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Binding Site on Human C-Reactive Protein (CRP) Recognized by the Leukocyte CRP-Receptor

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Abstract C-reactive protein (CRP), the prototypical inflammatory acute phase reactant in humans, interacts with monocytes and neutrophils via a specific receptor. To map the site on CRP recognized by the CRP receptor (CRP-R), synthetic peptides corresponding to the surface region on each of the five identical subunits were tested as competitors vs. [^{125}I]-CRP for cell binding. A peptide of residues 27–38 (TKPLKAFTVCLH) efficiently inhibited CRP binding when compared to other nonoverlapping peptides. This peptide was termed the cell-binding peptide (CB-Pep). The F(ab′)$_2$ of an IgG Ab to the CB-Pep specifically inhibited CRP binding upon reacting with the ligand. Competitive binding studies with synthetic peptides truncated from either the NH_2- or COOH-terminus of the CB-Pep revealed that the minimum length recognized by the CRP-R consisted of residues 31–36: KAFTVC. Conservative substitutions of residues within the CB-Pep indicated that the four residues AFTV were critical for CRP-R binding. The CB-Pep also inhibited induced superoxide generation by HL-60 granulocytes. The minimum length required for the inhibition was also KAFTVC; however, only Phe-33 and Leu-37 were critical residues in this assay. Anti-CB-Pep IgG Ab reacted more extensively with heat-modified CRP, suggesting that an altered conformation of CRP is preferentially recognized by the CRP-R. The results suggest that this contiguous sequence on a β-strand on one face of each of five subunits of the CRP pentamer serves as a unique recognition motif for inflammatory leukocytes. J. Cell. Biochem. 64:140–151.

Key words: human; C-reactive protein; acute phase reactant; monocytes; leukocyte

Homeostatic mechanisms for maintaining tissue integrity are altered dramatically after microbial invasion or inflammation. Such events initiate a cascade of cytokine-driven reactions that both amplifies and reorchestrates the pattern of protein synthesis by the liver, which results in increased blood levels of a diverse group of proteins (e.g., proteinase inhibitors, complement components, coagulation factors) termed acute phase reactants [Kushner, 1988]. The increased synthesis is initiated at the transcriptional level by the inflammatory cytokines interleukin (IL)-1 and IL-6 [Steel and Whitehead, 1994; Li et al., 1990; Majello et al., 1990; Zhang et al., 1995]. C-reactive protein (CRP) is the prototype acute phase reactant in humans and most other mammals since its blood level can increase several thousandfold. CRP is a member of the pentraxin family since it is composed of five identical, noncovalently linked 23 kDa subunits arranged in a flat pentameric disk with cyclic symmetry [Gewurz et al., 1995]. The single copy gene for human CRP on chromosome 1 encodes a 206 amino acid, nonglycosylated polypeptide chain that makes up each of the CRP subunits [Lei et al., 1985; Woo et al., 1985]. All CRPs display Ca^{2+}-dependent lectin-like binding reactivity specific for phosphocholine (PC), several other monophosphate esters, chromatin, histones, and sm/RNP [Kilpatrick and Volanakis, 1991]. The biological activities triggered by CRP-substrate complexes such as complement activation and opsonization are identical to those of IgG antibodies and suggest

Abbreviations used: a, anti-; CB, cell-binding; CRP, C-reactive protein; CRP-R, CRP receptor; fMLPP, formyl-methionyl-leucyl-phenylalanine-phenylalanine; G, granulocytic; [^{125}I], iodine-125; O_2^-, superoxide; PC, phospholcholine; Pep, peptide; PMN, polymorphonuclear neutrophils; SAP, serum amyloid P-component.

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a nonspecific host protective function [Mortensen, 1993]. Since the inducible CRP response is conserved among the vertebrates, an essential role for this protein has been proposed.

Specific receptors for CRP on monocytes/macrophages [Barna et al. 1987; Zeller et al., 1986b, 1989; Ballou et al., 1989; Zahedi et al., 1989; Tebo and Mortensen, 1990], neutrophils [Zeller et al., 1986a; Buchta et al., 1987a,b; Robey et al., 1987], and a subset of lymphocytes [James et al., 1981] have a measured K_d of 10^{-7}–10^{-8} M. Although there is evidence that a portion of the specific CRP binding occurs through the FcγRI on monocytes [Tebo and Mortensen, 1990; Crowell et al., 1991], more recent findings clearly show that the FcγRs and CRP receptors (CRP-R) are distinct [Marnell et al., 1995]. Receptor-bound CRP is internalized during phagocytosis via the endosomal route and partially degraded [Tebo and Mortensen, 1990; Zahedi et al., 1989]. Intact pentameric CRP is also cleaved by neutrophil membrane proteinases into peptides that activate macrophages [Robey et al., 1987; Shephard et al., 1989]. The interaction of CRP, as well as CRP-derived peptides, with leukocytes at inflammatory sites may serve as a mechanism to focus and modify the activities of cellular infiltrates.

In previous studies, a CRP peptide of amino acids 27–38 from each subunit was shown to mediate cell attachment of fibroblasts in vitro and was therefore designated the CRP cell-binding peptide (CB-Pep) [Fernandez et al., 1992]. In the experiments described here we tested several CRP synthetic peptides, including the CB-Pep, to identify and map the site(s) or epitope(s) on intact CRP recognized by the leukocyte CRP-R. We show that the CB-Pep selectively binds to the CRP-R on human U937 monocytic cells and HL-60 granulocytic cells and inhibits the respiratory burst of the latter. In addition, the CB-Pep contains a unique recognition motif that may be displayed more prominently by CRP at sites of inflammation.

MATERIALS AND METHODS

Reagents

N-formyl-methionyl-leucine-phenylalanine (fMLP) or fMLP-phe (fMLPP), PMA, DMSO, and ferricytochrome C (horse heart) were purchased from Sigma (St. Louis, MO). The PMA and fMLPP were stored concentrated in DMSO at −70°C and diluted with buffer just before use. The p-Aminophenyl phosphorylcholine (PC)-Sepharose for CRP purification and the 1,3,4,6-tetrachloro-3,6α-diphenylglycouril (Iodogen) for iodination were purchased from Pierce Chemical (Rockford, IL).

