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Analysis of the linear epitope for Fc-binding on the mouse IgG Fc receptor (moFcγRI) by synthetic peptide

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ABSTRACT. To identify the linear epitope for Fc-binding to the mouse immunoglobulin G (IgG) Fc receptor (moFcγRI), peptides derived from the membrane-distal extracellular domain (EC2) of moFcγRI, corresponding to the homologous region of human FcγRI (huFcγRI) and huFcγRII, were synthesized. Using a dot-blot assay, six peptides were tested. The results showed that the moRI3 peptide (CVFYRNGKSFQFS) could combine with mouse IgG efficiently. A competitive enzyme-linked immunosorbent assay (ELISA) showed that the IC_{50} value of the moRI3 peptide was 38.03 μM. The moRI3 peptide could inhibit the combination of mouse IgG to the transfected COS 7 cells significantly with an IC_{50} value of 72.68 μM. The IgG-binding region of moFcγRI was also localized in the C'-E loop of the EC2 domain as predicted according to huFcγRI and huFcγRII. We predicted that the minimum effective IgG-binding region of moFcγRI may be the peptide ^{153}SFQFSS^{158}. The linear epitope for immunoglobulin-binding to mouse FcγR is also described. Thus, we generated a peptide that

targets a fundamental aspect of ligand recognition by this receptor class.

Key words: Fc-binding linear epitope; IgG; FcγRI; Synthetic peptide

INTRODUCTION

Fcγ receptors (FcγRs), the receptors for the Fc portion of immunoglobulin G (IgG), are essential for antibody-dependent immune responses (Ravetch and Kinet, 1991). There are two types of Fc receptors (FcRs), including activating receptors and inhibitory receptors, which transmit their signals via immunoreceptor tyrosine-based activation motifs and immunoreceptor tyrosine-based inhibitory motifs, respectively (Ravetch and Lanier, 2000). Mouse FcγRI (moFcγRI) is a key receptor involved in the development of immune responses (Gavin et al., 1998; Barnes et al., 2002; Ioan-Facsinay et al., 2002) and links innate and adaptive immunities via its dual roles as a low-affinity receptor for the T-independent IgG3 and as a high-affinity receptor for the T-dependent (Th1) IgG2a (Gavin et al., 1998; Barnes et al., 2002).

Linking the humoral and cellular responses, the mouse high-affinity receptor for IgG, FcγRI (CD64), is expressed on monocytes and macrophages and induced by interferon-γ on neutrophils (Sivo et al., 1993). Although functions mediated by moFcγRI are less well-characterized, cross-linking of human FcγRI (huFcγRI) on myeloid cells leads to events such as tyrosine phosphorylation, Ca^{2+} flux, superoxide generation, inflammatory mediator release, antibody-dependent cellular cytotoxicity, and internalization of small immune complexes (Scholl et al., 1992; Harrison et al., 1994) and have been intensively studied. Many of these sequelae may also be true in mice. Mouse FcγRI is structurally homologous to huFcγRI and exhibits high-affinity binding of monomeric IgG (Scholl et al., 1992).

Protein engineering approaches suggest that interaction sites for cellular FcγRs are localized to the hinge proximal region of the C_H2 domain (Sarmay et al., 1992; Shields et al., 2001). Crystal structures have been reported for extracellular portions of FcγRIIA (Maxwell et al., 1999), FcγRIIB (Sondermann et al., 1999), FcγRIIIB (Sondermann et al., 2000), FcεRI (Takai et al., 1994), and FcαRI (Morton et al., 1999). Peptides derived from human IgG have exhibited the capacity to interact with FcRs (Medgyesi et al., 2004; Uray et al., 2004). With respect to FcRs, ligand-binding regions in the receptors have been proposed based on structural and molecular analyses (Woof and Burton, 2004).

FcRs are important in the immune response; therefore, resolving the molecular interaction involved in their ligand binding may open up possibilities for modulating FcR-mediated mechanisms. In this study, we analyzed novel peptides designed by aligning the EC2 domain protein sequences of moFcγRI, huFcγRI, and huFcγRIIA, and identified a consensus peptide sequence that binds FcγRI at a related site to IgG. Such an approach may be relevant for potential therapeutic interventions in inflammatory and autoimmune processes.

