Pentraxins and IgA share a binding hot-spot on Fc α RI

Jinghua Lu,¹ Kristopher D. Marjon,^{2,3} Carolyn Mold,^{2,3}
Lorraine Marnell,^{2,3} Terry W. Du Clos,^{2,4} and Peter Sun^{1*}

¹Structural Immunology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852

²Department of Internal Medicine, University of New Mexico, Albuquerque, New Mexico 87131

³Department of Molecular Genetics and Microbiology, University of New Mexico, Albuquerque, New Mexico 87131

⁴Department of Medicine, VA Medical Center, Albuquerque, New Mexico 87108

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Abstract: The pentraxins, C-reactive protein (CRP), and serum amyloid P component (SAP) have previously been shown to function as innate opsonins through interactions with Fc γ receptors. The molecular details of these interactions were elucidated by the crystal structure of SAP in complex with Fc γ RIIA. More recently, pentraxins were shown to bind and activate Fc α RI (CD89), the receptor for IgA. Here, we used mutations of the receptor based on a docking model to further examine pentraxin recognition by Fc α RI. The solution binding of pentraxins to six Fc α RI alanine cluster mutants revealed that mutations Y35A and R82A, on the C- and F-strands of the D1 domain, respectively, markedly reduced receptor binding to CRP and SAP. These residues are in the IgA-binding site of the receptor, and thus, significantly affected receptor binding to IgA. The shared pentraxin and IgA-binding site on Fc α RI is further supported by the results of a solution binding competition assay. In addition to the IgA-binding site, pentraxins appear to interact with a broader region of the receptor as the mutation in the C'-strand (R48A/E49A) enhanced pentraxin binding. Unlike Fc γ receptors, the H129A/I130A and R178A mutations on the BC- and FG-loops of D2 domain, respectively, had little effect on Fc α RI binding to the pentraxins. In conclusion, our data suggest that the pentraxins recognize a similar site on Fc α RI as IgA.

Keywords: surface plasmon resonance; site-directed mutagenesis; competition

Introduction

C-reactive protein (CRP) and serum amyloid P component (SAP) are two major acute phase proteins in humans and mice, respectively.^{1–4} Structurally they

belong to the pentraxin family of proteins, which are characterized by a pentameric ring-like structure bearing five identical subunits or protomers. The two faces of the pentamer are involved in ligand binding and effector recognitions, respectively, during inflammation. Although CRP and SAP display distinct ligand binding affinities,^{5–10} both activate complement component C1q.^{11–13}

CRP and SAP are structurally different from antibodies.^{14,15} Yet, the pentraxins were shown to interact with Fc γ receptors.^{16,17} Recently, we characterized the structural recognition between pentraxins and Fc γ receptors through the cocrystallization of SAP and Fc γ RIIA.¹⁸ The Fc γ receptors are type I transmembrane proteins with two or three extracellular domains.¹⁹ Unlike IgG recognition that

Abbreviations: CRP, C-reactive protein; SAP, serum amyloid P component.

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*Correspondence to: Peter D. Sun, Ph.D., 12441 Parklawn Drive, Rockville, MD 20852. E-mail: psun@niaid.nih.gov

Figure 1. (A) Pentraxin contacting residues on FcγRIIIa in the SAP-FcγRIIIa complex structure (PDB ID:3D5O); (B) The location of mutation sites on FcαRI structure. The IgA-binding sites on FcαRI are represented by surface drawing; and (C) Structure-based sequence alignment between FcαRI and FcγRIIIa. The secondary structure elements (arrow for β-strands and cylinder for helices) are indicated above the sequence. The IgA contacting residues in FcαRI are shaded in red, and the SAP contacting residues in FcγRIIIa are shaded in green, respectively. The mutation sites on FcαRI in (B) are highlighted in black boxes.

