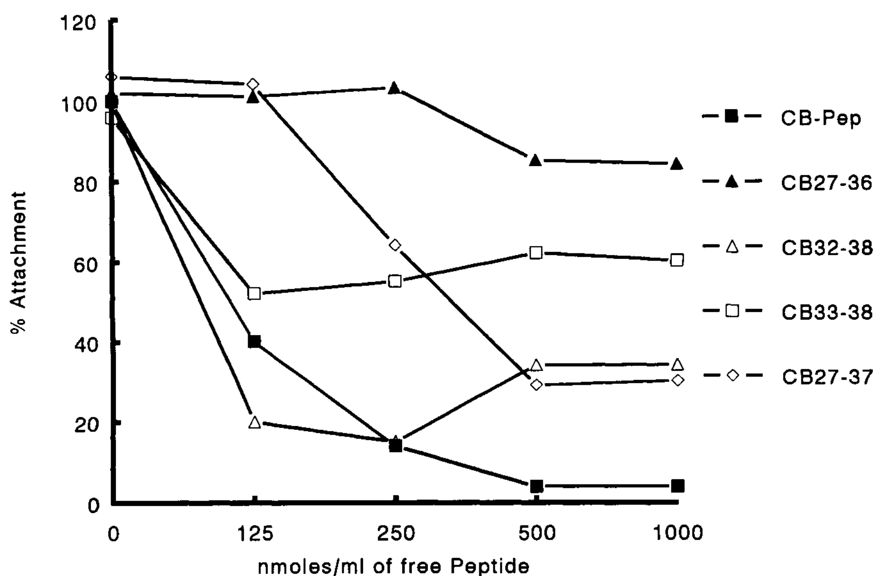

Fig. 4. Inhibition of cell attachment to the CB-Pep by modified peptides with residues deleted from the N- or C-terminus. Serial twofold dilutions of the soluble peptides were added to 10^5 fibroblasts before addition to wells coated with 10 nmoles/well of the CB-Pep. The percentage of the cell attachment to 10 nmoles of CB-Pep is indicated. The ability of a peptide to inhibit cell attachment by > 50% was considered significant. Data are mean values of three experiments.

Inhibition of Cell Attachment to CB-Peptide by Truncated Peptides

Since attaching the truncated peptides to the plate may force a nonnative conformation and may not result in a quantitative attachment of the peptide to the well, an inhibition assay of cell attachment was performed to test each peptide in solution. The data in Figure 4 demonstrate that peptides containing residues 33–37 inhibited cell attachment to the immobilized CB-Pep, whereas any peptide truncated to within residues 33–37 failed to inhibit attachment. Peptide 33–38 partially inhibited cell attachment, whereas peptide 32–38 completely inhibited attachment, indicating that residue 32 may also be involved in cell attachment (Fig. 4). As expected, the longer N-terminally truncated peptides CB28-38, CB29-38, CB30-38, and CB31-38 also inhibited cell attachment. The shorter N-terminally truncated peptides CB32-38 and CB33-38 still significantly inhibited attachment. The two shorter C-terminally truncated peptides CB27-34 and CB27-35 did not inhibit attachment to CB-Pep (Table II). The findings from both the direct attachment and attachment inhibition assays suggests that the minimal sequence required for cell attachment consist of residues 33–37: **Phe-Thr-Val-Cys-Leu.**

TABLE II. Inhibition of Cell Attachment to CB-Peptide by Peptides Truncated From the *N*-Terminus and C-Terminus

Peptide[a]	Sequence	% of attachment to fibronectin[b]
CB28-38	K-P-L-K-A-F-T-V-C-L-H	13
CB29-38	P-L-K-A-F-T-V-C-L-H	NS[c]
CB30-38	L-K-A-F-T-V-C-L-H	0
CB31-38	K-A-F-T-V-C-L-H	14
CB32-38	A-F-T-V-C-L-H	23
CB33-38	F-T-V-C-L-H	55
CB27-37	T-K-P-L-K-A-F-T-V-C-L	28
CB27-36	T-K-P-L-K-A-F-T-V-C	89
CB27-35	T-K-P-L-K-A-F-T-V	98
CB27-34	T-K-P-L-K-A-F-T	91

[a]The synthetic peptides were tested for inhibition of cell attachment at 125–1,000 nmoles/ml against CB-Pep coated at 10 nmoles/well. The data shown is for 1,000 nmoles/ml.
[b]Results are mean values of three experiments.
[c]Peptides that were not soluble under the conditions of the experiment.

