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Epitope Mapping of Fc gamma RIIa Monoclonal Antibodies

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Abstract

FcγRIIa (CD32) is an IgG receptor which has been shown to be important in autoimmune disease pathology. IV.3, 8.7, and 7.30 are anti-FcγRIIa monoclonal antibodies (mAbs), which block the interaction between FcγRIIa and complex IgG. In this study, the three mAbs were demonstrated to inhibit FcγRIIa function. The determination of the precise epitopes of the IV.3, 8.7, and 7.30 mAbs may become a potential approach for designing inhibitors for FcγRIIa. The epitope of IV.3, 8.7, and 7.30 were determined using chimeric receptors based on the extracellular domains of FcγRIIa and the FcεRI a chain. The epitopes for IV.3 was found to be mapped on amino acid residues 132-137, while 8.7 and 7.30 were on amino acid residues 112-119 and 157-162. Based on the crystal 3D model of FcγRIIa molecule, these amino acid sequences are clustered together forming a contiguous region within the ligand binding site of the receptor.

Key words: epitope mapping, monoclonal antibody, IgG receptor, FcγRIIa

Introduction

FcγRIIa is a low affinity receptor for monomeric IgG which only binds avidly to complexed IgG (Hogarth, 2002). FcγRIIa contains two extracellular Ig-like domains, a transmembrane domain, and a cytoplasmic domain (Powell, 2008; Hogarth, 2002). The ligand binding site of FcgRIIa has been revealed to be located within the second extracellular domain, away from the cell surface enabling engagement of the ligand; antibody Fc region (Powell, 2008; Hogarth, 2002). Monoclonal antibodies against FγcRIIa have been extensively used in the classification and functional analysis of this receptor (Hogarth, 1992; Ierino, 1993), and IV.3, 8.7, and 7.30 monoclonal antibodies (mAbs) have contributed in the understanding the role of FcγRIIa in several immunological responses during physiologic or pathologic conditions. These responses are including phagocytosis, antibody-dependent cell mediated-cytotoxicity (ADCC), superoxide generation, lysosomal enzyme release and TNF-a secretion (Ierino, 1993; McKenzie, 1999). As FcγRIIa plays an important role in the development and pathogenesis of antibody-mediated autoimmune diseases, specific blocking of FcγRIIa function is a potential strategy for a treatment for these diseases.

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Materials and Methods

Monoclonal antibodies and IgG reagents

The anti-FcγRIIa monoclonal antibodies (mAbs) 8.7 (mouse IgG1) and 7.30 (mouse IgG1) were made by Ierino *et al* (Ierino, 1993). IV.3 (mouse IgG2b) was produced from a hybridoma cell line obtained from the American Type Culture Collection (Looney, 1986). MAbs were purified using a protein A affinity chromatography (Ierino, 1993). To prevent non specific binding of the Fc portion of antibody to FcγRs, the Fab and $F(ab)'_2$ fragments were generated by an enzyme digestion using pepsin (Boehringer Mannheim) at an enzyme:substrate ratio of 1:10 to 1:50 at 37°C for 1 hour in 0.1M sodium citrate pH 3.5. The antibody cleavage was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of each samples of different ratios, and appropriate digested antibody was pooled for purification. The Fc fragments were removed from the samples by adsorption to protein A and purified $F(ab)'_2$ fragments (8.7 and 7.30) and Fab fragments (IV.3) were confirmed to be free from whole IgG and Fc fragments by SDS-PAGE analysis.

Chimeric FcγRIIa/FceRI receptors cDNAs and expression

The two extra cellular Ig-like domains of FcγRIIa and FcεRI are structurally related, sharing 38% amino acid identity (Hulett, 1994). However FcγRIIa and FcεRI are functionally different and it is also known that there is no cross-reaction of the mAbs in the binding to the two receptors. This made the two receptors ideal to use for generation of the chimeras to identify the epitopes of the anti-FcγRIIa mAbs. Sequence alignment of FcγRIIa and FcεRI is shown in Figure 1.