Cells

The promonocytic cell line U937 (DMS10.6 variant) was provided by Dr. Clark Anderson (Department of Medicine, Ohio State University) and grown in RPMI-1640 (M.A. Bioproducts, Walkersville, MD) supplemented with 2 mM glutamine, 0.1 mM MEM nonessential amino acids, 10% horse serum (Gibco-BRL, Gaithersburg, MD), and gentamicin sulfate (50 μg/ml). The promyelocytic cell line HL-60 was obtained from the ATCC (Rockville, MD) and grown in RPMI-1640 plus 10% defined FCS (HyClone, Logan, UT). The HL-60 cell line was differentiated into granulocytic (G) cells by incubating 2×10^5 cells/ml with 1.2% DMSO for 6 or 7 days, after which 85–95% of the cells rapidly reduce NBT dye and stain for cytoplasmic esterase [Collins, 1987]. The HL-60 cells were washed into Earle's balanced salt solution (EBSS) containing 10 mM HEPES (pH 7.4) and adjusted to 10^7 cells/ml at 4°C before running the functional assays.

Synthetic Peptides

Synthetic peptides numbered according to the derived sequence for the 206 amino acids present in each of the subunits of human CRP were used [Woo et al., 1985]:

Pep 1–15: Q-T-D-M-S-R-K-A-F-V-F-P-K-E-S;
Pep 27–38: T-K-P-L-K-A-F-T-V-C-L-H;
Pep 47–63: R-G-Y-S-I-F-S-Y-A-T-K-R-Q-D-N-E-I;
Pep 134–148: I-L-G-Q-E-Q-D-S-F-G-G-N-F-E-G;
Pep 152–176: L-V-G-D-I-G-N-V-N-M-W-D-F-V-L-S-P-D-E-I-N-T-I-Y-L;
Pep 191–206: K-Y-E-V-Q-G-E-V-F-T-K-P-Q-L-W-P.

A peptide of residues 65–85 (VWKERVGEWSLWIGRHKVTPK) of human SAP that corresponds to the only nonhomologous contiguous stretch between SAP and CRP was also synthesized. The peptides were synthesized by The Ohio State University Biochemical Instrument Center using t-boc synthesis on a model 9500 peptide synthesizer (Milligen/Biosearch; Millipore, Burlington, MA). The purity and biological activities of these peptides are described by

us elsewhere [Swanson et al., 1991a; Mullenix and Mortensen, 1994]. A peptide of residues 174–185 (IYLGGPFSPNVL) of CRP was a gift from Dr. Barbara P. Barna (Cleveland Clinic Research Foundation, Cleveland, OH) and has been shown to be an activator of macrophages for tumoricidal activity [Thomassen, et al., 1993]. All of these peptides were stored lyophilized at −20°C and dissolved in 10% acetic acid just prior to use and diluted in 10 mM HEPES-buffered EBSS. A series of truncated and substituted peptides were synthesized based on the most active of the peptides in the CRP cell-binding assays, which has previously been termed the cell-binding peptide (CB-Pep) or Pep 27–38 [Mullenix et al., 1994]. The truncated peptides with amino acids deleted from the C- and N-terminus are designated by the remaining residues—for example, CB28–38: KPLKAFTVCLH. The substituted peptides are designated by the altered residue in bold and its position as indicated: CB30**I:** TKP**I** KAFTVCLH. Small scale synthesis (1–3 mg) of the substituted and truncated peptides was done by a FMoc-*tert*-butyl solid phase synthesis strategy on an advanced Chemtech model 350 multiple peptide synthesizer [Mullenix et al., 1994]. The full-length substituted peptides were N-terminally sequenced on a Milligen prosequencer model 6600 to confirm the sequence accuracy. The substituted and truncated peptides were >85% pure on the basis of reverse phase HPLC profiles. The modified peptides were stored, dessicated, and dissolved as described above.

Purification, Radiolabeling of CRP

CRP was purified from human ascitic fluid using Ca^{2+}-dependent affinity chromatography on PC-phenyl Sepharose [Tebo and Mortensen, 1990]. The CRP concentration was determined by radial immunodiffusion against a high titer sheep anti-human CRP using a single serum CRP standard. The concentration of endotoxin in the purified CRP was 0.1–0.2 endotoxin units/milligram of protein as measured by the chromogenic limulus assay (M.A. Bioproducts) and corresponds to an LPS concentration of <0.05 ng/mg of CRP. The purified CRP was radiolabeled with 1.0 mCi Na[^{125}I] (Amersham, Arlington Heights, IL) per 1.0 mg of protein using glass tubes coated with Iodogen. The [^{125}I]-CRP was reisolated by Ca^{2+}-dependent affinity chromatography on a PC-phenyl Sepharose column. The [^{125}I]-CRP preparations had specific activities in the range of 0.3–0.5 μCi/μg of protein; >95% of the radioactivity was precipitated with cold 10% TCA. The [^{125}I]-CRP retained its antigenicity when tested with either polyclonal Ab or mAb in a competitive ELISA.

CRP Receptor Binding Assay

[^{125}I]-CRP was added to U937 cells at 10×10^6/ml in RPMI-1640 containing 10 mg/ml purified BSA, sodium azide (10 mM), and 10 mM HEPES buffer (pH 7.2) on ice with mixing for 30 min. Cell-bound ligand was measured in a 200 μl aliquot by layering the cells onto 0.2 ml of a 1:1 mixture of phthalate and dibutyl phthalate oils (Eastman Kodak Company, Rochester, NY), centrifuging at 4°C at 14,000*g* for 30 s and freezing in liquid nitrogen. The microfuge tube tip containing the cell pellet was immediately cut off and counted in a Beckman Gamma 4000 counter. This procedure minimizes dissociation of the ligand. Nonspecific binding was assessed by measuring the binding of [^{125}I]-CRP in the presence of a 100-fold excess of unlabeled CRP. Since modified or neo-CRP binds more efficiently to monocytic cells [Potempa et al., 1988], the [^{125}I]-CRP at 100 μg/ml in a 200 μl volume was heated for 10 min at 60°C. This procedure did not result in the formation of aggregates that precipitate or as detected by HPLC. Inhibition of the binding of [^{125}I]-CRP to U937 cells was evaluated by incubating the cells with various synthetic peptides for 2 h on ice prior to the addition of the [^{125}I]-CRP. Inhibition of binding by Ab CRP peptides was measured by allowing the [^{125}I]-CRP to react with the Ab on ice for 2 h prior to adding the mixture to the cells.