MATERIAL AND METHODS

Peptide design and synthesis

Based on the crystal structure of human FcγRIIa, we analyzed the EC2 domain protein sequences of moFcγRI, huFcγRI, and huFcγRIIA (NCBI Protein Accession Nos. EDL38886,

NP_000557 and NP_067674) with DNASIS 3.0 (MiraiBio, South San Francisco, CA, USA). Six peptides were designed corresponding to the sequence 117-192 of moFcγRI (Figure 1). The peptides were synthesized using a Symphony Multiplex Peptide Synthesizer (Protein Technologies, Inc., Tuscon, AZ, USA). Peptide purities were greater than 90% as assessed by high-performance liquid chromatography (HPLC) and mass spectrometry.

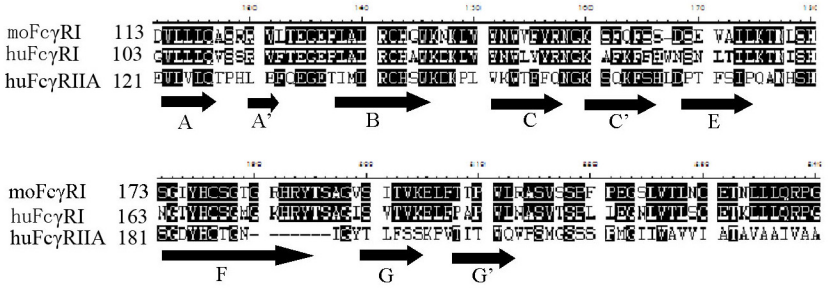

Figure 1. Alignment of the protein sequences of moFcγRI, huFcγRI and huFcγRIIA (NCBI protein accession Nos. EDL38886, NP_000557 and NP_067674). The black highlights indicate that two or three sequences are identical, and dotted lines represent deletions of amino acids.

Dot-blot assay

According to the method of Zhang et al. (2006), a dot-blot assay was developed to test the binding of mouse IgG to different moFcγRI peptides. The bovine serum albumin (BSA)-coupled peptides were blotted onto nitrocellulose membranes (Millipore, Billerica, MA, USA) at different concentrations and the soluble moFcγRI protein (smoFcp) and carrier protein BSA were used as positive and negative controls, respectively. After air drying, the membrane was blocked with 0.2% gelatin in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 (PBST) at 37°C for 1 h, and then incubated with 10 μg/mL horseradish peroxidase (HRP)-conjugated mouse IgG (HRP-IgG) in PBST containing 0.2% gelatin at 37°C for 1 h followed by thorough washing with PBST. Color was developed by using a 3-amino-9-ethylcarbazole staining kit (Zhongshan Goldenbridge, Beijing, China) according to manufacturer instructions.

Competitive enzyme-linked immunosorbent assay (ELISA)

The inhibitory capacity of the moRI3 peptide was measured using the competitive ELISA (Zhang et al., 2006). High-binding ELISA plates were coated with the recombinant moFcγRI protein at 10 μg/mL at 4°C overnight. Next, the plates were blocked with 0.2% gelatin at room temperature for 2 h. In the competition assay, 50 nM HRP-IgG was pre-incubated with various concentrations (5-640 μM) of moRI3 peptide at 4°C for 2 h. The mixed samples were then transferred to the plates for IgG binding with an incubation of 1 h at 37°C. 3,30,5,50-Tetramethylbenzidine (Sigma, St. Louis, MO, USA) was used as the chromogen for the color development. The reaction was stopped by the addition of 1 M sulfuric acid, and the absorbance values of the samples were measured at 450 nm. The moRI2 peptide and BSA were used as negative controls, and all experiments were carried out in triplicate. Binding of mouse IgG to the coated recombinant moFcγRI protein without the inhibiting peptide (B_0) was considered to be 100% binding. For each peptide

concentration, the inhibition percentage (Ic%) was calculated as Ic% = (Bn - Bi) / B0 x 100%, where *Bi* and *Bn* are the IgG binding of the inhibiting and negative peptides, respectively, at corresponding peptide concentrations. The amount of added peptide required to inhibit IgG binding by 50% (IC_{50}) was calculated based on peptide concentration.

