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Affinity of C-Reactive Protein toward Fc γ RI Is Strongly Enhanced by the γ -Chain

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C-reactive protein (CRP), the prototype human acute phase protein, is widely regarded as a key player in cardiovascular disease, but the identity of its cellular receptor is still under debate. By using ultrasensitive confocal imaging analysis, we have studied CRP binding to transfected COS-7 cells expressing the high-affinity IgG receptor Fc γ RI. Here we show that CRP binds to Fc γ RI on intact cells, with a k_d of $10 \pm 3 \mu\text{mol/L}$. Transfection of COS-7 cells with a plasmid coding for both Fc γ RI and its functional counterpart, the γ -chain, markedly increases CRP affinity to Fc γ RI, resulting in a k_d of $0.35 \pm 0.10 \mu\text{mol/L}$. The affinity increase results from an ~ 30 -fold enhanced association rate coefficient. The pronounced enhancement of affinity by the γ -chain suggests its crucial involvement in the CRP receptor interaction, possibly by mediating interactions between the transmembrane moieties of the receptors. Dissociation of CRP from the cell surfaces cannot be detected throughout the time course of several hours and is thus extremely slow. Considering the pentameric structure of CRP, this result indicates that multivalent binding and receptor clustering are crucially involved in the interaction of CRP with nucleated cells. (Am J Pathol 2007, 170:755–763; DOI: 10.2353/ajpath.2007.060734)

C-reactive protein (CRP), an ancient immune molecule, is the prototype human acute phase protein.^{1–3} In recent years, CRP has emerged as a powerful cardiovascular risk marker.⁴ Its potential pathogenic role in cardiovascular disease is at present controversially discussed.^{3,5–7} Nevertheless, CRP is already being considered a promising target for cardiovascular therapy.^{6,8}

Understanding the role of CRP in health and disease requires the identification of its specific receptor—if it indeed exists. Research aimed at identifying human CRP receptors has yielded conflicting results.^{9–14} Early reports proposed the existence of specific CRP receptors,⁹ but later studies pointed to interactions with transmembrane Fc γ receptors. However, reports suggesting that the low-affinity IgG receptor Fc γ RIIa, which binds IgGs with micromolar affinity,^{15,16} is the major CRP receptor,¹⁰ were inconclusive because antibodies were used in the CRP binding assay. Consequently, several authors claimed that CRP may not interact with Fc γ RIIa at all and that the observed effects resulted rather from an interaction of the anti-CRP antibody's Fc portion with Fc γ RIIa itself.^{3,11} Nonetheless, indisputable evidence of CRP binding to Fc γ receptors was provided by the demonstration that Fc γ RIIa signaling was triggered by CRP in the HL-60 monocytic cell line.¹² Moreover, phagocytosis of CRP-opsonized erythrocytes was observed for COS-7 cells co-transfected with Fc γ RIIa and Fc γ RI.¹³ Interestingly, potential binding epitopes for interaction with Fc γ RI and Fc γ RIIa were recently identified on the CRP molecule by site-directed mutagenesis.¹⁴

To assess the interaction between CRP and Fc γ receptors on the surface of live cells, we have developed a highly sensitive, fluorescence-based assay that avoids the use of antibodies and affords a precise, quantitative analysis in addition to plain visualization of the interaction. We have chosen Fc γ receptor-transfected COS-7 cells as an established model for our study because this cell line does not naturally express Fc receptors.^{10,17–19} The binding of CRP to receptors on the COS-7 cell surfaces is observed by using confocal fluorescence microscopy with single-molecule sensitivity.²⁰ For fluorescence monitoring, the CRP ligands are labeled with only a single fluorescent dye (Cy3) molecule, thereby avoiding adverse effects of excessive dye labeling on CRP

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structure and function. In our initial study we showed that CRP indeed binds to Fc γ RIIa with micromolar affinity, $k_d = 3.7 \pm 1 \mu\text{mol/L}$.²¹ Interestingly, the affinities of low-affinity Fc γ receptors for their cognate ligands also reside in this range. The extremely slow dissociation of bound CRP indicates a substantial stability of the CRP-receptor complex and suggests that CRP binds to multiple receptors. Indeed, the unusual pentraxin structure of CRP provides as many as five receptor-binding sites.

In addition to Fc γ RIIa, the high-affinity receptor for IgG, Fc γ RI, which is expressed on the surface of neutrophils and monocytes/macrophages,^{15,16} has been implicated as a CRP receptor.^{18,22} Fc γ RI is known to bind monomeric IgG, with k_d values ranging from 2×10^{-10} mol/L to 5×10^{-10} mol/L. It contains an α -chain that associates with a γ -chain homodimer in the plasma membrane. The γ -chain is essential for surface expression of Fc γ RI in the case of *in vivo* models.²³ However, it does not affect the transient expression of Fc γ RI *in vitro* but moderately (two-fold to fivefold) increases the IgG affinity to Fc γ RI and is required for the proper signaling function.^{13,17} Apart from the original report that used metabolically labeled CRP in a control experiment,¹⁸ only functional assays or assays with Fc γ RI extracted from the cell membrane^{13,22} and experiments involving anti-CRP antibodies in the detection of CRP binding¹⁸ have been reported to date. Moreover, a potential involvement of the γ -chain has not yet been considered.