Polyclonal Ab to CRP Peptides

For each peptide NZW rabbits were injected subcutaneously on the dorsum with 500 μg of the peptide covalently linked to the carrier KLH and emulsified in CFA. After 4 weeks, the rabbits were rechallenged i.m. with an additional 200 μg of peptide-KLH. After 3 weeks, the serum was collected and the IgG fraction purified using protein A affinity chromatography (Pharmacia, Gaithersburg, MD). Specific binding of the Ab to both the peptide and intact purified CRP was determined by a direct ELISA. Preparation of $F(ab')_2$ of the rabbit IgG was done by digestion with pepsin-sepharose (Pierce Chemical) and repurification of the antigen-binding fragment.

Superoxide Production

Superoxide production by HL-60(G) was measured by cytochrome *c* reduction in a cuvette assay [Mayo and Curnette, 1990]. Briefly, HL-60(G) cells at 5×10^6 cells/ml in EBSS with 20 mM HEPES buffer (pH 7.4) were kept on ice with peptides for 30 min. The cuvettes were placed in a heated (37°C) cuvette tray at 2×10^6/ml in a total volume of 500 µl, and a triggering stimulus of fMLP (0.1–1.0 µM) was added after temperature equilibrium. Mineral oil (40 µl) was layered onto the surface to prevent oxidation. The change in absorbance at 550 nm vs. the control was recorded over a 30–60 min period in a Beckman DU recording spectrophotometer. At the end of the reaction, 100 units of superoxide dismutase (Sigma) was added to measure the amount of dismutase-inhibitible reduced cytochrome *c* (>95% of the total). The results are expressed as nanomoles O_2^- produced, based on nanomoles cytochrome *c* reduced, per 10^6 cells during a specific time interval using an extinction coefficient of 21.1 M^{-1} cm^{-1} for cytochrome *c*.

RESULTS

Effect of Synthetic Peptides on Receptor Binding of CRP

To map the site(s) on intact, pentameric CRP recognized by leukocyte CRP-R, a series of nonoverlapping synthetic peptides corresponding to regions of either high surface probability or functional regions within each CRP subunit was screened for ability to inhibit specific binding of [^{125}I]-CRP. When a 500-fold molar excess of each of the peptides was allowed to compete against 2 pmoles (10 pmoles/ml) of [^{125}I]-CRP per 10^6 U937 monocytic cells, only two of the eight peptides tested significantly inhibited binding (Table I). These two peptides were Pep 27–38, which was previously shown to mediate cell (fibroblast) attachment activity in vitro and thus designated CB-Pep [Fernandez et al., 1992], and Pep 134–148, which corresponds to a highly conserved region among all the pentraxins and is responsible for the binding of one of the two Ca^{2+} ions per CRP subunit [Kinoshita et al., 1989; Mullenix and Mortensen, 1994].

To compare the relative efficiency of inhibition of ligand binding by the peptides, each peptide was titrated vs. [^{125}I]-CRP in the cell-binding assay. Both Pep 27–38 (CB-Pep) and Pep 134–148 inhibited CRP binding in a dose-dependent manner; however, only 10 pmoles/ml of the CB-Pep was required to obtain 25% inhibition vs. 400 pmoles/ml of the Pep 134–148 (Fig. 1). The magnitude of the inhibition obtained with a mixture of these two peptides was additive (Fig. 1). The results suggest that both peptides contain residues that contribute to the site, or sites, on CRP recognized by the CRP-R.

TABLE I. Effect of Synthetic Peptides of C-Reactive Protein (CRP) on the Binding of ^{125}I-CRP to U937 Human Monocytic Cells

Residues[a]	Designation	% inhibition of specific binding (±SD)[b]
1–16	N-terminal	−4.4 (±5.8)
27–38	Cell-binding (CB-Pep)	56.1 (±7.6)
47–63	PC-binding	−8.5 (±3.1)
134–148	Ca^{++}-binding	48.9 (±8.2)
152–176	Ca^{++}-binding	8.5 (±2.2)
174–185	Macrophage-activating	−5.0 (±6.3)
191–206	C-terminal	5.1 (±9.4)
63–83	SAP unique sequence	6.2 (±2.2)

[a]Residue number is based on the derived sequence for the mature polypeptide sequence of human CRP.
[b]A 500-fold molar excess of each peptide was allowed to compete against 2 pmoles of labeled CRP per 10^6 U937 cells.

Effect of Antibody to the CB Peptide on CRP Binding

Since the CB-Pep was an effective inhibitor of ligand binding, a polyclonal (rabbit) IgG Ab generated against the CB-Pep and which displays specific reactivity with intact CRP was tested for its ability to inhibit binding of the labeled ligand to U937 cells. IgG Abs specific for several other CRP peptides (Pep 47–63, Pep 134–148, Pep 152–176, and Pep 191–206) were also evaluated since they react with determinants on the surface of the pentraxin. $F(ab')_2$ fragments of each of the IgG anti-peptide Abs were used to circumvent any interaction between the [^{125}I]-CRP-IgG complex and the two classes of human FcγRs (FcγRI and FcγRII) on U937 cells [van de Winkel and Anderson, 1991]. The $F(ab')_2$ to Pep 27–38, Pep 134–148, and Pep 152–176 inhibited binding of CRP, whereas the same Ab fragment to the PC-binding peptide (Pep 47–63) and the C-terminal peptide (Pep 191–206) did not (Fig. 2). In addition, $F(ab')_2$ of the anti-CRP mAb HD2-4, which reacts with an epitope on the plane of the pentraxin opposite the PC-binding site [Roux et al., 1983], also inhibited CRP binding (Fig. 2). The results suggest that the MO CRP-R recognizes