Chimeric receptor constructs used as templates in the construction of this series

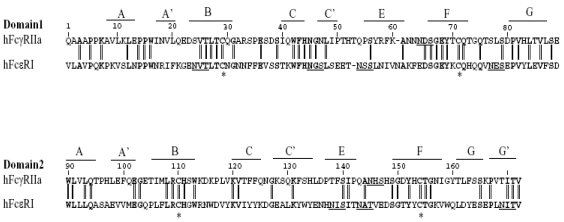

Figure 1. Sequence alignment of extracellular domains of FcγRIIa and FcεRI

of FcγRs were previously described (Hulett, 1993; Rigby, 2000). These include chimeras comprised of the domain one (D1) and domain two (D2) from either FcγRIIa or FcεRI, with the transmembrane region and cytoplasmic membrane anchor of FcγRIIa. The sequences of the FcγRIIa-based chimeras are listed in Figure 2 and that of the FcεRI-based chimeras in Figure 3.

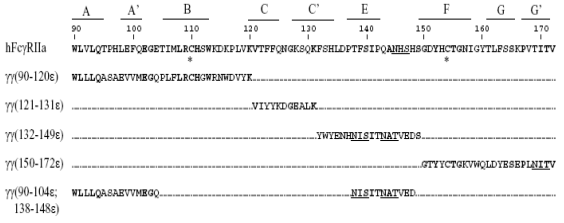

Figure 2. Domain 2 amino acid sequences of FcγRIIa and the FcγRIIa-based chimeras sequence

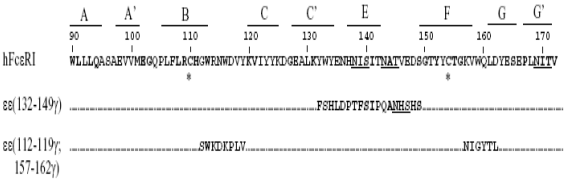

Figure 3. Domain 2 amino acid sequences of FcεRI and the FcεRI -based chimeras sequence

Generation of the chimeric receptors

Chimeric receptor cDNAs were generated by Splice Overlap Extension polymerase chain reaction (SOE-PCR) described previously (Hogarth, 1994). The reactions were performed on 100ng of the appropriate cDNA clone in the presence of 500ng of oligonucleotides, 50mM KCl, 10mM Tris-Cl, pH8.3, 1.5mM $MgCl_2$ and 1.25mM dinucleotide triphosphates using 0.5U of *Taq*

polymerase (Amersham) for 25 amplification cycles. A second PCR reaction was performed to splice the two fragments together and amplify the spliced product. 100ng of each gel purified fragment were combined and amplified with the appropriate primers under the same PCR conditions. Oligonucleotides used in the construction of the chimeric DNAs are listed on Table 1.

Chimeric and mutant receptor expression constructs were produced by subcloning the DNA in to the eukaryote expression vector pKC3. Each PCR product was engineered to have an *EcoRI* site at their 5′ end (the 5′ flanking primers NR1 and EG6, contain an *EcoRI* recognition site), and a *SalI* site at their 3′ end (the 3′-flanking primer EG5, contains a *SalI* recognition site), which enabled the final PCR products to be cloned into *EcoRI* and *SalI* sites of pKC3.

Transfections

Transfection of COS-7 cells with the chimeric cDNAs were performed as described previously (18). Briefly, COS-7 cells were maintained in RPMI-1640 (Commonwealth Serum Laboratories) supplemented with 10% heat-inactivated Foetal Calf Serum, 2mM glutamine, 100U/ml penicillin, 100mg/ml streptomycin (Commonwealth Serum Laboratories) and 50mM 2-mercaptoethanol (Koch Light Ltd.). Cells were cultured at 37°C with 10% CO_2. For the binding studies using a FACS analysis, COS-7 cells at 30-50% confluent in 10-cm petri-dishes were transiently transfected with 10mg DNA using FuGENE 6 (Boehringer Mannheim) according to the manufacturer's protocols. Cells were harvested after 36-60 hour for subsequent assays.