Here, we have applied ultrasensitive confocal laser-scanning fluorescence microscopy to the study of CRP binding to Fc γ RI. By quantitative assessment of both the kinetics and equilibrium of the CRP-Fc γ RI interaction, we provide clear evidence that CRP indeed binds to Fc γ RI in intact cells, with an affinity in the range typical of low-affinity Fc γ receptor-antibody interaction, similar to our previous results with Fc γ RIIa.²¹ When the γ -chain is present, a pronounced affinity enhancement is observed. This detailed characterization of the interaction of CRP with Fc γ receptors is a key step toward a molecular understanding of the physiological role of CRP and its potential role in cardiovascular disease.

Materials and Methods

Cell Culture and Viability

COS-7 cells were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and maintained in Dulbecco's modified Eagle's medium/10% fetal calf serum with 1% penicillin/streptomycin/L-glutamine. Cell viability assays (trypan blue) revealed 99% viable transfected cells. To ensure cell viability during confocal scanning experiments, we frequently applied 75 nmol/L propidium iodide (Molecular Probes, Eugene, OR) *in situ* after completion of the measurement. Even after several hours on the microscope, the cells were always found to be viable. As a positive control, subsequent permeabilization with 0.1% Triton X-100 (Sigma-Aldrich, Taufkirchen, Germany) yielded bright nuclear staining.

Reagents and Antibodies

Recombinant CRP was obtained from Calbiochem (Bad Soden, Germany) and human ascites CRP (haCRP) from Merck Biosciences (Schwalbach, Germany). Mouse anti-CD64, clone 10.1, horseradish peroxidase-conjugated anti-human IgG (Fc-specific), and mouse IgG₁ were purchased from Dako Cytomation (Glostrup, Denmark); anti-CD64-FITC, clone 10.1, was from BD Biosciences Pharmingen (San Diego, CA); anti-V5 was from Invitrogen (Groningen, The Netherlands); and PE-goat F(ab')₂ anti-mouse IgG (PE-GAM) was from Caltag Labs (Hamburg, Germany). Human IgG for inhibition experiments was purchased from Sigma-Aldrich. Anti-NGFR for control experiments with low-affinity NGF receptor was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Fluorescence Labeling of CRP and Antibodies

Recombinant and highly purified CRPs were labeled with Cy3-NHS (Amersham, Freiburg, Germany) by coupling the succinimidyl ester derivative of the dye to amine groups at pH 8.2 in phosphate buffer. Anti-CD64 (clone 10.1) antibody was conjugated with the fluorescent dye Alexa Fluor 647 (Molecular Probes) using maleimide coupling to free thiol groups. Unreacted dye was removed by gel filtration. The degree of labeling was kept minimal (one to two fluorescent labels per protein molecule) to exclude interference of the dye with CRP-receptor interactions.

Characterization of CRP

CRP was proven free of IgG contamination by Western blot analysis, using a 4 to 12% ProGel-Tris-Glycin-Gel (Anamed, Darmstadt, Germany). The gel was blotted, and the membrane Hybond C extra (Amersham) was blocked in a phosphate-buffered saline (PBS) solution with 4% milk powder and 1% bovine serum albumin. The membrane was stained with anti-human IgG-horseradish peroxidase and developed with ECL Western blotting substrate (Pierce, Bonn, Germany). The endotoxin (lipopolysaccharide, LPS) content of our recombinant CRP was quantified by using the Limulus amoebocyte lysate test kit QCL 1000 (Cambrex, Walkersville, MD). It yielded an LPS content of 40 EU/(mg CRP), corresponding to 1 molecule LPS per $\sim 10,000$ CRP pentamers (Cambrex). As a control, LPS (Sigma-Aldrich) from *Escherichia coli* strain 0111:B4 (identical to the endotoxin standard in the kit) was added to the CRP preparations in the binding assays at a ratio of 1000 EU/(mg CRP) to investigate the influence of LPS on CRP binding to Fc γ RI. No LPS effect on CRP binding was observed. We also examined CRP from human ascites fluid (Calbiochem), which had a LPS content of 5 EU/(mg CRP), corresponding to 1 LPS molecule per $\sim 80,000$ CRP pentamers. This CRP preparation exhibited Fc γ RI binding essentially identical to recombinant CRP. To ensure the structural integrity of the CRP in the fluorescently labeled CRP preparations, we used fluorescence correlation spectroscopy. Both the

measured correlation amplitudes and diffusion coefficients confirmed the pentameric structure of CRP in the solutions. Finally, we also verified the physiological activity of CRP using phosphocholine binding assays, as described.²⁴ The CRP preparations showed dose-dependent phosphocholine binding, demonstrating that the protein was in a physiologically competent state.

Fc γ RI, γ -Chain, and Fc γ RI- γ Vector Cloning and Transfection

Human Fc γ RI and γ -chain cDNA were generated by means of reverse transcriptase-polymerase chain reaction from human macrophage RNA using Cloned *Pfu* DNA Polymerase (Stratagene, La Jolla, CA). Sequences were cloned without stop codon, with the V5 epitope tag sequence at the 3' end. They were cloned separately into pcDNA3.1 using the directional TOPO expression kit (Invitrogen). Both Fc γ RI and γ -chain cDNA were cloned together in the same vector pBudCE4.1 (Invitrogen), thus generating a double construct further referred to as Fc γ RI- γ . The γ -chain in the double construct was cloned without stop codon, also with the V5 sequence at the 3' end.