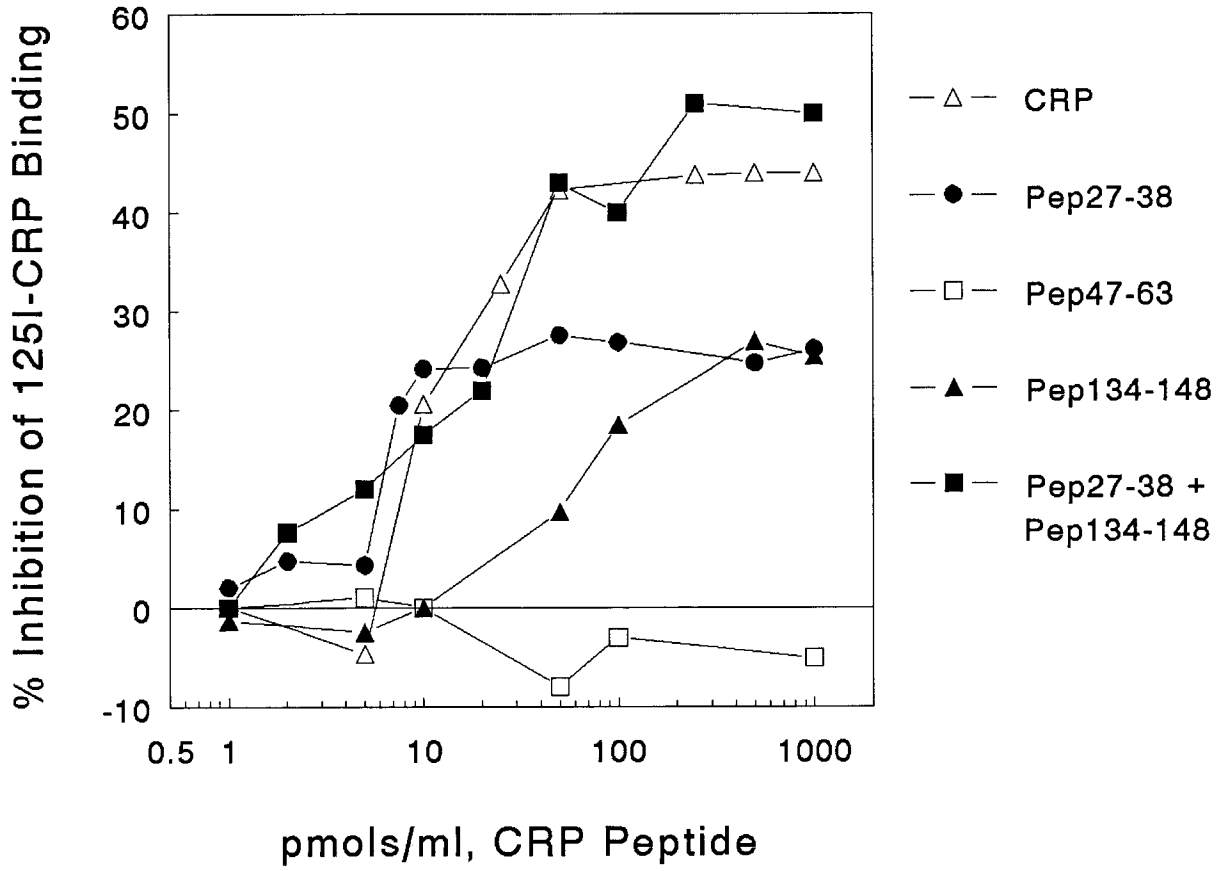

Fig. 1. Inhibition of specific binding of [^{125}I]-CRP to U937 monocytic cells by synthetic CRP peptides. Cells (10^6) were allowed to interact with up to a 500-fold molar excess of either unlabeled CRP or one of the peptides prior to the addition of 2.0 pmoles (10 pmoles/ml) of [^{125}I]-CRP. The percentage inhibition of total binding is shown. Specific binding was 40–50% of the total binding in all assays. Data are the mean values from five experiments.

a site on the CRP pentraxin on the face or plane opposite the PC-binding site but on the same plane as the residues involved in Ca^{2+} binding.

Effect of Modifications of the CB-Pep on CRP Binding

Since the most efficient inhibition of CRP binding was obtained with Pep 27–38, both truncated versions of the CB-Pep and Pep 27–38 with substitutions of a single residue were tested for their ability to inhibit ligand binding. A series of synthetic peptides truncated from either the NH_2- or the COOH-terminus of Pep 27–38 was tested, and the minimum length needed for receptor binding was found to consist of residues 31–36 of the peptide (Table II). Therefore, the CRP-R appears to be capable of recognizing and binding the KAFTVC sequence or motif. Since dimer formation of the peptides can occur via the Cys-SH at position 36, reduced CB-Pep was also tested and found to have the same inhibitory effect on CRP binding as the nonreduced peptide.

To identify critical amino acids in the CB-Pep, synthetic peptides, each with a single conservative substitution (based on size and charge) at positions 30–37 were compared with the CB-Pep itself. Residues 32 through 35 (AFTV) within the CB-Pep were needed to obtain significant inhibition of specific [^{125}I]-CRP binding (Table III). Peptides with substitutions for each of the residues at positions 27–29 (TKP), a tuftsin (TKPR)-like sequence in the CB-Pep, did not alter CRP-binding to U937 cells (data not shown). The results suggest that the critical residues are also contained within the minimal length peptide recognized by the CRP-R.

Effect of Modifying CRP on Binding of Anti-CB-Pep

The recent derivation of the secondary structure of CRP based on the crystal structure of