Direct binding study of mAbs to the chimeric receptors

Flowcytometry studies

Transfected COS-7 cells were incubated with 1mg IV.3 Fab, 1mg 8.7 $F(ab)'_2$, or 5mg 7.30 $F(ab)'_2$ mAbs for 30 minutes on ice. Unbound antibodies were removed by washing the cells with 0.5% Bovine serum albumin (BSA) in Phosphate Buffered Saline (PBS). FITC-conjugated anti-mouse IgG (Silenus) was then added for further 30 minutes on ice in the dark. Unbound conjugated antibody was removed and cells were analysed on a FACSCalibur flowcytometer (Becton Dickinson). The level of expression of the chimeric receptors were determined by one of the anti-FcγRIIa mAbs or when appropriate by 47 mAb, a murine IgG1 antibody against domain 2 of FcεRI (Rigby, 2000; Rigby, 2000a).

Results

Fab and $F(ab)'_2$ fragments of IV.3, 8.7 and 7.30

In testing the mAbs as inhibitors of FcγRIIa, it was important to exclude the possibility of the mAb Fc binding to the receptor. This was achieved by removal of the Fc portion by pepsin digest resulting in the $F(ab)'_2$ fragments of the mIgG1 (8.7 and 7.30 mAbs) and Fab fragments of mIgG2b (IV.3 mAb). Remaining Fc portion or intact mAb was removed by absorption to protein A and monitored by SDS-PAGE analysis. Purified Fab fragments were shown to be free from

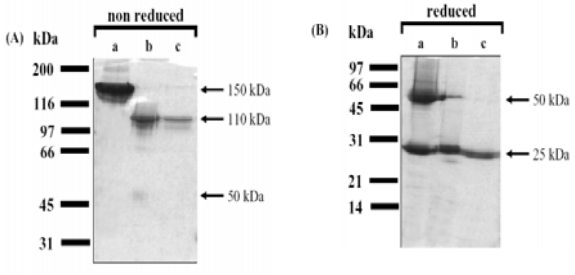

Figure 4. SDS-PAGE analysis of whole and $F(ab)'_2$ fragments of 8.7 mAb.

Fc fragments or intact antibody by SDS-PAGE analysis (Figure 4).

Purified mAb 8.7 was seen in the non reduced SDS-PAGE to migrate at 150kDa (prior to the pepsin digestion) (Figure 4A.a), and reduction of the disulphide bonds with Dithiothreitol (DTT) resulted in the free heavy chain (50kDa) and light chains (25kDa) (Figure 4B.a). Following pepsin digestion, 8.7, $F(ab)'_2$ fragment was seen at 110kDa in the non reduced samples with no Fc fragment apparent at 50kDa presumably because of the complete digestion of this fragment (Figure 4A.b), with the major component of the Fab fragment (25kDa) (Figure 4B.b) and some traces of intact heavy chain indicating some undigested antibody was present (Figure 4B.b). The Protein A purification resulted the $F(ab)'_2$ fragments free from intact antibody (Figure 4A.c and Figure 4B.c). Unless otherwise stated, IV.3, 8.7 and 7.30 mAbs used in this study were Fab or $F(ab)'_2$ fragments.

