Molecular Characteristics of IgA and IgM Fc binding to the Fcα/μR

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Abstract

Fca/m receptor (Fca/mR), a novel Fc receptor for IgA and IgM, is a type I transmembrane protein with an immunoglobulin (Ig)-like domain in the extracellular portion. Although IgA and IgM bind to Fca/mR, the molecular and structural characteristics of the ligand-receptor interactions have been undetermined. Here, we developed twelve monoclonal antibodies (mAbs) against mouse Fca/mR by immunizing mice deficient in Fca/mR gene. Eight mAbs totally or partially blocked IgA and IgM bindings to Fca/mR. These blocking mAbs bound to a peptide derived from the Ig-like domain of mouse Fca/mR, which is conserved not only in human and rat Fca/mR but also in polymeric Ig receptor (poly-IgR), another Fc receptor for IgA and IgM. These results suggest that IgA and IgM bind to an epitope in the conserved amino acids in the Ig-like domain of Fca/mR as well as poly-IgR.

Keywords; Fca/mR, Fc receptor, IgM, IgA, polymeric Immunoglobulin receptor, Immunoglobulin-like domain

Introduction

The receptors for Fc portions of immunoglobulins (FcR) mediates various immune responses upon binding to antibodies or immune complexes [1, 2]. Mice deficient in FcR genes for IgG (*FcgRI, FcgRII, FcgRIII*) and IgE (*FceRI, FceRII*) have revealed their physiological roles in immune responses in vitro and in vivo [3, 4]. Although functional Fc receptors for IgM had been reported on subpopulations of human and rodent T, B and NK cells [5] [6] [7] [8] [9] [10], a gene encoding an Fcμ receptor had not previously been identified. We finally cloned a novel Fc receptor for IgM, which was found to be also an Fc receptor for IgA, designated Fca/m receptor (Fca/mR) [11, 12]. The Fca/mR is a type I transmembrane protein with an immunoglobulin (Ig)-like domain in the extracellular portion, and is expressed on B cells and macrophages [11, 12]. The Fca/mR is an only IgM receptor identified on human and murine hematopoietic cells to date, and is thought to play a central role for immune responses mediated by IgM and IgA [13].

The Fca/mR genes were mapped to syntenic regions of mouse chromosome

1 (1F) and human chromosome 1 (1q32.3), near several other Fc receptors, including Fcγ receptors I, II, and III, Fcε receptor, and the polymeric Ig receptor (poly-IgR) [11]. The poly-IgR gene, another Fc receptor for IgM and IgA expressed on mucosal epithelial cells [14], is only 12 kbp apart from the Fca/mR in the Fc receptor gene cluster on chromosome 1 [15], suggesting that these two receptors seem to be closely related in their phylogenies. The polyIgR contains five Ig-like domains in the extracellular portion. We found a motif in the immunoglobulin (Ig)-like domain of human and mouse Fca/mR that is conserved in the first Ig-like domain of human, mouse and rat poly-IgR [11]. Although this region is important for IgM and IgA binding to the poly-IgR [14], the molecular and structural characteristics of IgA and IgM binding to Fca/mR has been unclear.

We have recently established twelve monoclonal antibodies (mAbs) against mouse Fca/mR (mFca/mR), some of which efficiently block the IgM and IgA bindings to Fca/mR. By using these mAbs, we analyzed the molecular characteristics of the ligand binding by Fca/mR.

Materials and Methods

Cells, mice, antibodies and peptides

Ba/F3 and BW5147 is mouse pro-B and thymoma cell lines, respectively. The Ba/F3 and BW5147 transfectants stably expressing mFca/mR were established, as described [11]. Mice deficient in Fca/mR gene were established by using gene targeting strategy of ES cells (manuscript for preparation). Fluorescein isothiocyanate (FITC)-conjugated streptavidin, mouse IgM and IgA were purchased from PharMingen (San Diego, CA). The peptides generated based on a motif in the Ig-like domain of mFca/mR (VTIHCHYAPSSVNRHQRKYW)[11] and on chicken egg ovalbumin (OVA) (ISQAVHAAHAEINEAGR) were purchased from Peptide Institute, Inc. (Osaka, Japan)

Establishment of monoclonal antibodies against mFca/mR

1 x 10^7 Ba/F3 transfectant expressing mFca/mR emulsified with complete Freund adjuvant (Sigma, St. Louis, MO) were injcetd into both foot pads of an Fca/mR-deficient mouse on day 0 and 7. On day 10, draining lymph node cells were

harvested and fused with Sp2/0 myeloma cells. Hybridomas were grown on methyl cellurose plate (ClonaCell-HY^{R}, Stem Cell Technologies, Seattle, WA), according to the manufacture's instruction.