Cell Attachment to Substituted Peptides

To identify the critical residues within the CB-Pep responsible for direct cell attachment activity, experiments were done using synthetic peptides containing a single substitution at each residue. Only the CB33Y and CB34S peptides of

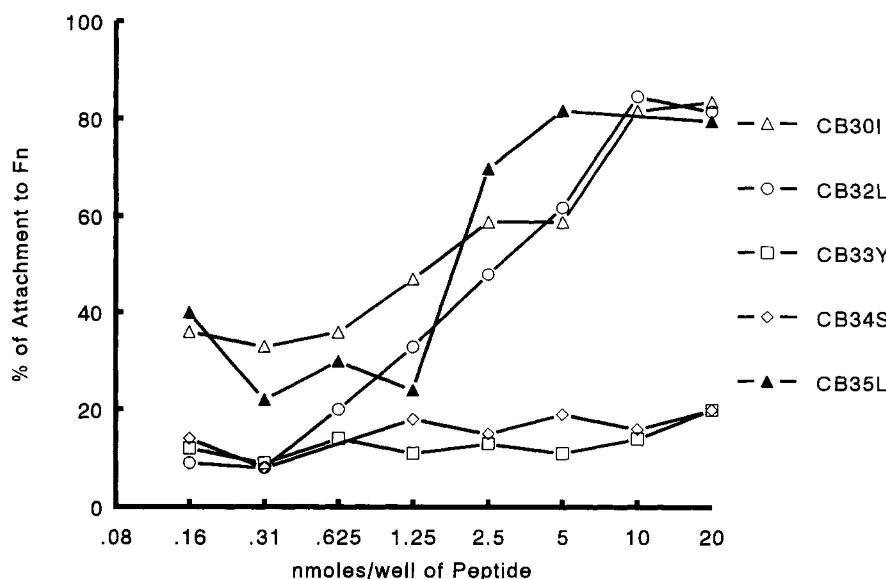

Fig. 5. Direct cell attachment to peptides containing a single conservative substitution at each of several different residues within the CB-Pep. The substituted amino acid is indicated by the number of the residue. Fibroblasts (10^5/well) were added to wells coated with peptide. The percentage of the cell attachment for each peptide vs. attachment to 0.5 pmole of Fn is indicated. Results are mean values of three experiments.

the eight substituted peptides failed to support cell attachment (Table III; Fig. 5). Titration of the substituted peptides over a range of relevant concentrations revealed that the loss of activity did not reflect a significant change in the dose-response curve (Fig. 5). The results clearly indicate a requirement for both Phe and Thr at positions 33 and 34 of the CB-Pep.

Substitutions for each of the residues between 33 and 37 of the CB-Pep were chosen to maintain charge, hydrophobicity, and size. The substitutions were Phe to Tyr at residue 33, Thr to Ser at residue 34, Val to Leu at residue 35, and Leu to Ile at residue 37. The Cys at residue 36 was not substituted since it is part of the intrachain disulfide bond with the Cys-97. The tuftsin tetrapeptide (TKPR) which is identical to the first three residues of CB-Pep was previously shown by us not to affect cell attachment to CB-Pep (26), and therefore residues 27–29 were not substituted. The conservative nature of these substitutions may have prevented identification of additional critical residues.