primarily involves the second Ig-like domain of FcγRs,²⁰ SAP interacts with both the first and second domains of FcγRIIIA. However, the pentraxin-binding site on FcγRs overlaps with the antibody binding region on the receptors.¹⁸ More recently, we identified FcαRI as another cell surface receptor for pentraxins.²¹ FcαRI (CD89) is the major receptor for IgA in humans and no murine counterpart is known.^{22,23} It is also a transmembrane protein with two extracellular domains and a short intracellular domain, which is associated with the FcRγ-chain. The structure of an FcαRI in complex with IgA revealed that one IgA molecule was bound to two receptor molecules.²⁴ FcαRI is distinct from other Ig-domain Fc receptors, such as FcεRI, FcγRII, and FcγRIII, in that the relative D1–D2 orientation of FcαRI is opposite to that of FcRγs and FcεRI. Unlike FcγRs and FcεRI that mainly use their receptor D2 domain for antibody binding, FcαRI exclusively uses its D1 domain to interact with IgA. However, the recognition of the pentraxins by FcαRI remains to be defined. Given the fact that FcγRs use topologically identical secondary structures on the receptor D1 and D2 domains to contact with two diagonally positioned subunits of pentraxins, a docking model of FcαRI–pentraxin interaction was generated.²¹ In the current work, we mutated residues in FcαRI

that lie at the contact region as predicted by the model and tested the binding of these mutants to CRP, SAP, and IgA. The results showed that the pentraxin recognition residues of FcαRI primarily reside in the receptor D1 domain in a region shared with the IgA-binding site. The combined mutational and binding studies identified critical FcαRI residues for pentraxin recognition and provided further information for the understanding of the physiological role for pentraxin-mediated FcαRI activation on neutrophils.

Results

Design of FcαRI mutations

Previously, we determined the crystal structure of SAP in complex with FcγRIIIA.¹⁸ FcγRIIIA binds the effector face along the diagonal of the SAP pentamer, interacting with the A and C subunits [Fig. 1(A)]. Both the A and C subunits of SAP use their ridge helix and C-terminus to contact topologically identical D1-and D2-domains of FcγRIIIA, respectively. Compared to FcγRIIIA, FcαRI also contains two Ig-like domains except the D1–D2 orientation is flipped by almost 180°,^{21,24} leaving the possibility that the receptor may bind to pentraxins in a similar way as FcγRIIIA. A model of CRP binding to

Figure 2. Characterization of Fc α RI wild type (WT) and mutant proteins. (A) SDS-PAGE analysis of the mutant Fc α RI receptors; (B) Concentration-dependent surface plasmon resonance (SPR) binding between MIP8a and Fc α RI wild type (WT) (black square), Y35A/L36A/Q38A (green triangle), R48A/E49A (magenta triangle), R82A (blue triangle), G103A/K104A (yellow triangle), H129A/I130A (red circle), and R178A (cyan diamond); (C) and (D) Representative sensorgrams for wild-type Fc α RI and mutants at a concentration of 50 nM binding to the receptor specific antibodies MIP7c (C) or MIP68b (D).

Fc α RI, generated based on the SAP-Fc γ RIIA cocrystal structure, suggests regions of the IgA receptor that may be involved in pentraxin interaction.²¹ To investigate the individual contributions of these structural elements and map the binding interface of Fc α RI–pentraxin recognition, we designed six alanine cluster or single mutations [Fig. 1(B,C)]. Three of them are located in the C-strand (Y35A/L36A/Q38A), C'-strand (R48A/E49A), and F-strand (R82A) of the D1 domain, respectively, to probe the Fc α RI region homologous to the SAP-binding site on the D1-domain of Fc γ RIIA. Two are located on the D2-domain of Fc α RI in BC-loop (H129A/I130A) and FG-loop (R178A), respectively, to probe the Fc α RI region homologous to the SAP-binding site on the D2-domain of Fc γ RIIA. G103A/K104A probes the contribution of the receptor hinge region to pentraxin binding.

Purification and characterization of mutant proteins

Mutations of Fc α RI were generated by site-directed mutagenesis and confirmed by DNA sequencing. The soluble receptors were expressed in *E. coli* and

refolded using procedures similar to those used for Fc γ RIIA.¹⁸ After refolding, mutant Fc α RI proteins were purified by nickel affinity and gel-filtration chromatography. All purified receptors showed a single band at approximately 25 kDa by SDS-PAGE [Fig. 2(A)]. To further characterize the structural integrity of Fc α RI mutants, we tested their binding to the receptor-specific monoclonal antibodies, MIP8a, MIP7c, and MIP68b. MIP8a and MIP7c are known to block IgA binding to Fc α RI.²⁵ All six mutant Fc α RI receptors exhibited similar binding to MIP68b and MIP7c [Fig. 2(C,D)], suggesting the soluble mutant receptors are properly folded.