Flow cytometry analysis

Cells were harvested using 1 mM EDTA, washed, and resuspended in cold serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) containing 0.1% sodium azide. Fifty microliter diluted aggregates were added to cells, mixed, and incubated at 4°C for 2 h. Cells were washed twice with cold PBS containing 0.1% sodium azide and transferred to 500 μL cold sheath fluid per sample. For the inhibition experiments, fluorescein isothiocyanate (FITC)-labeled IgG aggregates were pre-incubated, respectively, with various concentrations of moRI3 peptide (5-640 μM) or control at 4°C for 2 h. All experiments were carried out in triplicate. Fluorescent intensity of the samples (counting 10,000 cells per sample) was measured on a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ, USA).

RESULTS AND DISCUSSION

Depending on the alignment of the EC2 domain protein sequences of moFcγRI, huFcγRI, and huFcγRIIA, six peptides (moRI1-moRI6) were designed covering the loops of A-B, B-C, C-C', C'-F, F, and F-G, respectively (Table 1). Binding of mouse IgG to the six peptides was tested using a dot-blot assay. The results showed that HRP-IgG only bound to the third peptide, moRI3 (^{146}(C)VFYRNGKSFQFS^{156}). Different moRI3 concentrations were tested for binding to mouse IgG (Figure 2).

Table 1. Details of six peptides covering the loops of A-B, B-C, C-C', C'-F, F, and F-G.

Code	Sequence[1]	Length (aa)	Mass (Da)	p*I*	IgG binding[2]	Predicted position
moRI1	^{117}(C)QASRRVLTEGEPLALR^{133}	17	1899.10	9.54	-	A-B loop
moRI2	^{134}CHGWKNKLVYNV^{145}	12	1460.67	9.98	-	B-C loop
moRI3	^{146}(C)VFYRNGKSFQFS^{156}	13	1582.74	10.14	+	C'-E loop
moRI4	^{157}(C)SSDSEVAILK^{166}	11	1151.28	4.11	-	E region
moRI5	^{167}(C)TNLSHSGIYH^{176}	11	1281.80	7.81	-	E-F loop
moRI6	^{177}CSGTGRHRYTSAGVSI^{192}	16	1651.74	10.44	-	F-G loop

[1]The Cys residue added to the N-terminus of the peptides for conjugation is listed in parentheses. [2]The binding of mouse IgG to the BSA-conjugated peptide was tested by the dot-blot assay. "+" represents specific binding of mouse IgG bound to the peptide, and "-" means that binding was not detected.

The inhibitory effect of moRI3 on mouse IgG binding to moFcγRI was evaluated by competitive ELISA. The moRI3 peptide inhibited the combination of mouse IgG to soluble moFcγRI coated on the plate (Figure 3). The IC_{50} value of the moRI3 peptide was 38.02 μM after non-specific inhibition of the control peptide was subtracted.

Inhibition by the moRI3 peptide was determined by calculating the percentages of cells showing fluorescence. Figure 4 shows that the peptide moRI3 inhibited the binding of mouse IgG on the transfected COS 7 cells. The IC_{50} value of the moRI3 peptide was 72.68 μM. Because there was no inhibition effect at each concentration, the moRI2 peptide and BSA were used as negative controls.

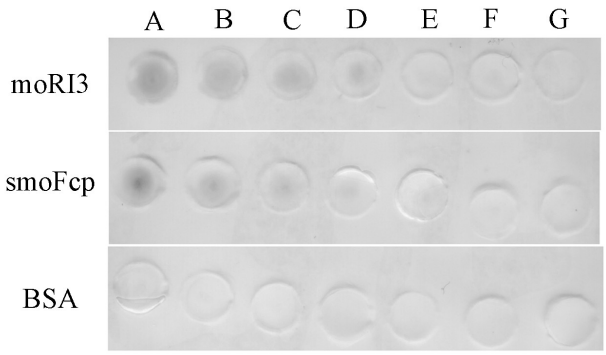

Figure 2. Binding of mouse IgG to the BSA, moRI2 and moRI3 peptides. The BSA-coupled peptides were blotted onto nitrocellulose membranes at different contents and the soluble moFcγRI protein (smoFcp) and carrier protein BSA were used as positive and negative controls, respectively. A-G represents the different contents (20, 10, 5, 2.5, 1.25, 0.625, and 0 μg, respectively) of BSA, smoFcp and moR3 peptides.