Competitive binding analyses

1 x 10^{6} BW5147 transfectants expressing mFca/mR were incubated with a variable dose of each anti-mFca/mR mAb generated for 30 min at 4^{0}C. To compare recognition sites by each anti-mFca/mR mAb generated one another, the transfectant were then washed with PBS and incubated with 0.05 ug/ml of a biotin-conjugated anti-mFca/mR mAb, followed with FITC-conjugated streptavidin. To examine blocking ability for IgM and IgA bindings to the mFca/mR, 1 x 10^{6} BW5147 transfectants expressing mFca/mR were incubated with 0.5 ug/ml of each purified anti-mFca/mR mAb for 30 min at 4^{0}C, washed with PBS twice and then stained with FITC-conjugated mouse IgM or IgA. To examine binding ability of anti-Fca/mR mAbs to the motif peptide conserved in Fca/mR and poly-IgR, 0.05 ug of each biotin-conjugated anti-mFca/mR mAb was incubated with a variable dose of the motif

peptide for 1 hour at 4^0C. Then 1 x 10^6 BW5147 transfectants expressing mFca/mR were added into the mAbs-peptide mixture and incubated for 30 min at 4^0C, followed by FITC-conjugated streptavidin. The transfectant was analyzed by FACS Calibur^R (BD, San Diego, CA).

Results and Discussion

Establishment of mAbs against the mFca/mR

Although we had been trying to generate mAbs against mFca/mR by immunizing rats with the Ba/F3 transfectant expressing mFca/mR several times, we could generate only one anti-mFca/mR mAb (TX6) [11]. Furthermore, we could never generated anti-human Fca/mR (hFca/mR) mAb by immunizing mice with the hFca/mR antigens. Because amino acid sequences of extracellular domain of Fca/mR are highly conserved among human, mouse and rat (80.2% identity between mouse and rat and 51.3% between mouse and human) [11], we considered that the immunological tolerance against Fca/mR antigens may be a cause of difficulty for establishing anti-human and mouse Fca/mR mAbs. In order to avoid immunological tolerance against mFca/mR antigens, we immunized a mouse deficient in *Fca/mR* gene, instead of rat or other species of animals, for generation of anti-mFca/mR with the mFca/mR-expressing Ba/F3 transfectant and successfully generated twelve anti-mFca/mR mAbs, designated TX57 to TX68 (**Table 1**).

Characterization of recognition sites by the anti-mFca/mR mAbs

To characterize recognition sites by the anti-Fca/mR mAbs, we performed competitive binding analysis, by using the BW5147 transfectant expressing mFca/mR. The BW5147 transfectant was incubated with either mAb out of 12 mAbs generated and then stained with either biotinylated mAb, followed with FITC-conjugated streptavidin. Analyses by flow cytometry demonstrated that any of mAbs, except TX57, did not interfere in the binding of TX57 to the transfectant **(Fig 1A**). Similarly, TX64 binding to the transfectant was not inhibited by any of mAbs other than TX64 itself (**Fig 1B**). These results suggest that TX57 and TX64 mAbs recognize epitopes different from those recognized by the other mAbs. In contrast, TX58, TX59, TX60, TX61 and TX66 mAbs and TX67 and TX68 mAbs, but not the other mAbs, completely or partially inhibited the binding of these mAbs one another to the transfectant, respectively (**Fig 1C, D and Table 2**). Similarly, TX62, TX63 and TX65 mAbs, but not the other mAbs, interfered in the binding of these mAbs one another to the transfectant (**Fig 1E and Table 2**). It was of interest that TX58, TX59, TX60, TX61 and TX66 mAbs, but not

the others, cross-reacted with hFca/mR (**Table 1 and data not shown**), suggesting that these mAbs recognize an epitope conserved also in human Fca/mR. Based on these results, we divided the mAbs into five groups that differed from each other in their recognition sites (**Table 1**).

Blocking of ligand binding to the Fca/mR by the anti-Fca/mR mAbs

Next, we examined whether IgM and IgA bindings to the mFca/mR could be blocked by the anti-mFca/mR mAbs. The BW5147 transfectant expressing the mFca/mR was incubated with either anti-mFca/mR mAb and then further incubated with FITC-conjugated IgM or IgA. As demonstrated in **Fig 2 and Table 1**, analyses by flow cytometry demonstrated that TX57 mAb in the group I totally inhibited the binding of both IgM and IgA to the transfectant. All the mAbs in the groups III and IV (TX58, 59, 60, 61, 66, 67 and 68) partially inhibited the ligand binding. In contrast, the mAbs in the groups II and V did not affect IgM and IgA bindings to the transfectant. These results suggested that the epitopes in the Fca/mR recognized by the mAbs in the groups I, III and IV are either the IgA and IgM binding site of the Fca/mR or physically

related to the ligand binding site.