IgA binding to mutant Fc α RI

The crystal structure of an IgA-Fc in complex with Fc α RI revealed the D1 domain of the receptor bound to the C_{H2}–C_{H3} interface of IgA heavy chain in a 2:1 stoichiometry.²⁴ The IgA-binding site on the D1-domain of Fc α RI involves Tyr 35 in the BC loop, the D strand (Arg 52–Lys 55), the DE loop (Phe 56 and Trp 57), and the FG loop (Arg 82–His 85). Among the six mutants we generated, Y35 and R82 are part

Figure 3. Surface plasmon resonance (SPR) analysis of the binding of FcαRI wild type (WT) and mutants to IgA, CRP, and SAP. (A) Representative sensorgrams of the interaction between WT or mutant FcαRI analytes and chip-immobilized IgA, CRP, or SAP were staggered in the order of a twofold serial dilution of analyte concentrations ranging from 6.8 to 0.4 μM and (B) The concentration-response units (RU) plot of the corresponding binding sensorgrams as shown in (A) between FcαRI wild type (WT) (black square), Y35A/L36A/Q38A (green triangle), R48A/E49A (magenta triangle), R82A (blue triangle), G103A/K104A (yellow triangle), H129A/I130A (red circle), and R178A (cyan diamond). The response units at the steady state were used in the plot.

Table I. *BIAcore Affinity between Mutant FcαRI and Pentraxins or IgA*

			Binding affinity K _D (μM)		
			CRP	SAP	IgA
FcγRI mutations			IgA contact		
Wild type			2.6 ± 0.4	2.5 ± 0.3	0.2 ± 0.2
D1-domain mutations					
C-strand	Y35A/L36A/Q38A	L257	4.4 ± 1.4	4.1 ± 1.0	7.2 ± 1.4
C'-strand	R48A/E49A		1.1 ± 0.3	0.9 ± 0.1	0.35 ± 0.3
F-strand	R82A	L256	6.2 ± 1.3	17.8 ± 3.2	23.6 ± 3.0
D2-domain mutations					
D1–D2 hinge	G103A/K104A		2.1 ± 0.8	2.8 ± 0.5	0.2 ± 0.2
BC-loop	H129A/I130A		2.8 ± 1.0	2.2 ± 0.6	0.4 ± 0.4
FG-loop	R178A		3.7 ± 1.1	3.1 ± 0.8	0.3 ± 0.2

of the IgA contacting residues of the receptor and mutations at these residues have been shown to disrupt immunoglobulin binding [Fig. 1(C)].^{26,27} To determine the binding capacity of FcαRI mutants to pentraxins and IgA, we carried out binding assays using surface plasmon resonance (SPR). To avoid the effects of different orientations on BIAcore chips, we immobilized pentraxins and IgA on CM5 chips and used wild type and mutant FcαRI receptors as the analytes. As expected, both Y35A/L36A/Q38A and R82A mutations affected IgA binding resulting in a ~30 – 100-fold lower affinity compared to the wild type [Fig. 3(A,B)]. In addition, reduced binding was also observed in two mutations for the IgA-blocking antibody MIP8a, indicating they might be part of the binding epitope of MIP8a [Fig. 2(B)]. In contrast, both IgA and MIP8a exhibited binding to the other four mutant receptors that was similar to binding to the wild-type receptor. This is consistent with the IgA–FcαRI complex structure, which shows that these regions are not in contact with IgA.

Pentraxin binding to mutant FcαRI receptors

Among the six Ala patch mutations, the C- and F-strand mutations, Y35A/L36A/Q38A and R82A, respectively, on the D1-domain of the receptor resulted in the most profound reductions in CRP and SAP binding (Fig 3, Table I). The R82A mutation, in particular, reduced the pentraxin binding affinities by three – eightfold. The nearly complete loss of binding of these two mutant FcαRI receptors to IgA suggests pentraxins and IgA share a binding site on FcαRI. Furthermore, the affinity rank of pentraxin binding to Y35A/L36A/Q38A and R82A mutant receptors is the same as that of IgA, suggesting similar energetic distributions for FcαRI binding to pentraxins and IgA. Although Arg 82 is part of the IgA-binding site, it is outside of the SAP-binding site on FcγR [Fig. 1(C)], illustrating a difference between pentraxin binding to the two Fc receptors. Another mutation, R48A/E49A, resides outside the observed IgA-binding site and did not affect IgA binding. R48A/E49A, however, exhibited twofold enhanced binding to both CRP and SAP