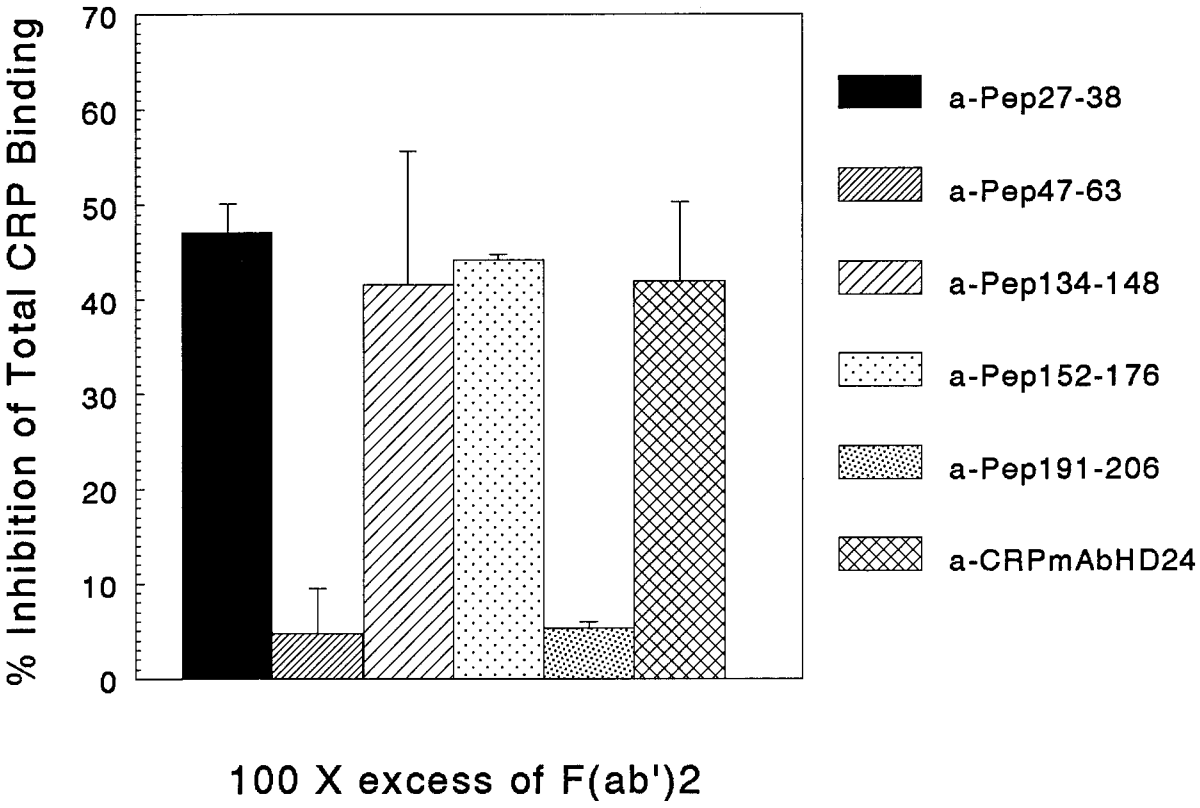

Fig. 2. Inhibition of binding of [^{125}I]-CRP to U937 cells after reacting the ligand with F(ab′)$_2$ fragments of polyclonal IgG anti-CRP peptides. [^{125}I]-CRP was allowed to react with a 100-fold excess of one of the anti-CRP peptide Ab or the mAb HD2-4 just prior to addition to U937 cells in a cell-binding assay. The percent inhibition (±SD) of total binding was measured in four experiments. a = anti-.

TABLE II. Inhibition of Specific Binding of ^{125}I-CRP to U937 Cells by Truncated Cell-Binding Peptides of the CB-Pep (CB 27–38)[a]

Peptide	Sequence	% inhibition (±SD)
CB 27–38	TKPLKAFTVCLH	61.0 ± 5.6
CB 28–38	KPLKAFTVCLH	58.6 ± 1.8
CB 29–38	PLKAFTVCLH	50.6 ± 16.5
CB 30–38	LKAFTVCLH	57.3 ± 15.9
CB 31–38	KAFTVCLH	56.6 ± 14.8
CB 32–38	AFTVCLH	3.5 ± 3.0
CB 33–38	FTVCLH	9.1 ± 4.5
CB 27–37	TKPLKAFTVCL	42.3 ± 8.6
CB 27–36	TKPLKAFTVC	47.5 ± 7.1
CB 27–35	TKPLKAFTV	8.0 ± 3.9

[a]Each peptide assayed at a 500-fold excess (10 nmoles/ml) vs. [^{125}I]-CRP in the cell-binding assay.

TABLE III. Inhibition of Specific Binding of ^{125}I-CRP to U937 Cells by CB-Peptide Homologues with a Single Substituted Residue

Peptide[a]	Sequence	% inhibition (±SD) of specific binding
CB 30I	TKP<u>I</u>KAFTVCLH	68.2 ± 17.1
CB 31R	TKPL<u>R</u>AFTVCLH	58.4 ± 11.4
CB 32L	TKPLK<u>L</u>FTVCLH	−0.3 ± 2.3
CB 33Y	TKPLKA<u>Y</u>TVCLH	−4.5 ± 3.4
CB 34S	TKPLKAF<u>S</u>VCLH	2.3 ± 1.1
CB 35L	TKPLKAFT<u>L</u>CLH	−5.7 ± 2.8
CB 37I	TKPLKAFTVC<u>I</u>H	60.2 ± 10.2

[a]Peptide is designated by the residue number that is substituted, and the substituted amino acid is underlined.

SAP predicts that amino acids 32–38 are present on one of the 14 β strands in each CRP subunit and that at least part of this stretch is not solvent-accessible [Srinivasan et al., 1994; Emsley et al., 1994]. Therefore, we tested whether a mild heat treatment of CRP, a procedure known to increase CRP binding to the leukocyte CRP-R [Potempa et al., 1988], would result in any change in the recognition of the soluble [^{125}I]-CRP by the anti-CB-Pep polyclonal IgG Ab. Heating of labeled CRP for 5–10 min resulted in up to a threefold increase in the