The gene coding for the truncated form of low-affinity nerve growth factor receptor (Δ LNGFR) was cloned in pBudCE4.1. Δ LNGFR was used as a nonrelated receptor in control experiments. All vectors were sequenced. 60-mm plates were seeded at 90 to 95% confluence, and after 24 hours, they were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Mock-transfected cells were treated with transfectant reagent only.

Fluorescence-Activated Cell Sorting (FACS) Detection of Fc γ RI and Fc γ RI- γ Expression

Forty-eight hours after transfection, cells were detached with Accutase (PAA Labs, Linz, Austria) and washed three times in ice-cold Dulbecco's PBS (without Ca²⁺ and Mg²⁺; PAA Labs) containing 0.1% bovine serum albumin. Fc γ RI-transfected cells were incubated with anti-CD64-FITC antibody in the same buffer for 0.5 hours on ice in the dark, washed twice, and then subjected to FACS analysis. Fc γ RI- γ -transfected cells were permeabilized in BD Cytofix/Cytoperm solution (BD Biosciences Pharmingen) for 20 minutes on ice and then washed and stained with anti-V5 antibody in a BD Perm/Wash buffer (BD Biosciences Pharmingen) for 0.5 hours on ice. Cells were washed three times with the same buffer and then stained with PE-GAM F(ab)₂ for 0.5 hours on ice in the dark. Finally, cells were washed and resuspended in stain buffer (BD Biosciences Pharmingen) and subjected to FACS analysis. Mock-transfected cells were treated identically with antibodies and solutions. FACS data were analyzed using the CellQuest software (BD Biosciences, Heidelberg, Germany). Thirty thousand cells were gated by fluorescence-1 (green) and fluorescence-2 (red).

Ninety-nine percent of mock-transfected cells were assessed as background.

Confocal Imaging and Analysis

Confocal images were collected using a home-built confocal laser-scanning fluorescence microscope with single-fluorophore sensitivity.^{20,25} An Ar⁺/Kr⁺ ion laser (Spectra Physics 164; Spectra Physics, Mountain View, CA) and a HeNe laser (Polytec, Waldbronn, Germany) were used for fluorescence excitation at 514.5 nm and 632.8 nm, respectively. The excitation light was focused into the sample, and the resulting fluorescence emission was collected by a water immersion objective (C-Apochromat 63 $\times$ /1.2W; Zeiss, Göttingen, Germany). Highly efficient detection in two spectral channels (green, 557 to 607 nm; red, 665 to 850 nm) was accomplished by splitting the fluorescence light using custom-made band pass filters in conjunction with dichroic mirrors (AHF, Tübingen, Germany) and subsequent detection with photon-counting detectors (AQR-14; Perkin-Elmer, Vaudreuil, QC, Canada). Confocal fluorescence images consisting of 128 $\times$ 128 pixels were acquired in a field of 80 $\times$ 80 μ m² with a depth resolution of $\sim$ 2 μ m. For both excitation wavelengths, a laser power of 1 μ W was incident on an area of 0.3 μ m². For quantitative analysis, the fluorescence emitted by membranes of selected cells was examined as a function of time after incubation with fluorescently labeled CRP or anti-CD64 or after equilibration with these proteins at different concentrations. Within a single series of measurements, the membrane fluorescence was quantified by the average fluorescence of the same number (typically 100) of brightest pixels from the membrane of a chosen cell. Co-localization was evaluated quantitatively using CoLocalizer Express (Colocalization Research Software, Boise, ID).

Co-Localization and Inhibition Measurements

Buffer solutions (Dulbecco's PBS without Ca²⁺ and Mg²⁺; PAA Labs) containing transfected COS-7 cells were transferred to a sandwich chamber consisting of two glass coverslips separated by 200- μ m-thick Mylar spacers. After 15 minutes, the cells were exposed to solutions of 17 nmol/L Alexa 647-labeled anti-CD64 antibodies. After washing with buffer, the cells were incubated with CRP-Cy3 at 0.87 μ mol/L. Subsequently, confocal images were taken. For the inhibition measurements, the cells were first stained with 7 nmol/L Alexa 647-labeled anti-CD64, then incubated for 20 minutes with 70 nmol/L antibodies (anti-CD64 or human IgG), and finally incubated with 8.7 μ mol/L CRP-Cy3 for 20 minutes, applying washing steps after each incubation.

Association Kinetics and Equilibrium Binding

The kinetics of association of Cy3-labeled CRP to Fc γ RI-transfected COS-7 cells was studied by acquiring confocal images as a function of time. For studies of equilibrium binding, incubation times were adjusted in accord

Figure 1. FACS analysis of transfected COS-7 cells. **A:** Staining with anti-CD64-FITC antibody showed 62% positivity for FcγRI and 55% for FcγRI-γ-transfected cells. **B:** Staining with anti-V5/PE revealed 53% positivity for γ-chain-transfected cells and 46% for FcγRI-γ-transfected cells. Of identically treated mock-transfected cells, 99% were excluded as background. The histogram plot of the anti-CD64-FITC staining (**C**) shows strong similarity of CD64 expression for both transfection with FcγRI alone (thin line, MFI 204.6) and transfection with FcγRI-γ (bold line, MFI 202.51).

with the kinetic data. COS-7 cells were exposed to different concentrations of Cy3-labeled CRP. After washing, confocal images were acquired to assess the degree of saturation of the receptors.