[Fig. 3(A,B)], illustrating a difference between the receptor binding to IgA and pentraxins. Three cluster mutations on the D2-domain of FcαRI were generated to probe the involvement of the D1–D2 hinge region (G103A/K104A), BC-loop (H129A/I130A), and FG-loop (R178A) in the receptor recognition of pentraxins. Both the BC- and FG-loops are part of the SAP binding interface of FcγRIIA, and FG-loop of Fcγ receptor, in particular, is critical for IgG binding.^{20,28,29} However, the G103A/K104A and H129A/I130A mutations of FcαRI did not affect pentraxin binding while a mild but reproducible reduction in pentraxin binding was observed in the R178A mutation (Fig. 3). Thus, although the FG-loop on the D2-domain of FcαRI is likely part of the receptor interface with the pentraxins, the overall effects of the three D2-domain mutations are quite marginal compared to the D1-domain mutations. The lack of mutational effects on pentraxin binding suggests that the FcαRI D2-domain BC-loop region is not part of the pentraxin-binding site. This is in contrast to FcγRIIA, where the BC-loop is in contact with SAP.¹⁸ Collectively, the mutational analysis showed that while both D1- and D2-domains of FcαRI are involved in pentraxin recognition, the critical pentraxin binding interface resides in the N-terminal D1 domain of the receptor that overlaps with the IgA-binding site.

Competition between IgA and CRP for the binding of FcαRI

The overlapping of the IgA and pentraxin-binding sites on the D1-domain of FcαRI predicts a competition between pentraxins and IgA for binding to the receptor. Indeed, a solution-based competition assay showed that soluble IgA inhibited FcαRI binding to both immobilized CRP and IgA in a concentration-dependent manner [Fig. 4(A)]. The shared CRP and IgA-binding site on FcαRI was further investigated by competition-binding assays using another IgA-blocking antibody (MIP7c) and the nonblocking antibody MIP68b.²⁵ MIP7c that recognizes the receptor D1 domain and blocks IgA binding also blocks the binding of CRP to FcαRI. In contrast, MIP68b

Figure 4. (A) Blocking of CRP or IgA binding to FcαRI by IgA. CRP or IgA was immobilized on the CM5 chip, and the analytes consisted of a mixture of FcαRI (2.2 μ M) with a twofold serial dilution of human IgA (2.3 μ M). (B) Blocking of CRP or IgA binding to FcαRI by MIP7c or MIP68b (2.3 μ M).

augmented the binding of the receptor to both IgA and CRP [Fig. 4(B)].

Discussion

Previous mutational analysis of CRP showed that FcαRI binds to the same effector face of pentraxins as Fcγ receptors.²¹ To further define the pentraxin-binding site on FcαRI, we generated six FcαRI cluster mutations based on modeling of the crystal structure of SAP in complex with FcγRIIA. Solution binding measurements between pentraxins or IgA and the mutated FcαRI receptors showed that the two IgA-binding site mutations, Y35A/L36A/Q38A and R82A, drastically reduced pentraxin (both CRP and SAP) binding to the receptor, demonstrating an overlap between the IgA and pentraxin-binding site on the receptor. There are, however, unique regions of the receptor, such as the C'-strand in the D1 domain, that affected pentraxin but not IgA binding as shown by the R48A/E49A mutations. The crystal structure of SAP and FcγRIIA complex shows both the D1- and D2-domains of FcγRIIA contribute

equally to SAP recognition. For this reason, we designed three mutants for each domain. Although the three D1-domain mutants affected pentraxin binding, to our surprise, the effects of the three D2-domain mutants on pentraxin binding, are much less with only a mild reduction in binding observed in the FG-loop mutation, R178A. The D2-domain FG-loop on Fcγ receptors contributes critically to IgG recognition and is part of the SAP-binding epitope.^{18,20} The observed partial loss in CRP and SAP binding to R178A mutation shows that while the FG-loop of FcαRI is involved in pentraxin recognition, the hot-spot for pentraxin binding appears to be located in the IgA-binding site on the D1 domain. It is possible that the critical binding sites on the D2-domain of FcαRI were missed due to the model inaccuracy or our limited mutational analysis. Nevertheless, the mutational results suggest that residues on the D2-domain of FcαRI contributed little to pentraxin binding. Overall, the preferential pentraxin binding to the D1-domain of FcαRI but D2-domain of FcγRIIA shows that pentraxin