Binding to a peptide conserved in both Fca/mR and poly-IgR by the anti-mFca/mR mAbs

We previously found a motif in the Ig-like domain of human and mouse Fca/mR that is conserved in the first Ig-like domain of human, mouse and rat poly-IgR (**Fig 3A**[11]). Previous reports suggested that this motif is important for IgM and IgA bindings to the poly-IgR [16, 17]. Thus, this region is predicted to be also important for IgA and IgM bindings to the Fca/mR. To evaluate this possibility, we synthesized the peptides corresponding to this motif sequence (motif peptide) (**Fig 3A**) and to the chicken egg ovalbumin (OVA) as a control for competitive binding analyses. Each anti-mFca/mR mAb was preincubated with the motif or control peptides and then used for staining of the BW5147 transfectant expressing the Fca/mR. As demonstrated in **Fig 3B and Table 1**, pre-incubation of the mAbs in the groups I, III and IV with the motif peptide significantly decreased the antibody binding to the transfectant. In contrast, the binding of any of the mAbs in the groups II and V to the transfectant were

not affected by pre-incubation with the motif peptide. These results suggested that the mAbs in the groups I, III and IV recognize epitopes in the motif peptide in the Ig-like domain of Fca/mR.

Although the mAbs in the groups I, III and IV block the IgA and IgM bindings to mFca/mR as a result of competitive binding to the motif peptide which is conserved also in human Fca/mR, only the mAbs in group III cross-reacted to human Fca/mR (Table 1). There is one amino acid difference between the motif peptide sequences of mouse and human Fca/mR (**Fig 3A**), which might be involved in epitopes recognized by the mAbs in groups I and IV. It should be noted that, while the motif peptide is also highly conserved in mouse, human and rat poly-IgR (**Fig 3A**), no mAbs in the group I to V cross-reacted to the mouse poly-IgR (data not shown).

In conclusion, by using mice deficient in *Fca/mR* gene, we have successfully developed anti-mFca/mR mAbs recognizing epitopes within the highly conserved peptide in mouse, human and rat Fca/mR. These mAbs efficiently block the IgA and IgM bindings to mFca/mR, suggesting the presence of IgA and IgM binding site in the peptide sequence of Fca/mR as well as poly-IgR. These mAbs should be helpful for

molecular and functional characterization of IgA and IgM interaction with Fca/mR.

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Figure legends

Figure 1. Competitive binding analyses between anti-mFca/mR mAbs

BW5147 transfectant expressing mFca/mR was pre-incubated with each mAb and then stained with biotinylated mAb indicated, followed with FITC-conjugated streptavidine and analyzed by flow cytometry. Data are shown in relative mean fluorescence intensity (MFI) of transfectant stained with biotynyated antibodies after preincubation with each mAb, as compared with that stained with biotynyated antibodies alone. Data are representative from more than three independent experiments.

Figure 2. Competitive binding analyses between anti-mFca/mR mAbs and IgA or IgM

BW5147 transfectant expressing mFca/mR was pre-incubated with each mAb indicated (bold lines) or control Ig (dotted lines) and then stained with FITC-conjugated mouse IgA or IgM and analyzed by flow cytometry. Shaded histograms indicate autofluorescence of the transfectant. Data are representative from more than three

independent experiments.

Figure 3. Competitive binding analyses between mFca/mR mAbs and a peptide from the conserved amino acids sequency

(A) The conserved amino acid sequences in the Ig-like domain of Fca/mR and in the first Ig-like domain of poly-IgR are shown in each species indicated. The peptide in the box was used for the competitive binding analyses for (B).

(B) Biotin-conjugated anti-mFca/mR mAb indicated was incubated with the peptide derived from the conserved amino acid sequences, as shown in box (A), or OVA peptide at variable concentrations indicated. BW5147 transfectant expressing mFca/mR was stained with the antibody-peptide mixtures, followed by FITC-conjugated streptavidin and analyzed by flow cytometry. Data are shown in relative MFI of the transfectant stained with antibody-motif peptide mixtures, as compared with that stained with antibody-control peptide (OVA) mixtures. Data are representative from more than three independent experiments.