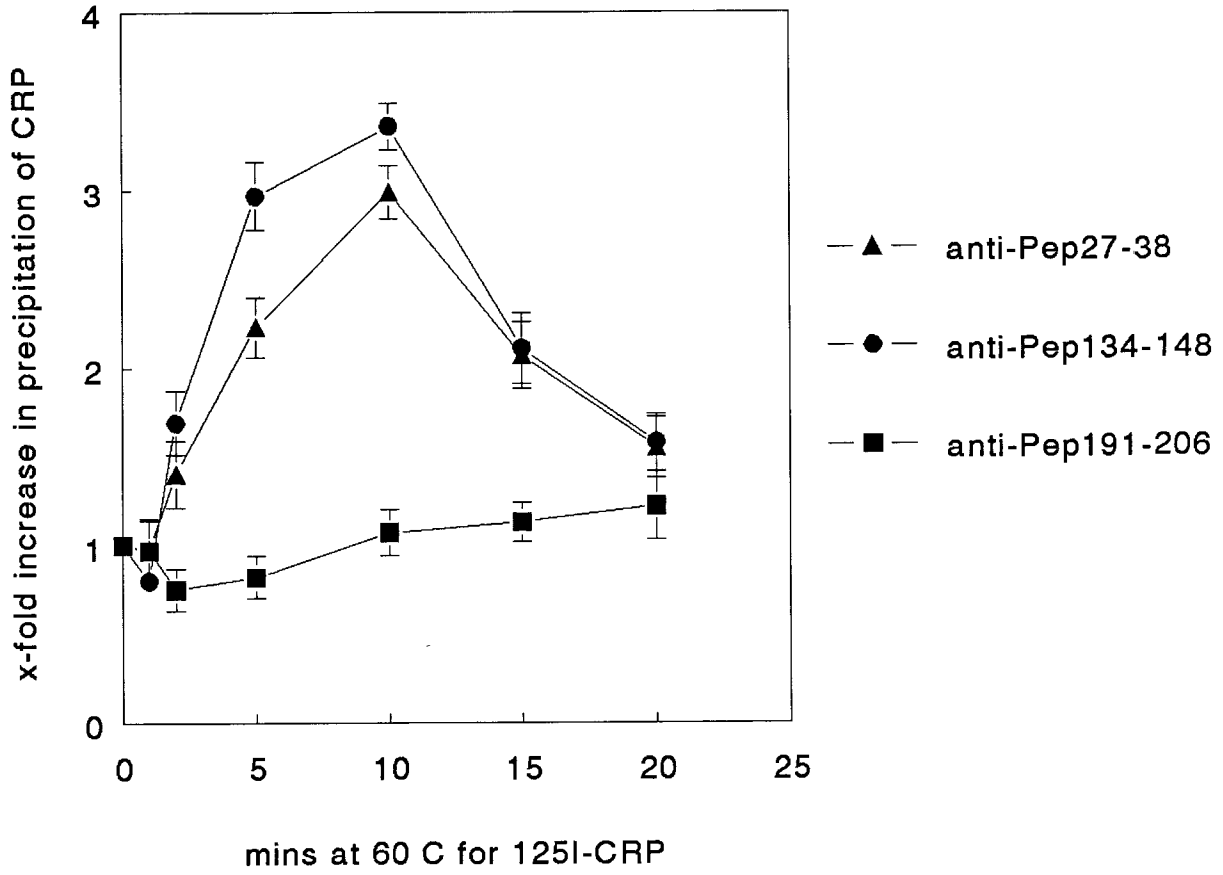

Fig. 3. Immunoprecipitation of [^{125}I]-labeled CRP with polyclonal IgG anti-CRP peptide antibodies vs. time of exposure of the labeled CRP to 60°C. The [^{125}I]-CRP reacting with each of the antibodies was captured with an excess of protein A-sepharose beads. The times-fold increase in CRP precipitated was calculated on the basis of the amount of labeled CRP bound in the absence of heating. The anti-peptide IgG Abs all reacted to a similar extent with unheated, labeled CRP. Mean values (±SD).

amount of CRP recognized by Abs to both Pep 27–38 and Pep 134–148 (Fig. 3). By contrast, the anti-Pep 191–206 IgG that binds to a solvent-accessible region failed to immunoprecipitate more CRP after heating of the ligand (Fig. 3). The heat treatment was at a temperature (60°C) low enough so that the CRP did not become aggregated when assayed by HPLC, nor did it precipitate [Potempa et al., 1987]. This heat modification of CRP has previously been shown to generate "neo-CRP" since it reveals additional epitopes defined by reactivity with certain mouse mAbs to CRP [Ying et al., 1989].

Effect of the CRP Peptides on Superoxide Production

Since previous studies showed that purified human CRP at elevated concentrations of >50 µg/ml inhibited the respiratory burst of neutrophils triggered by a variety of stimuli [Buchta et al., 1987a; Kew et al., 1990; Shephard et al., 1992], we examined the effect of the CRP synthetic peptides on the O_2^- response of HL-60(G) cells, which bind CRP with the same affinity, specificity, and number of binding sites/cell as U937 cells [Tebo and Mortensen, 1990]. Of the peptides listed in Table I, only Pep 27–38, inhibited O_2^- production when HL-60(G) cells were exposed to the peptide just prior to triggering the respiratory burst with the bacterial chemotactic peptide, fMLPP. A dose-dependent inhibition of the fMLPP-driven response was observed over a range of Pep 27–38 concentrations of 3.0–16,000 pmoles/ml, with the threshold for inhibition occurring at 400 pmoles/ml, or the equivalent of ~10 µg/ml of the intact CRP pentraxin, a modest acute phase level of CRP in the blood (Fig. 4). HL-60(G) cells stimulated by 1.0 µM fMLPP generated 7.5 (±1.7) nmoles O_2^-/