Figure 5. Schematic representation of the interaction between SAP- Fc γ RIIa, IgG1 Fc-Fc γ RIII or IgG1 Fc-Fc γ RIIa, CRP-Fc α RI, and IgA Fc-Fc α RI. The interfaces are highlighted in shaded red circles.

recognition occurs primarily at the site of immunoglobulin binding irrespective of the topological location of the immunoglobulin-binding site or the Ig specificity of the receptor (Fig. 5). These shared binding sites with antibodies result in competition between pentraxins and IgG or IgA binding to their respective Fc receptors.

Neutrophils are early responders of microbial infections. They are often among the first cell types recruited to the site of inflammation. Likewise, the expression of the acute phase pentraxin, CRP, is upregulated systemically in early stage of infections. In addition to Fc γ receptors, human neutrophils also express abundant Fc α RI. The ability of CRP to bind and opsonize bacterial pathogens and its ability to activate both Fc α RI and Fc γ Rs suggest a potential acute phase pentraxin-mediated neutrophil activation through synergistic engagement of Fc α RI and Fc γ Rs by pentraxin-opsonized microbial pathogens.

Materials and Methods

Reagents

CRP was purified by affinity and ion-exchange chromatography from human pleural fluid as described previously.³⁰ Purity was determined by SDS-PAGE gels stained with SYPRO Ruby protein gel stain (Invitrogen).

SAP was isolated from human pleural fluids, as previously described.¹⁸ Mouse anti-CD89 mAb were purchased from Serotec (clone MIP8a), BD Biosciences (clone A59), and Thermo Scientific (clone MIP7c and MIP68b). Human IgA and F(ab')₂ goat anti-IgA were purchased from Jackson ImmunoResearch.

Site-directed mutagenesis and purification of Fc α RI proteins

The recombinant Fc α RI with mutated regions were generated by site-directed mutagenesis. Briefly, the wild-type ectodomains of Fc α RI (1–208) were cloned into a pET30a vector with a His₆-tag at the C terminus. Six surface-exposed regions of Fc α RI, three on D1 and two on D2 domains, and one at the D1-D2 hinge, were replaced with alanine residues individually using a QuikChange II site-directed mutagenesis kit (Agilent) according to the manufacturer's instruction. The three D1-domain mutations are on the C-strand (Y35A/L36A/Q38A), C'-strand (R48A/E49A), and F-strand (R82A). The hinge mutation is G103A/K104A, and the two D2-domain mutations are H129A/I130A on the BC-loop and R178A on the FG loop. All mutations were confirmed by DNA sequencing (ACGT, Wheeling, IL). Both wild type and the mutated Fc α RI receptors were expressed and purified as previously reported.³¹

Surface plasmon resonance

SPR measurements were performed using a BIAcore 3000 instrument and analyzed with BIAevaluation 4.1 software (Biacore AB). To measure the binding affinity to Fc α RI and its mutants, CRP and SAP were immobilized on carboxylated dextran CM5 chips (Biacore AB) using primary amine-coupling and 10 μ L/mL flow rate in 10 mM HEPES (pH 6.8), according to the protocol from the manufacturer. In addition, human IgA and anti-CD89 antibodies, clone MIP8a, MIP7c, or MIP68b were coupled to CM5 chips. Binding analyses were performed using serial dilutions of wild type and mutant receptors from 6.8 to 0.4 μ M in a buffer containing 10 mM HEPES (pH 7.4), 0.15M NaCl, 1.0 mM CaCl₂ at a flow rate of 50 μ L/min. To measure the competition between human IgA and CRP on Fc α RI binding, CRP or IgA was immobilized onto a CM5 sensorchip with analytes consisting of 2.2 μ M of Fc α RI with various concentrations of human IgA ranging from 0 to 2.3 μ M in the buffer described above. The dissociation constants were obtained by either steady state or kinetic curve fittings using BIAevaluation (Biacore, NJ).

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