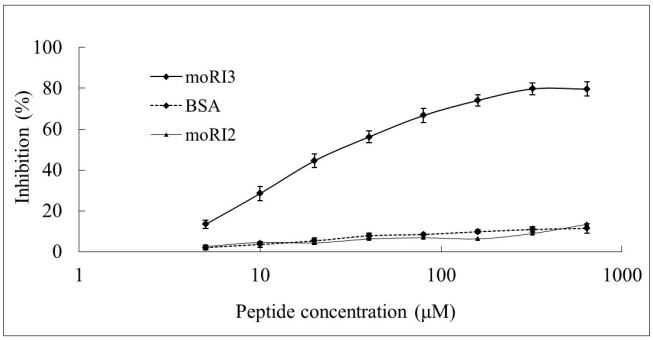

Figure 3. Inhibition of IgG binding to soluble moFcγRI. HRP-IgG was pre-incubated, respectively, with the peptide moRI3 at different concentrations (5-640 μM) at 4°C for 2 h using control peptide (moRI2) and BSA as negative control.

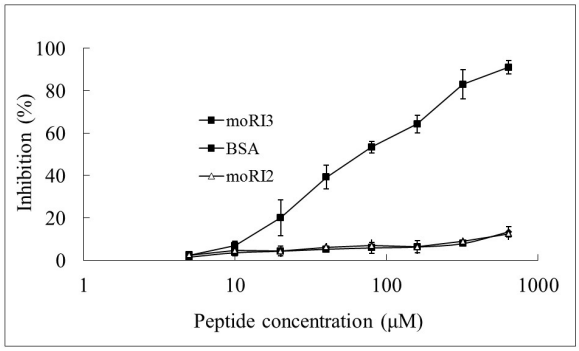

Figure 4. Inhibition of IgG/receptor interaction by the moRI3 peptide. FITC-labeled IgG aggregates were pre-incubated with various concentrations of the moRI3 peptide (5-640 μM) or control at 4°C for 2 h, and then applied to the transfected COS 7 cells. Fluorescent spectra were analyzed by flow cytometry (BD, FACSCalibur) counting 10,000 cells per sample.

In this study, we developed a linear epitope for Ig-binding on mouse FcγR. The moRI3 peptide (^{146}(C)VFYRNGKSFQFS^{156}) possesses binding capability to mouse IgG. According to sequence alignment, we found that moRI3 is located in the C'-E loop of the EC2 domain. The extracellular regions of FcγRs share the same overall heart-shaped structure in which the two domains are positioned at an acute angle to each other and share a large interface. Each of the EC1 and EC2 domains of all the receptors are arranged into an identical overall Ig-fold composed of β-strands labeled as AA'BCC'EFG from the amino-terminus (Zhang et al., 2006). The overall fold of two IgG-like domains in a sharply bent structure is shared by FcγRIII, FcγRIIa/b, and FcεRIα, as observed in the crystal structures (Sondermann et al., 2000). The binding region of the FcR to the Fc fragments has been identified in the FcγRIII/hFc1 and FcεRIα/IgE-Fc-fragment complex structures. It consists mainly of rather flexible loops that rearrange upon complex formation. The B/C, C'/E, and F/G loops, as well as the C' strand, carry the contact residues in the FcγRIII/hFc1 structure and contain conserved amino acid residues that share common binding principles as well as sequence variations for the generation of specificity (Sondermann et al., 2000). The structure of FcγRIIa was determined previously and shows the same overall fold as described for FcγRIII. The reported structure originates from crystals of space group $P2_12_12$, and the molecules form a dimer about one of the crystallographic dyads. This arrangement results in a dominant crystal contact, indicating that a dimer of FcγRIIa recognizes IgG. In this dimer, the molecules contact each other via the CC'FG sheets and the C/C' loop of the C-terminal domain. Our results showed that the IgG-binding region of moFcγRI was also localized in the C'-E loop of the EC2 domain. This is in agreement with the structures of huFcγRI and huFcγRII.

To effectively examine the IgG-binding region, the peptides were first designed to be longer. According to the minimum effective IgG-binding region of huFcγRI and huFcγRIIA (C'-E), we predicted that the effective IgG-binding region of moFcγRI was likely the peptide ^{153}SFQFSS^{158}. This will be confirmed in future studies. Thus, we generated a peptide that targets a fundamental aspect of ligand recognition by this receptor class. We hypothesize that a peptide derived from the Fc-binding site of the receptor would be a better candidate for the downregulation of FcR-initiated inflammatory responses.

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