Results

FACS Detection of FcγRI and γ-Chain Expression

To ensure simultaneous expression of FcγRI and the γ-chain in the same cell, we constructed a combined plasmid and determined the levels of expression by using FACS. Staining with an antibody against FcγRI (anti-CD64-FITC) showed 62% positivity for FcγRI and 55% for FcγRI-γ-transfected cells (Figure 1A). Ninety-nine percent of identically treated, mock-transfected cells were excluded as background. Staining with anti-V5/PE revealed 53% positivity for γ-chain and 46% for FcγRI-γ-transfected cells (Figure 1B). Here, only the γ-chain gene in the double vector contains the V5 sequence at the 3' end (see Materials and Methods). Again, 99% of identically treated mock-transfected cells were excluded as background. The histogram plot of anti-CD64-FITC staining (Figure 1C) indicates that co-expression of the γ-chain has no influence on the FcγRI expression. The mean fluorescence intensity (MFI) was very similar for cells transfected with FcγRI alone (MFI, 204.6) and cells

Figure 2. Confocal laser-scanning fluorescence microscopy images showing co-localization of FcγRI and CRP on the plasma membranes of COS-7 cells. **A:** Background fluorescence arising mainly from the cell bodies. **B:** Incubation with 0.87 μmol/L CRP-Cy3 causes high background fluorescence and strong fluorescence from surfaces of cells expressing FcγRI. **C:** After washing, the background fluorescence is removed, whereas the fluorescence on the cell surfaces remains because of tight binding of CRP. **D:** The receptor expression pattern of individual cells, visualized by weak staining with anti-CD64 labeled with Alexa Fluor 647, closely resembles the pattern of green CRP-Cy3 fluorescence in **C**.

transfected with a vector carrying both FcγRI and γ-chain (MFI, 202.51).

Co-Localization of Ligands and Receptors

By using confocal imaging on FcγRI-transfected COS-7 cells, we consistently observed co-localization of CRP-binding sites and receptor positions, which strongly supports our claim that CRP binds to FcγRI. An example is shown in Figure 2. The background fluorescence of the FcγRI-transfected COS-7 cells was weak and mainly localized within the cells (Figure 2A). The addition of Cy3-labeled CRP to the buffer solution produced a strong background signal because of the dissolved protein and an accumulation of CRP at sites on the plasma membranes, as indicated by the intense green fluorescence from these locations (Figure 2B). After washing with PBS, the background disappeared but the fluorescence from the cell surfaces remains (Figure 2C). Receptor staining with anti-CD64 labeled with Alexa Fluor 647 created red surface fluorescence proportional to the receptor expression level (Figure 2D). Comparison of Figure 2, C and D, revealed a clear correlation between the spatial distributions of CRP and receptors on transfected cells, which strongly supports our interpretation that CRP binds to FcγRI. In the center of the scanned area in Figure 2, we show a cell that binds neither CRP nor anti-CD64 to any significant extent. It apparently does not express FcγRI and thus serves as an intrinsic negative control, excluding apoptosis or unspecific binding as possible reasons

Figure 3. Confocal laser-scanning fluorescence microscopy images showing inhibition of CRP binding by different antibodies. COS-7 cells were minimally stained with anti-CD64 labeled with Alexa Fluor 647 (**A–D**) to identify Fc γ RI- γ -transfected cells. Binding of CRP-Cy3 was strongly inhibited after incubation with solutions containing 70 nmol/L anti-CD64 (**E**) and human IgG (**F**), whereas no inhibition was observed after incubation with mouse IgG₁ (**G**). **H**: Strong CRP-Cy3 binding without inhibition is shown as a control. For all experiments, a CRP-Cy3 concentration of 8.7 μ mol/L was used.

for the observed CRP-binding. For several transfected cells we performed a quantitative analysis of co-localization according to Manders and colleagues²⁶ and found an average overlap coefficient of $r = 0.89$, which indicates a strong co-localization of CRP and Fc γ RI.

Although the well-known LPS contamination of recombinantly produced CRP, which amounts to ~ 1 LPS molecule per 10,000 CRP pentamers, is unlikely to influence CRP binding to Fc γ RI, we verified experimentally that we could safely exclude an effect of LPS. To this end, we added LPS up to a final concentration of 1000 EU/(mg CRP), thereby increasing the initial LPS contamination of recombinant CRP 25-fold without observing any effect on CRP binding to Fc γ RI. Furthermore, when using CRP from human ascites fluid with a lower LPS content of 5 EU/(mg CRP), binding to Fc γ RI was essentially identical as with recombinant protein.

Inhibition of CRP Binding

To verify that CRP indeed binds to Fc γ RI, we performed competitive inhibition experiments using monomeric IgG as well as anti-CD64 monoclonal antibodies to block different interaction sites on the receptor. In both cases, efficient inhibition of CRP binding was found after incubation with 70 nmol/L (10 μ g/ml) antibody solutions (Figure 3). The average fluorescence from CRP-Cy3 bound to the cell surface was reduced by a factor of 5 ± 1 for anti-CD64 and 6 ± 2 for IgG (Figure 3, E and F) compared with a control experiment without inhibition (Figure 3H). We used mouse IgG₁ as a negative control for inhibition; it showed no inhibitory effect on CRP binding (Figure 3G). For the analysis, the fluorescence signals were normalized to the same surface concentration of

receptors, as judged from the levels of receptor staining (Figure 3, A–D).