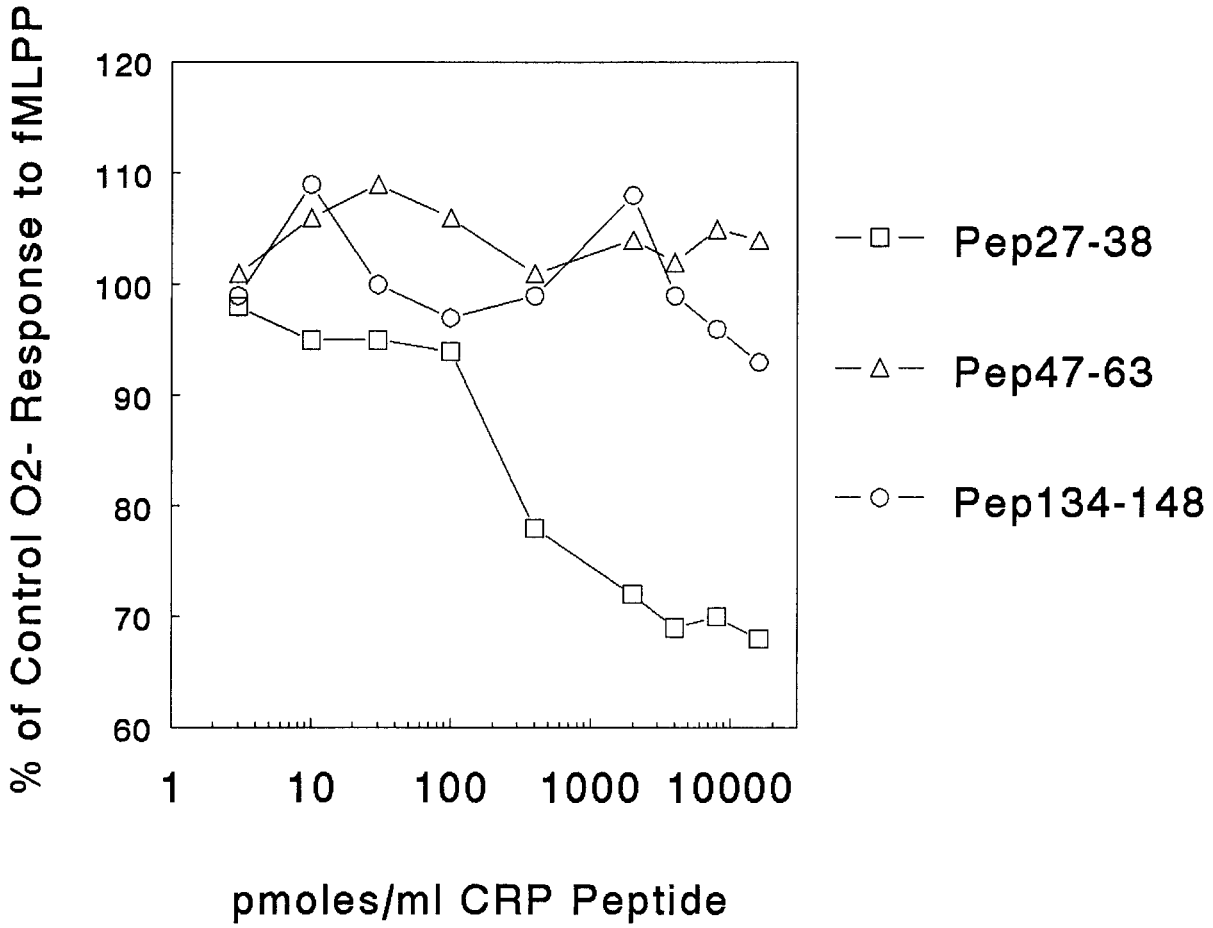

Fig. 4. Effect of CRP peptides on the fMLPP-induced respiratory burst of HL-60(G) cells. The cells were exposed to different concentrations of various peptides for 30 min prior to triggering O_2^- production with 1.0 μM of fMLPP. The data represent the mean percentage inhibition from five experiments. The SD for each value was ≤6.0%.

10^6 cells/15 min. Pep 27–38 inhibited both the rate and extent of O_2^- produced. None of the peptides listed in Table I by themselves were capable of inducing a significant respiratory burst. Thus, the CRP peptide that selectively binds to the CRP-R also altered a basic functional response of granulocytes associated with inflammation.

Effect of Modifications of the CB-Pep on Superoxide Production

Each of the truncated and substituted forms of Pep 27–38 listed in Tables II and III was also evaluated for its ability to inhibit O_2^- production in response to fMLPP. HL-60(G) cells were exposed to the various synthetic peptides just prior to triggering the respiratory burst. A reproducible, significant decrease in the response was observed with some of the truncated peptides, and an assessment of their relative inhibitory activity revealed that the minimum length required for significant inhibition required residues 31–37, or KAFTVC (Table IV).

Synthetic peptides that correspond to the 12 residue CB-Pep that contains a single, conservative substituted amino acid were also compared to Pep 27–38 for their ability to inhibit the fMLPP-driven respiratory burst. Peptides with substitutions at residues 33 and 37 lost their ability to significantly inhibit the O_2^- response (Table V). Therefore, only the Phe-33 and Leu-37 in the CB-Pep appear to be critical for leukocyte CRP-R signaling.

DISCUSSION

The most significant new finding revealed by the experiments described herein is that the CRP-R on human monocytic and granulocytic cell lines recognizes a unique peptide sequence on each of the identical protomer subunits of

TABLE IV. Effect of Truncated CRP Cell-Binding Peptides on fMLPP-Induced Superoxide Production by HL-60(G) Cells*

Peptide	Sequence	% of control (±SD)
CB 27–38	TKPLKAFTVCLH	72.7 ± 5.5
CB 28–38	KPLKAFTVCLH	74.0 ± 6.9
CB 29–38	PLKAFTVCLH	83.1 ± 7.1
CB 30–38	LKAFTVCLH	86.0 ± 7.7
CB 31–38	KAFTVCLH	81.0 ± 7.2
CB 32–38	AFTVCLH	108.0 ± 9.0
CB 33–38	FTVCLH	110.1 ± 8.8
CB 27–37	TKPLKAFTVCL	62.3 ± 6.4
CB 27–36	TKPLKAFTVC	63.5 ± 7.1
CB 27–35	TKPLKAFTV	95.8 ± 8.5

[a]Each peptide was tested at 400 and 2,000 pmoles/ml with 10^6 granulocytes triggered with 1.0 μM of fMLPP. Data were obtained from four experiments with the peptide at 2,000 pmoles/ml.

pentameric CRP, an acute phase reactant. This peptide was previously designated as the cell-binding peptide (CB-Pep) on the basis of its ability to mediate attachment of fibroblasts in vitro and consists of amino acids 27–38: TKPLKAFTVCLH [Mullenix et al., 1994]. The minimum length peptide for binding to the leukocyte CRP-R consists of residues 31–36: KAFTVC. Since this sequence is not present in the nonpentraxin proteins based on searches of the gene data banks, it represents a novel recognition motif that probably requires a distinct receptor. A peptide of this same size also inhibited the induced respiratory burst of HL-60(G), a cellular function particularly relevant to events at inflammatory sites where CRP selectively accumulates [Kushner, 1988; Gewurz et al., 1995]. The minimum length peptide within CB-Pep required for activity in the cell attachment assays was found to be ^{33}FTVCL [Mullenix et al., 1994], which is slightly shorter at the NH_2-terminus than the ^{31}KAFTVC peptide required for the interaction with the leukocyte CRP-R. The difference may reflect the influence of flanking residues on peptide conformation. These two very different assays (attachment vs. ligand binding) both revealed that the two critical residues are the hydrophobic Phe-33 and polar Thr-34. Overall, these findings explain numerous earlier observations showing that both the intact CRP pentamer and CRP-derived peptides mediate a variety of activities through their interaction with specific receptors on both monocytes and neutrophils [reviewed in Mortensen, 1993].