TABLE III. Cell Attachment to CB-Pep with Single Amino Acid Substitutions*

Peptide[a]	Sequence	% of attachment to fibronectin
CB-Pep	T-K-P-L-K-A-F-T-V-C-L-H	99
CB30I	T-K-P-**I**-K-A-F-T-V-C-L-H	82
CB31R	T-K-P-L-**R**-A-F-T-V-C-L-H	96
CB32L	T-K-P-L-K-**L**-F-T-V-C-L-H	82
CB33Y	T-K-P-L-K-A-**Y**-T-V-C-L-H	14
CB34S	T-K-P-L-K-A-F-**S**-V-C-L-H	16
CB35L	T-K-P-L-K-A-F-T-**L**-C-L-H	81
CB37I	T-K-P-L-K-A-F-T-L-C-**I**-H	100
CB38K	T-K-P-L-K-A-F-T-L-C-L-**K**	83

*The peptides were coated at 10 nmoles/well. Fibroblasts were added at 10^5 cells/well and allowed to adhere for 30 min. The percentage of the cell attachment vs. attachment to 10 nmoles/well of CB-Pep was measured for each peptide. Results are mean values of three experiments.

[a]Peptides are designated by the substituted residue which is in bold.

Inhibition of Cell Attachment to CB-Pep by Substituted Peptides

Synthetic peptides with single conservative substitutions at each of the residues beginning with amino acid 30 of the CB-Pep were tested for their ability to inhibit fibroblast attachment to the CB-Pep. All the substituted peptides inhibited cell attachment to the CB-Pep except peptide CB33Y which has a Tyr replacing the Phe at position 33 (Table IV; Fig. 6). The inability of peptide CB33Y to inhibit cell attachment confirms that the Phe at 33 is critical for cell attachment. Titration of the substituted peptides in the attachment inhibition assay showed a dose-

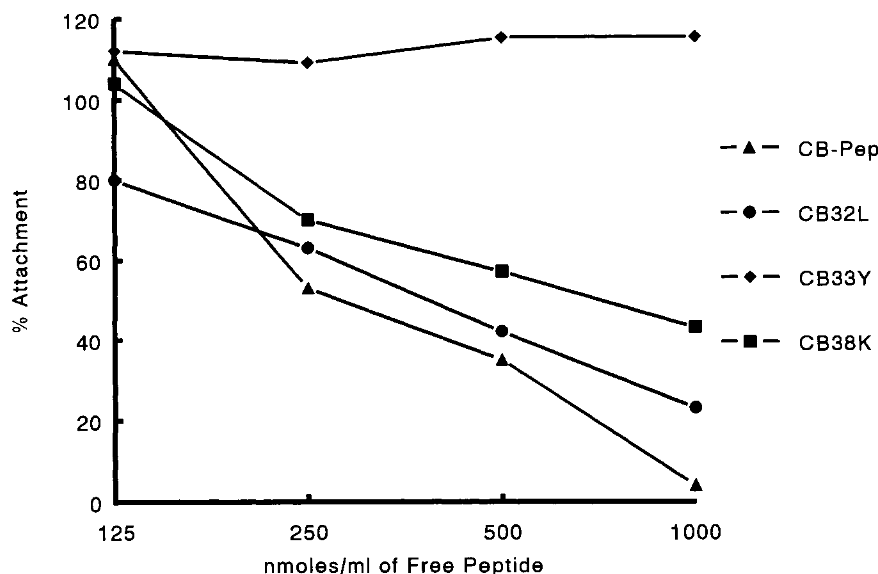

Fig. 6. Inhibition of cell attachment to CB-Pep by peptides with substituted residues. Serial twofold dilutions of soluble peptides were added along with 10^5 fibroblasts to wells coated with 10 nmoles of CB-Pep. The percentage of cell attachment to 10 nmoles of CB-Pep is shown.

response effect (Fig. 6). The peptides with conservative substitutions for residues 34–37 inhibited cell attachment as well as the CB-Pep itself (Table IV). The results from both the direct attachment and attachment inhibition experiments with substituted peptides reveal that Phe and Thr at residues 33 and 34 are critical residues within the CB-Pep.