Expression of receptor chimeras

All the FcγRIIa/FcεRI chimeras were expressed at the cell surface. The level of expression of the chimeric receptors were determined by one of the anti-FcγRIIa mAbs or when appropriate by 47 mAb, a murine IgG1 antibody against domain 2 of FceRI (Rigby, 2000). As IV.3, 8.7 and 7.30 mAbs are known to block FcγRIIa binding to its ligand, it was suggested that their epitopes were likely to be adjacent to, or within the FcγRIIa ligand binding site. As expected, IV.3, 8.7, and 7.30 recognised the chimera eg consisting of FcεRI domain1 and FcγRIIa domain2. Furthermore, none were bound to the chimera ge consisting of FcγRIIa domain1 and FcεRI domain2 (Figure 5) indicating the epitopes of IV.3, 8.7, and 7.3 mAbs were likely to be within the domain 2 of FcγRIIa

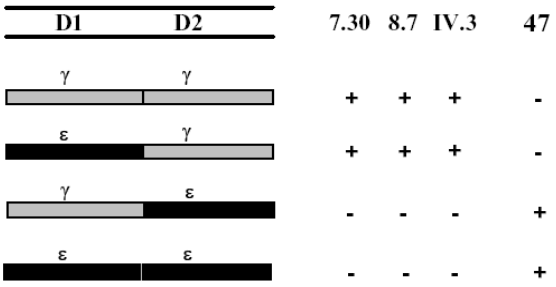

Figure 5. Summary of binding profiles of 8.7, 7.30 and IV.3 to FcγRIIa.

All the chimeras bound at least one mAb at levels equivalent to the γγ receptor, this indicated all chimeras were surface expressed, and were likely to be correctly folded as they had progressed through the secretory pathway with its mechanisms for retardation and degradation of misfolded proteins.

Mapping of the 8.7 and 7.30 mAbs epitopes

Transient expression of the receptor constructs resulted in of 20–35% of cells positive for receptor expression. Flowcytometry analysis of 8.7 mAb binding to transfected cells is shown in Figure 6.

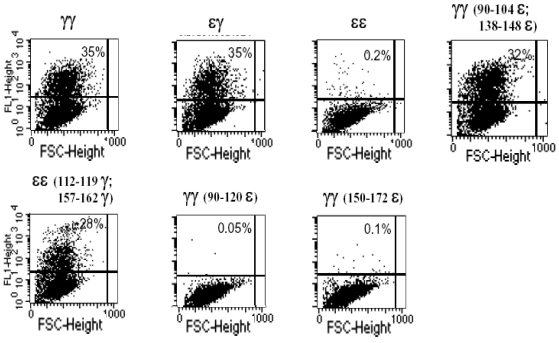

Figure 6. Dot plots of 8.7 mAb direct binding assay to FcγRIIa/FcεRI chimeric receptors by flow cytometry.

In this experiment, 35% of cells transfected with the wild type FcγRIIa (denoted as gg) were positive for 8.7 binding. Likewise, 35% of the εγ transfected cells bound 8.7. In contrast, 0.2% of events were in the upper quadrant for the cells transfected with

the εε construct. This non binding control indicated that 0.2% of the cells within the upper quadrant were the background for nonspecific cell staining in this experiment. The lack of binding of 8.7 to chimera γ(90-120ε) was shown as background staining at 0.05% (Figure 6). Likewise, the lack of 8.7 binding to chimera gg(150-172e) was shown as background staining at 0.1% (Figure 6). In contrast, 8.7 was able to bind chimera γγ(90-104e; 138-148e) as shown by 32% stained positive cells (Figure 6). Inspection of the FcγRIIa structure combined with the binding site mutagenesis data guided the choice of the amino acid residues 112-119 and 157-162 to be exchanged in the construction of Chimera εε(112-119γ; 157-162γ). Unlike the other chimeric receptors, Chimera εε(112-119γ; 157-162γ) is a gain of function mutant, where only these residues (112-119 and 157-162) from the FcγRIIa extracellular domains are present in what is otherwise FcεRI. The binding of 8.7 to Chimera εε(112-119γ; 157-162γ) was demonstrated in the 28% stained positive cells (Figure 6) shows that these regions, comprising the BC (residues 112-119) and FG (residues 157-162) loops of FcγRIIa, described the 8.7 epitope (Figure 7).