Controls for Binding Specificity and Cell Viability

To establish that binding of CRP is receptor-related and not an artifact of transfection, we performed studies on COS-7 cells transfected with the receptor Δ LNGFR, which is naturally expressed only on membranes of neuronal cells and is not related to immune receptors.²⁷ Strong expression of the control receptor was detected by antibody staining (Figure 4A), yet no binding of CRP could be observed (Figure 4, B and C). In contrast, the level of expression of Fc γ RI correlated strongly with CRP-Cy3 binding (Figure 2, C and D). Thus, we can safely exclude that the binding of CRP to Fc γ RI is nonspecific and caused by the transfection itself. To exclude that IgG contaminations in the CRP preparation are responsible for binding, we verified CRP purity by Western blot analysis (Figure 4D).

The viability of the cells studied in the CRP-binding experiments was frequently examined, as shown by the example depicted in Figure 4, E–G. We never observed cell penetration and nuclear staining by propidium iodide for the investigated cells (Figure 4F). However, subsequent co-incubation with 0.1% Triton X-100 led to cell permeabilization and bright yellow nuclear staining within a few minutes because of the accumulation of propidium iodide in the nucleus (Figure 4G).

Association Kinetics

We have analyzed the increase of membrane-associated fluorescence with incubation time to characterize the ki-

Figure 4. Control experiments verifying the binding specificity of CRP and cell viability after CRP binding. **A:** COS-7 cells transfected with the receptor ΔLINGFR were minimally stained with anti-NGFR labeled with Alexa Fluor 647; **B:** autofluorescence background level; **C:** insignificant change in fluorescence emission from background despite incubation with 8.7 μmol/L CRP-Cy3. **D:** Western blot to test CRP preparations for IgG contamination. Both rCRP and haCRP (lanes 1 and 3, 20 μg each) are shown to be free of IgG, as indicated by the positive controls with human IgG (lanes 5 to 10, from 100 to 3.125 ng). **E–G:** Viability of FcγRI-γ-transfected COS-7 cells after CRP binding, demonstrated by overlay images of the green and red detection channel. **E:** The fluorescence from a representative CRP-Cy3-binding cell on 514-nm laser excitation occurs as a green pattern from the cell surface. **F:** This pattern remains unchanged even after 10 minutes of incubation with 75 nmol/L propidium iodide, demonstrating the viability of the cell. **G:** As a positive control, an image after 10 minutes of incubation with a mixture of propidium iodide and Triton X-100 (0.1%) shows penetration of the dye into the nucleus, indicated by the bright yellow nuclear pattern from propidium iodide emitting into both the green and the red emission channels.

netics of association of CRP with FcγRI (Figure 5A). For incubation with 30 and 90 nmol/L CRP, we obtained apparent association rate coefficients of $3.1 \times 10^{-4} \text{ seconds}^{-1}$ and $1.1 \times 10^{-3} \text{ seconds}^{-1}$ from fitting exponential model functions to the data. After essentially complete loading of the receptors with CRP, we washed the cells with buffer to study CRP dissociation. However, we were unable to detect any CRP dissociation from the cells throughout the period of several hours. Therefore, the dissociation rate coefficient was much smaller than the association rate coefficient, and consequently, we could convert the apparent rate coefficients into a second-order association rate coefficient, yielding $1.1 \pm 0.3 \times 10^4 \text{ Lmol}^{-1} \text{ seconds}^{-1}$. Figure 5B shows the association time course for cells co-transfected stoichiometrically with both FcγRI and the γ-chain in a single vector (FcγRI-γ). The association kinetics of CRP was significantly accelerated (note the different scale on the time axis). Here we find apparent association rate coefficients of $3.9 \times 10^{-3} \text{ seconds}^{-1}$ and $1.1 \times 10^{-2} \text{ s}^{-1}$ for CRP concentrations of 10 and 30 nmol/L, respectively, yielding a second-order association rate coefficient of $3.8 \pm 1.0 \times 10^5$

Figure 5. Association kinetics of CRP-Cy3 to FcγRI receptors. The measurements were performed in the absence (**A**) and presence (**B**) of the γ-chain. The normalized, membrane-associated fluorescence of representative cells is plotted as a function of incubation time (symbols) for two different CRP-Cy3 concentrations. The lines show best fits with exponential model functions. In **B**, the fluorescence signal from a receptor-negative cell in the same sample is displayed as a negative control.

$\text{Lmol}^{-1} \text{ seconds}^{-1}$. Therefore, ligand association speeds up by ~30-fold in the presence of the γ-chain.

Equilibrium Binding Studies

The affinity of CRP to FcγRI and FcγRI-γ was determined by quantitative analysis of the receptor-associated fluorescence as a function of the free ligand concentration. A pronounced shift of the binding curve toward higher affinity was found when the γ-chain was present (Figure 6). The binding of CRP to FcγRI is characterized by $k_d = 10 \pm 3 \text{ μmol/L}$; the presence of the γ-chain enhances the affinity by ~30-fold, yielding $k_d = 0.35 \pm 0.10 \text{ μmol/L}$. This affinity enhancement correlates nicely with the acceleration of the association kinetics in presence of the γ-chain, indicating that the γ-chain assists in forming a stable CRP-receptor complex. Because Ca^{2+} ions are known to affect the binding of ligands to CRP,^{1,2} we also investigated the dependence of CRP-FcγRI interaction on divalent ions. To this end, we added 0.5 mmol/L Ca^{2+} to the buffer solution while the cells were incubated with CRP at concentrations in the steep transition regions of the curves in Figure 6. However, we did not observe any significant changes in the fluorescence (data not shown).