Cell Attachment to CB33-37 and CB-Pep

Cell attachment to the truncated peptides indicated that the minimal sequence required for the cell attachment activity of the CB-Pep is F-T-V-C-L (residues 33–37). To confirm this prediction, the peptide itself was synthesized and compared to the CB-Pep in direct cell attachment assays. The pentapeptide (CB33-37) supported cell attachment over a range of concentrations similar to that of the CB-Pep (Fig. 7) although cell attachment to the CB33-37 was considerably less efficient than the cell attachment to CB-Pep. The limited solubility of peptide CB33-37 precluded its use in attachment inhibition experiments.

TABLE IV. Inhibition of Cell Attachment to CB-Pep by Synthetic Peptides With Single Amino Acid Substitutions*

Peptide	Sequence	% of attachment to CB-Pep[a]
CB-Pep	T-K-P-L-K-A-F-T-V-C-L-H	5
CB30I	T-K-P-**I**-K-A-F-T-V-C-L-H	10
CB31R	T-K-P-L-**R**-A-F-T-V-C-L-H	5
CB32L	T-K-P-L-K-**L**-F-T-V-C-L-H	23
CB33Y	T-K-P-L-K-A-**Y**-T-V-C-L-H	98
CB34S	T-K-P-L-K-A-F-**S**-V-C-L-H	8
CB35L	T-K-P-L-K-A-F-T-**L**-C-L-H	15
CB37I	T-K-P-L-K-A-F-T-L-C-**I**-H	0
CB38K	T-K-P-L-K-A-F-T-L-C-L-**K**	45

*Soluble peptides with single residue substitutions at 1,000 nmoles/ml were added along with 10^5 fibroblasts to wells coated with 10 nmoles/well of the CB-Pep. Peptides are designated by the substituted residue which is in bold.
[a]The percentage of the cell attachment to 10 nmoles of CB-Pep is indicated for each of the competing peptides. Results are mean values from three experiments.

DISCUSSION

Blood levels of the acute phase reactant CRP become greatly elevated during the first 24–48 h as part of the systemic inflammatory response to acute microbial infections, major trauma, vasculitis, and connective tissue diseases, reaching levels up to 1.0 mg/ml [Ballou and Kushner, 1992]. Thereafter, CRP levels rapidly decline, and the protein is deposited at inflamed sites [Kushner and Kaplan, 1961; Kushner et al., 1963; Gitlin et al., 1977; DuClos et al., 1981] where the CRP is presumably degraded by neutrophils based on observations of the in vitro degradation of CRP by PMNs [Robey et al., 1987; Shephard et al., 1988]. The CRP-derived

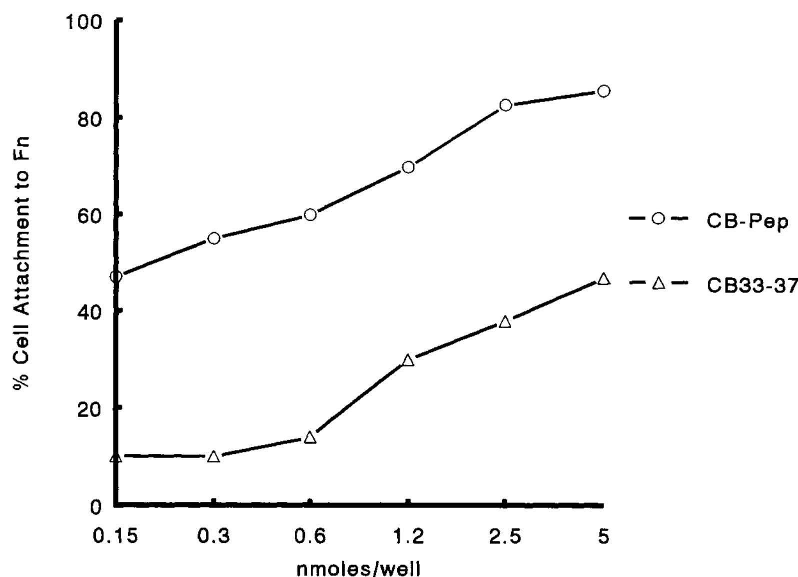

Fig. 7. Direct cell attachment to peptide CB33-37 and the CB-Pep. Serial twofold dilutions of the peptides were coated onto plates, and fibroblasts were added at 10^5 cells/well. The percentage of cell attachment vs. cell attachment to 0.5 pmoles of Fn was measured. Results are mean values of three experiments.