The same chimeras used for mapping the 8.7 epitope were used to identify the 7.30 epitope. FACS analysis of 7.30 mAb binding to transfected cells is shown in Figure 8. 7.30 mAb bound γγ transfectants (20% stained positive cells), εγ (33%), but not εε (0.5% stained positive cells) transfectants, the binding patterns of 7.30 to the other chimeric constructs were identical to those found in 8.7 mAb. 7.30 failed to bind Chimera γγ(90-120ε) with merely 0.2% stained positive cells as background stain and Chimera γγ(150-172ε) (0.02% stained positive cells), and bound to Chimera γγ(90-104ε; 138-148ε) (28% stained positive cells) and chimera εε(112-119γ; 157-162γ) (32% stained positive cells) (Figure 8).

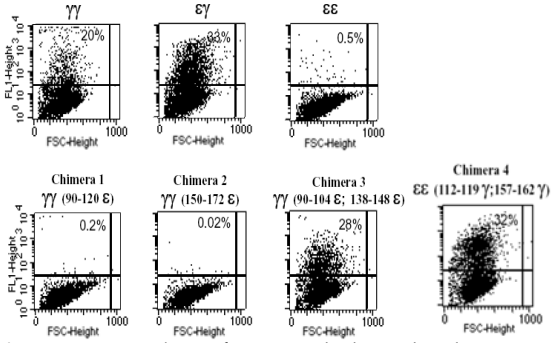

Figure 8. Dot plots of 7.30 mAb direct binding assay to FcγRIIa/FcεRI chimeric receptors by flow cytometry

Thus the 7.30 epitope is identical to that of 8.7 being defined by residues 112-119 and 157-162.

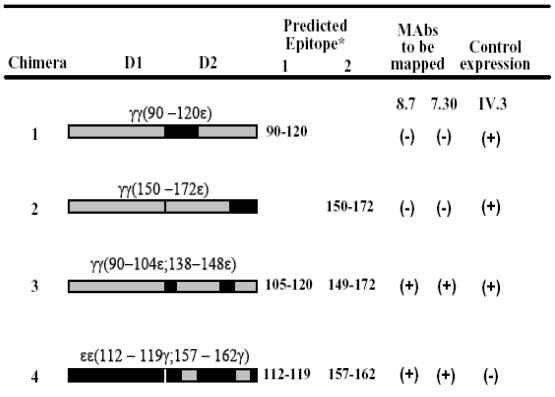

Figure 7. Elucidation of the 8.7 and 7.30 epitopes using FcγRIIa/FcεRI chimeric receptors binding assay.

Mapping of the IV.3 mAb epitope

Chimeras used for mapping the 8.7 and 7.30 mAbs were also used to map the epitope of IV.3, with the addition of Chimera γγ(121-131) and Chimera γγ(132-149e). The flowcytometry analysis of IV.3 mAb binding to the chimeras was shown in Figure 9. Similarly to 8.7 and 7.30 mAbs, IV.3 bound to γγ (28% positive cells) and εγ (24% positive cells) transfectants, and failed to bind ε (0.3% stained cells). In contrast to 8.7 and 7.30 mAbs, IV.3 was found to bind Chimera

γγ(90-120ε) (22% stained cells) and Chimera γγ(150-172ε) (20% stained cells), indicating that the predicted IV.3 epitope is within the amino acid residues 121 and 149. Furthermore, IV.3 was able to bind Chimera γγ(90-104ε; 138-148ε) (25% stained cells), which restricts the epitope to be predicted between amino acid residues 121-137.