Figure 6. Concentration dependence of CRP-Cy3 binding to Fc γ RI receptors. The degree of saturation with CRP of Fc γ RI without (circles) and with (squares) γ -chain is shown by plotting the normalized membrane fluorescence of representative cells as a function of the CRP concentration. The curves were normalized to their extrapolated saturation values. Best-fit curves for a bimolecular binding model are shown as lines.

These results suggest that the affinity of CRP toward Fc γ RI does not increase noticeably in the presence of 0.5 mmol/L Ca $^{2+}$.

Discussion

Using ultrasensitive confocal imaging analysis, we have observed CRP binding to Fc γ RI. The interaction can be competitively inhibited by human IgG as well as anti-CD64 antibodies. The quantitative analysis of the CRP concentration dependence of the binding equilibrium yields an equilibrium dissociation coefficient $k_d = 10 \pm 3$ μ mol/L, in agreement with earlier studies that used metabolically labeled CRP.¹⁸ The functional counterpart of Fc γ RI in receptor signaling on IgG binding, the γ -chain, increases the affinity of CRP to Fc γ RI by ~ 30 -fold. The extremely slow CRP dissociation suggests that receptor clustering causes extremely tight CRP binding to multiple Fc γ RI receptors. These findings offer several new insights into the understanding of CRP-Fc γ receptor interactions.

CRP shares several essential functional properties with antibodies.^{1–3} First, CRP binds small ligand molecules such as phosphocholine. Second, ligand-bound CRP activates the complement system via the classical pathway.²⁸ Third, CRP opsonizes biological particles for macrophages.²⁹ Considering these functional similarities, it may not be a surprise that CRP interacts with the same receptors as antibodies, despite the fact that the pentraxin structure of CRP is very different from the structure of antibodies. CRP appeared very early in the evolution of the immune system^{1–3}; it is already expressed by primitive organisms such as *Limulus polyphemus*. Therefore, it is intriguing to speculate that Fc γ receptors may have originated as CRP receptors and only later, in the evolution of the specific humoral immunity, have evolved into receptors for IgG proteins. Macrophages, which also appeared early in evolution, express Fc γ receptors in high quantities and are likely target cells for CRP. Possi-

bly, CRP, Fc γ -receptors, macrophages, complement, and complement receptors may have orchestrated the primitive humoral and cellular immunity and were only later supplemented by specific humoral and cellular immunity involving lymphocytes and antibodies.

The affinities of CRP binding to Fc γ RI and Fc γ RI α obtained from our live-cell imaging studies are in the same range as those of low-affinity Fc γ receptors for their Ig ligands, for example, Fc γ RI α for IgGs.^{15,16} Significantly higher affinities were reported from surface plasmon resonance (SPR) experiments on extracellular fragments of Fc γ RI that were cleaved from transfected COS-7 cells and immobilized individually on sensorchip surfaces.²² These apparent discrepancies arise from the different ways in which the experiments are performed, and the results reported by the two methods actually have very different meanings. At the heart of this issue lies the multivalent binding of the pentameric CRP molecule, allowing CRP to bind up to five individual Fc γ receptors. Consequently, five independent or energetically coupled binding reactions govern the overall CRP-Fc γ RI interaction. Such multivalent interactions are usually subsumed in an overall binding affinity, or avidity. In the SPR experiment, CRP binding to single Fc γ receptors is measured in the presence of free CRP ligand in solution; this procedure may yield well-defined equilibrium parameters for CRP binding to individual Fc γ receptor fragments.²² Note that the lifetime of a single, high-affinity receptor-ligand bond (nmol/L k_d values) is still only on the order of seconds, and thus, removal of free CRP from the solution will lead to immediate dissociation. Consequently, in assays involving removal of CRP from the solution before the analysis, such as FACS and fluorescent or radiometric cellular assays, only the avidly bound CRP molecules interacting with multiple receptors will stay bound during the entire duration of the experiment and are included in the analysis. Because signal transduction via Fc γ RI is initiated by cross-linking of individual receptors in the membrane,^{15,16} we assume that it is the avidly bound fraction of CRP that is responsible for physiological cell signaling. Therefore, we consider our ultrasensitive fluorescence microscopy studies on live cells more pertinent to the physiological process than the *in vitro* studies using individual receptor fragments.

Our investigations have established a pronounced effect of the γ -chain on CRP binding to Fc γ RI, namely a 30-fold increase of k_d on stoichiometric co-transfection of the γ -chain. By contrast, the γ -chain increases the IgG affinity to Fc γ RI only moderately (twofold to fivefold).¹⁷ Still, in both cases it is likely that the homodimeric γ -chain mediates interactions between the transmembrane parts of individual Fc γ RI receptors. Following this line of reasoning, the strongly enhanced binding of CRP in the presence of the γ -chain can be consistently explained by the increased probability of simultaneous interactions of CRP with several receptors.