peptides generated by PMNs elicit a monocyte chemotactic response, as well as cytokine and superoxide production [Robey et al., 1987; Tebo and Mortensen, 1991; Miyagawa et al., 1988]. Membrane receptors from human monocytic cell lines for both monomers of CRP and CRP-complexes that mediate binding, phagocytosis, and activation have been partially characterized [Tebo and Mortensen, 1990; Crowell et al., 1991]; however, the site(s) on CRP recognized by monocytes or neutrophils has not been defined. During the course of studies of the ligand specificity of the CRP-R we found that one of several synthetic peptides tested supported cell attachment in vitro [Fernandez et al., 1992]. The active peptide is composed of residues 27–38 and is therefore found in each of the five identical subunits of the intact pentameric protein. A peptide composed of residues 27–38 from the homologous human pentraxin SAP was reported by Dhawan et al. [1990] to mediate cell attachment. The cell binding activity of the CB-Pep of CRP was not inhibited by the cell adhesive peptides of the extracellular matrix proteins of Fn and laminin (i.e., the RGDS-bearing peptides of Fn and the YIGSR-bearing peptides of laminin) [Fernandez et al., 1992]. Attachment to the CRP-peptide does not occur via integrin receptors since the postattachment cell "spreading" is not observed, indicating that cell attachment to the CB-Pep does not involve the cytoskeleton [Hynes, 1992]. In addition, the cell binding to the CRP-derived peptide appears to involve recognition of a unique sequence motif since the 12 residue sequence of the CB-Pep was not found in a search of proteins in the sequence data banks at the level of $>55\%$ homology. The CRP cell attachment sequence is not similar to any of the known adhesive recognition sequences [Yamada, 1991]. Therefore, identification of the critical residues and the minimal recognition sequence within the 12 residue CB-Pep of CRP may allow us to eventually define a potentially novel receptor for selective cell adhesion at inflamed sites.

Since CRP is in general analogous to Fn in that both blood proteins are acute phase reactants and found at sites of tissue damage, we compared the efficiency of cell attachment of both the CB-Pep and intact CRP vs. Fn. Although Fn mediates cell attachment at least 10^4 times more efficiently than CRP on a molar basis, both the CB-Pep and intact CRP supported similar levels of cell attachment. Since the peptide blocked the activity of the intact CRP, the peptide residues are probably accessible on the intact, immobilized form of CRP. However, CRP in solution failed to block cell attachment to the CB-Pep, clearly suggesting distinct conformations for CRP in the fluid phase