TABLE V. Effect of Single Amino Acid Substitutions within the CRP CB-Pep on fMLPP-Induced Superoxide Response of HL-60(G) Cells

Peptide[a]	Sequence	% of control (±SD)
CB 27–38	TKPLKAFTVCLH	77.2 ± 6.9
CB 30I	TKP<u>I</u>KAFTVCLH	89.0 ± 7.1
CB 31R	TKPL<u>R</u>AFTVCLH	83.4 ± 7.4
CB 32L	TKPLK<u>L</u>FTVCLH	80.0 ± 2.3
CB 33Y	TKPLKA<u>Y</u>TVCLH	117.0 ± 13.4
CB 34S	TKPLKAF<u>S</u>VCLH	86.8 ± 11.1
CB 35L	TKPLKAFT<u>L</u>CLH	87.5 ± 12.8
CB 37I	TKPLKAFTVC<u>I</u>H	106.0 ± 10.2

[a]Peptide is designated by the residue number that is substituted, and the substituted amino acid is underlined.

The determination of the minimum number of amino acids and the identity of critical residues within the CB-Pep provides some insight into the location and structure of this region on pentameric CRP. First, the observation that the $F(ab')_2$ of IgG antibodies to some of the CRP peptides, including the CB-Pep, prevents binding of intact CRP to the CRP-R suggests that the binding site is located on the same plane of the pentraxin as the amino acid side chains implicated in Ca^{2+} binding and the epitope identified by the HD2-4 mAb [Swanson et al., 1991b; Mullenix et al., 1994; Kilpatrick et al., 1982]. Assuming that the V-domains comprising the Ag-binding site of the antibody fragment are large enough to sterically hinder receptor binding to the 25 kDa CRP subunits, we conclude that the CRP-R binds to the same plane or face of the pentraxin that bears the HD2-4 epitope. This face of the flat CRP pentamer is opposite the plane bearing the PC-binding site [Roux et al., 1983]. Previous reports clearly demonstrated that neither bound PC nor PC-bearing macromolecules complexed with CRP interfered with attachment or phagocytosis through the CRP-R but rather enhanced the activity [Mortensen and Duskiewicz, 1977].

The three-dimensional modeling of CRP provides additional information on the location of the CRP-R recognition sequence. The derived structure for the CRP protomer is that of a compact, flattened "jelly roll" composed of 14 antiparallel β strands [A strand → O strand] on two surfaces with a hydrophobic core [Emsley et al., 1994; Srinivasan et al., 1994]. The ^{31}KAFTVC^{36} minimal sequence implicated in receptor binding occurs as part of the third and longest of these β strands (strand C) which is

composed of residues 32–40. This strand is located in the center on one of the faces of the subunit, and these residues are not at sites involved in interprotomer interactions [Emsley et al., 1994]. The predicted location is consistent with our observation that binding of the anti-CB-Pep IgG is substantially enhanced by mild conditions of alteration of the structure of the CRP pentraxin. The mild denaturing conditions needed to modify CRP into a tertiary structure with neo epitopes ("neo-CRP") has previously been well documented [Potempa et al., 1987; Ying et al., 1989]. The neo-CRP or modified form of CRP occurs in CRP complexes or when CRP is present on cell surfaces and is thought to lead to enhanced binding to phagocytic cells [Potempa et al., 1988; Bray et al., 1988]. Therefore, it appears that the pentameric CRP, which mediates the Ca^{2+}-dependent binding reactions that trigger its biological activities, may be altered to permit a focused and amplified reaction with other molecular and cellular components at inflammatory sites.

The inhibition of the induced respiratory burst in HL-60(G) cells by the CB-Pep extends the findings of others reporting inhibition of the triggered production of reactive oxygen intermediates by neutrophils exposed to acute phase levels of purified human CRP (≥50–100 μg/ml) [Buchta et al., 1987a; Kew et al., 1990; Shephard et al., 1990, 1992]. One other group of investigators has examined CRP peptides in the same assays and reports that synthetic peptides of residues 77–82 and 201–206 inhibit the PMN respiratory burst [Shephard et al., 1992]; however, the use of these particular peptides was based on sequences of peptides obtained from PMN membrane proteinase digestion of CRP [Shephard et al., 1990]. Our measurement of inhibition of the fMLPP-induced O_2^- response differs from the reported augmentation by heat aggregated CRP of the intracellular respiratory burst by both PMNs and MOs initiated by aggregated IgG binding to the FcγRII [Zeller and Sullivan, 1993]. A critical difference between the latter study and most of the others is that usually the extracellular release of the reactive oxygen species was measured. Although the precise mechanism of inhibition of cellular activation by CRP is unknown, some reports show a decrease in cAMP levels, ATP levels, and protein phosphorylation in neutrophils, clearly suggesting that receptor signaling events are modified [Buchta et al., 1987a]. The modulation of the response of PMNs may be especially relevant in vivo since C5a-induced neutrophil alveolitis was greatly reduced in transgenic mice expressing the rabbit CRP gene [Heurtz et al., 1994] and by CRP-derived peptides [Heurtz et al., 1996]. Mice passively infused with purified human CRP are protected against a lethal challenge with streptococci by a mechanism that requires the uptake of bacteria by phagocytic cells in the spleen and liver [Mortensen, 1993]. The essential role of CRP in this inducible host protective process has been confirmed in mice expressing an inducible human CRP transgene [Szalai et al., 1995]. CRP, as well as peptides derived from its digestion, accumulating at sites of tissue damage may activate responses of cells in the monocyte/macrophage lineage but attenuate PMN activities.

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