There is general agreement that CRP is a powerful cardiovascular risk marker.⁴ CRP has also been implicated as a contributor to the pathogenesis of cardiovascular disease. Although the evidence is still controversial, CRP has already been considered as a promising target

for cardiovascular therapy.^{6–8} CRP is intimately involved in experimental atherogenesis in some^{6,30} although not in all animals.³¹ CRP deposits in atherosclerotic lesions in co-localization with macrophages, macrophage foam cells, and complement C5b-9.^{32,33} CRP stimulates monocyte chemotaxis³³ and induces low-density lipoprotein uptake into macrophages.³⁴ Both these pivotal processes in atherogenesis may be mediated via Fc γ receptors. In atherosclerotic lesions, CRP accumulates, and local CRP concentrations may be as high as in acute phase response, or even higher. The latter may facilitate CRP-Fc γ R interaction. We suggest that CRP, low-density lipoprotein, complement, and macrophage Fc γ receptors all act in concert to induce an inflammatory response that significantly contributes to the development of atherosclerotic lesions. Macrophages, not arterial wall cells such as endothelial or smooth muscle cells, express Fc γ receptors in high quantities and are thus the most likely target cells for CRP, especially in light of compelling evidence that the reported physiological effects of CRP on endothelial cells and smooth muscle cells^{35,36} are artifacts caused by LPS or azide contamination of commercially available CRP preparations.^{37–39}

In summary, we have used advanced biophysical methods to assess quantitatively the interactions of CRP with Fc γ RI and Fc γ RIIIa receptors expressed on the surfaces of live cells. Our findings reconcile conflicting prior studies and yield a consistent picture of CRP receptor interactions. This may be an important step toward the understanding of the role of CRP in health and disease.

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References

- Mortensen RF: C-reactive protein, inflammation, and innate immunity. *Immunol Res* 2001, 24:163–176
- Black S, Kushner I, Samols D: C-reactive protein. *J Biol Chem* 2004, 279:48487–48490
- Pepys MB, Hirschfield GM: C-reactive protein: a critical update. *J Clin Invest* 2003, 111:1805–1812
- Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, Pfeffer MA, Braunwald E: C-reactive protein levels and outcomes after statin therapy. *N Engl J Med* 2005, 352:20–28
- Manolov DE, Koenig W, Hombach V, Torzewski J: C-reactive protein and atherosclerosis. Is there a causal link. *Histol Histopathol* 2003, 18:1189–1193
- Torzewski J: C-reactive protein and atherogenesis: new insights from established animal models. *Am J Pathol* 2005, 167:923–925
- Scirica BM, Morrow DA: Is C-reactive protein an innocent bystander or proatherogenic culprit? The verdict is still out. *Circulation* 2006, 113:2128–2134; discussion 2151
- Pepys MB, Hirschfield GM, Tennent GA, Gallimore JR, Kahan MC, Bellotti V, Hawkins PN, Myers RM, Smith MD, Polara A, Cobb AJ, Ley SV, Aquilina JA, Robinson CV, Sharif I, Gray GA, Sabin CA, Jenvey MC, Kolstoe SE, Thompson D, Wood SP: Targeting C-reactive protein for the treatment of cardiovascular disease. *Nature* 2006, 440:1217–1221
- Tebo JM, Mortensen RF: Characterization and isolation of a C-reactive protein receptor from the human monocytic cell line U-937. *J Immunol* 1990, 144:231–238
- Bharadwaj D, Stein MP, Volzer M, Mold C, Du Clos TW: The major receptor for C-reactive protein on leukocytes is Fc γ receptor II. *J Exp Med* 1999, 190:585–590
- Saeland E, van Royen A, Hendriksen K, Vile-Weekhout H, Rijkers GT, Sanders LA, van de Winkel JG: Human C-reactive protein does not bind to Fc γ RIIIa on phagocytic cells. *J Clin Invest* 2001, 107:641–643
- Chi M, Tridandapani S, Zhong W, Coggeshall KM, Mortensen RF: C-reactive protein induces signaling through Fc γ RIIIa on HL-60 granulocytes. *J Immunol* 2002, 168:1413–1418
- Bodman-Smith KB, Gregory RE, Harrison PT, Raynes JG: Fc γ RIIIa expression with Fc γ RI results in C-reactive protein- and IgG-mediated phagocytosis. *J Leukoc Biol* 2004, 75:1029–1035
- Bang R, Marnell L, Mold C, Stein MP, Du Clos KT, Chivington-Buck C, Du Clos TW: Analysis of binding sites in human C-reactive protein for Fc γ RI, Fc γ RIIIa, and C1q by site-directed mutagenesis. *J Biol Chem* 2005, 280:25095–25102
- Raghavan M, Bjorkman PJ: Fc receptors and their interactions with immunoglobulins. *Annu Rev Cell Dev Biol* 1996, 12:181–220
- Ravetch JV, Bolland S: IgG Fc receptors. *Annu Rev Immunol* 2001, 19:275–290
- Miller KL, Duchemin AM, Anderson CL: A novel role for the Fc receptor gamma subunit: enhancement of Fc gamma R ligand affinity. *J Exp Med* 1996, 183:2227–2233
- Marnell LL, Mold C, Volzer MA, Burlingame RW, Du Clos TW: C-reactive protein binds to Fc gamma RI in transfected COS cells. *J Immunol* 1995, 155:2185–2193
- Stein MP, Edberg JC, Kimberly RP, Mangan EK, Bharadwaj D, Mold C, Du Clos TW: C-reactive protein binding to Fc γ RIIIa on human monocytes and neutrophils is allele-specific. *J Clin Invest* 2000, 105:369–376
- Wiedenmann J, Schenk A, Röcker C, Girod A, Spindler KD, Nienhaus GU: A far-red fluorescent protein with fast maturation and reduced oligomerization tendency from *Entacmaea quadricolor* (Anthozoa, Actinaria). *Proc Natl Acad Sci USA* 2002, 99:11646–11651
- Manolov DE, Röcker C, Hombach V, Nienhaus GU, Torzewski J: Ultrasensitive confocal fluorescence microscopy of C-reactive protein interacting with Fc γ RIIIa. *Arterioscler Thromb Vasc Biol* 2004, 24:2372–2377
- Bodman-Smith KB, Melendez AJ, Campbell I, Harrison PT, Allen JM, Raynes JG: C-reactive protein-mediated phagocytosis and phospholipase D signalling through the high-affinity receptor for immunoglobulin G (Fc γ RIIIa). *Immunology* 2002, 107:252–260
- van Vugt MJ, Heijnen AF, Capel PJ, Park SY, Ra C, Saito T, Verbeek JS, van de Winkel JG: FcR gamma-chain is essential for both surface expression and function of human Fc gamma RI (CD64) in vivo. *Blood* 1996, 87:3593–3599
- Hundt M, Zielinska-Skowronek M, Schmidt RE: Lack of specific receptors for C-reactive protein on white blood cells. *Eur J Immunol* 2001, 31:3475–3483
- Schenk A, Ivanchenko S, Röcker C, Wiedenmann J, Nienhaus GU: Photodynamics of red fluorescent proteins studied by fluorescence correlation spectroscopy. *Biophys J* 2004, 86:384–394
- Manders EMM, Verbeek FJ, Aten JA: Measurement of co-localization of objects in dual-colour confocal images. *J Microsc* 1993, 169:375–382
- Wiehe JMI, Niesler C, Torzewski J, Zimmermann O, Wiesneth M, Schmitt M, Schwarz K, Döhner H, Hombach V, Greiner G: Efficient transient genetic labeling of human CD34+ progenitor cells for in vivo application. *Regen Med* 2006, 1:223–234
- Volanakis JE: Complement activation by C-reactive protein complexes. *Ann NY Acad Sci* 1982, 389:235–250
- Mortensen RF, Osmand AP, Lint TF, Gewurz H: Interaction of C-reactive protein with lymphocytes and monocytes: complement-dependent adherence and phagocytosis. *J Immunol* 1976, 117:774–781
- Sun H, Koike T, Ichikawa T, Hatakeyama K, Shiomi M, Zhang B, Kitajima S, Morimoto M, Watanabe T, Asada Y, Chen YE, Fan J: C-reactive protein in atherosclerotic lesions: its origin and pathophysiological significance. *Am J Pathol* 2005, 167:1139–1148
- Reifenberg K, Lehr HA, Baskal D, Wiese E, Schaefer SC, Black S, Samols D, Torzewski M, Lackner KJ, Hüsman M, Blettner M, Bhakdi S: Role of C-reactive protein in atherogenesis: can the apolipoprotein E knockout mouse provide the answer. *Arterioscler Thromb Vasc Biol* 2005, 25:1641–1646