vs. the immobilized form. Furthermore, anti–CB-Pep antibodies bind to the intact CRP when it is bound to a substrate on a surface, but not when it is in solution (Mullenix and Mortensen, unpublished). These observations are consistent with those of others documenting the appearance of new epitopes on CRP upon binding to a solid phase [Shields et al., 1992]. Substrate adsorption artifacts with the CB-Pep were not a major consideration since soluble peptide also actively inhibited attachment and because similar levels of cell adhesion to a carrier (BSA)-CB-Pep conjugate were observed previously [Fernandez et al., 1992; Hynes, 1992]. Cell attachment to CRP deposited at sites of tissue damage might be substantial even if the process is much less efficient than with extracellular matrix proteins like Fn since CRP often attains μM levels in the blood [Robey et al., 1987], and therefore nmole amounts of CRP could easily accumulate at damaged sites [Kushner et al., 1963]. A recent study of the fate of radiolabeled CRP in patients with mild inflammatory conditions failed to detect CRP deposition at inflammed sites but did not exclude CRP localization to sites of tissue necrosis [Vigushin et al., 1993]. Nonetheless, if CRP deposits are degraded in vivo in a manner consistent with the neutrophil-mediated proteolysis documented in vitro [Robey et al., 1987; Shephard et al., 1988], then the active peptides generated may mediate cell adhesion at or near the site. The CRP cell attachment peptide could be generated since neutrophil elastase and tryptic cleavage sites exist at residues 25 and 31, respectively. Such cleavage would result in peptides of sufficient length to contain the critical residues for cell attachment. Recent findings using mAb that recognize neo-CRP, the conformation assumed by CRP when bound to a surface, also recognize residues 22–45 [Ying et al., 1992].

Our approach of using modified synthetic peptides to identify both the critical residues and minimal length of the CRP cell-attachment peptide was the same as that originally used to characterize the major cell recognition sequence of Fn [Pierschbacher and Ruoslahti, 1984]. Experiments using both direct cell attachment to immobilized peptides and inhibition of cell attachment by soluble peptides were conducted to minimize the structural constraints imposed by a "solid-phase" presentation of the peptide. The CRP CB-Pep in solution lacks definitive secondary structure measurable by circular dichroism analysis (Mullenix et al., unpublished); however, there is no evidence that any specific conformation is required for cell attachment activity. Definitive three-dimensional structural information on CRP is not yet available, and therefore it is not possible to determine whether the active sequence is on the surface of CRP or becomes available with a conformational change induced by either binding to a matrix [Shields et al., 1992] or physiological levels of Ca^{++} [Kilpatrick et al., 1982; Swanson et al., 1991]. Results from both experimental approaches indicated that the minimal sequence required for cell adhesion is (r33) **Phe-Thr-Val-Cys-Leu** (r37). The experiments with substituted peptides clearly indicated that the hydrophobic, nonpolar Phe at position 33 and the adjacent polar Thr residue at 34 are the critical residues within the peptide. The conservative substitutions for the small hydrophobic residues at 35 (Ile for Leu) and at 37 (Ile for Val) did not influence activity. Although the Cys at residue 36 of the CB-Pep allows homodimer formation, the reduced peptide supported the same level of cell attachment, indicating that cleavage within the CRP subunit is not necessary for activity and is consistent with the cell-binding activity observed with the intact pentraxin.

Since the homologous SAP peptide composed of residues 27–38 possesses similar cell-binding activity, the critical residues within the minimal sequence would be expected to be conserved. A comparison of the relevant sequence below shows that the only significant difference is the presence of the large, hydrophobic Phe at position 37 of SAP vs. the smaller, hydrophobic Leu in the CRP sequence:

SAP (33–37): F-T-L-C-F
CRP (33–37): F-T-V-C-L.

It is noteworthy that the C-terminal half of the SAP dodecapeptide was found to be the active portion of the peptide in cell-attachment assays, suggesting that this function may be conserved among the pentraxin family members [Liu et al., 1987; Dhawan et al., 1990]. The SAP peptide has recently been shown to bind heparin [Heegaard and Robey, 1992]; however, the CRP CB-Pep failed to bind heparin in preliminary studies, although the peptide may interact with other proteoglycans that are widely distributed on cell surfaces [Ruoslahti, 1989]. The receptor interacting with the CB-Pep may be similar or identical to the CRP-R on both monocytes and

neutrophils; however, the only basis for proposing that the CRP-R and the cell adhesion receptor for CB-Pep are similar is that CB-Pep itself partially inhibits specific ligand (CRP) binding to human monocytic and granulocytic cell lines [Zen and Mortensen, 1993]. The potential for cellular interactions with sites containing CRP and SAP deposits are much greater than heretofore appreciated and suggest a potential role for CRP in inflammatory responses and eventual wound healing.

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