32. Torzewski J, Torzewski M, Bowyer DE, Frohlich M, Koenig W, Waltenberger J, Fitzsimmons C, Hombach V: C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arterioscler Thromb Vasc Biol* 1998, 18:1386–1392
33. Torzewski M, Rist C, Mortensen RF, Zwaka TP, Bienek M, Waltenberger J, Koenig W, Schmitz G, Hombach V, Torzewski J: C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. *Arterioscler Thromb Vasc Biol* 2000, 20:2094–2099
34. Zwaka TP, Hombach V, Torzewski J: C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. *Circulation* 2001, 103:1194–1197
35. Pasceri V, Willerson JT, Yeh ET: Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation* 2000, 102:2165–2168
36. Verma S, Li SH, Badiwala MV, Weisel RD, Fedak PW, Li RK, Dhillon B, Mickle DA: Endothelin antagonism and interleukin-6 inhibition attenuate the proatherogenic effects of C-reactive protein. *Circulation* 2002, 105:1890–1896
37. Taylor KE, Giddings JC, van den Berg CW: C-reactive protein-induced in vitro endothelial cell activation is an artifact caused by azide and lipopolysaccharide. *Arterioscler Thromb Vasc Biol* 2005, 25:1225–1230
38. Swafford Jr AN, Bratz IN, Knudson JD, Rogers PA, Timmerman JM, Tune JD, Dick GM: C-reactive protein does not relax vascular smooth muscle: effects mediated by sodium azide in commercially available preparations. *Am J Physiol* 2005, 288:H1786–H1795
39. Oroszlan M, Herczenik E, Rugonfalvi-Kiss S, Roos A, Nauta AJ, Daha MR, Gombos I, Karadi I, Romics L, Prohaszka Z, Fust G, Cervenak L: Proinflammatory changes in human umbilical cord vein endothelial cells can be induced neither by native nor by modified CRP. *Int Immunol* 2006, 18:871–878