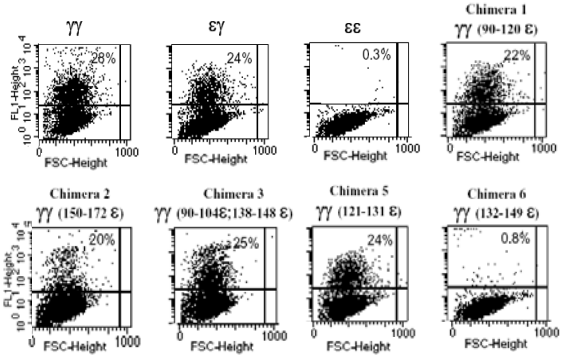

Figure 9. Dot plots of IV.3 mAb direct binding assay to FcγRIIa/FcεRI chimeric receptors by flow cytometry.

IV.3 bound to Chimera γγ(121-131e) (24% stained cells) which indicates a tight region between residues 132-137. Finally, IV.3 failed to bind Chimera γγ(132-149e) (0.8% stained cells) which confirmed the epitope of IV.3 to be mapped in the region between the amino acid residues 132-137 (Figure 10).

Chimera	D1	D2	Predicted Epitope*	MAbs to be mapped	Control expression
				IV.3	8.7
1	γγ(90 –120ε)		121-172	(+)	(-)
2	γγ(150 –172ε)		121-149	(+)	(-)
3	γγ(90–104ε;138–148ε)		121-137	(+)	(+)
5	γγ(121 –131ε)		132-137	(+)	(+)
6	γγ(132 –149ε)		132-137	(-)	(+)

Figure 10. Elucidation of the IV.3 mAb epitope using FcγRIIa/FcεRI chimeric receptors binding assay

This region is infact comprised of the C'E loop of FcγRIIa, which is one of the crucial loops of the FcγRIIa ligand binding site. Thus the inhibitory effect of IV.3 on FcγRIIa function is due to the IV.3 epitope being within the ligand binding site of FcγRIIa (Powell, 1999). The FcγRIIa ligand binding site is comprised of BC (aa113-119), C'E (aa134-137), and FG loop (aa 158-160) of the domain 2.

Epitopes for 8.7 and 7.30 mAbs are αα 112-119 and αα 157-162 which are in overlapped regions of the FcγRIIa ligand binding site, BC loop and FG loop respectively. The epitope for IV.3 mAb is αα132-137 which consisted of the C' strand and C'E loop of FcγRIIa, another crucial loop for the receptor ligand binding site.

Discussion

FcγRIIa has been shown to play a pivotal role in autoimmune disease processes (Sardjono, 2005). Monoclonal antibodies against FcγRIIa (IV.3) blocked FcγRIIa function and has been shown to be potential in down regulating inflammatory mediators (i.e. TNFa) released by macrophages activated through FcγRs. Furthermore, the generation of small molecule inhibitors which target FcγRIIa has been shown to inhibit arthritis development in FcγRIIa transgenic mice with Collagen-Induced Arthritis (Pietersz, 2008; Pietersz, 2002). A small molecule which mimic the specific antigen binding site of the blocking mAbs against FcγRIIa (i.e. IV.3, 8.7, and/or 7.30) may provide an alternative approach to design novel inhibitors for FcγRIIa. However, this approach required first the identification of the epitope of the mAbs. Because the steric effect of a small drug is much less than an antibody, it was crucial to elucidate whether the inhibitory effect of each of these mAbs resulted from its epitope coinciding with the receptor ligand binding site.

Previous study has suggested that the

epitopes of IV.3, 8.7 and 7.30 were located within domain 2 of FcγRIIa (Ierino, 1993). In this study, using the chimeric receptors containing regions of FcγRIIa/FcεRI the precise epitopes of IV.3, 8.7, and 7.30 were determined. Identical epitopes are shared by 8.7 and 7.30 mAbs and mapped on the amino acid residues 112-119 and 157-162.

IV.3 was found to recognize the epitope on residues 132-137 which mapped within C'E loop. Although residue 134 (Histidine or Arginine) is crucial in determining the High Responder/Low Responder polymorphism of FcγRIIa (Sardjono, 2003), IV.3 is not affected by the variation of His/Arg134, as it bound equally to chimeras containing His134 or Arg134. The approach using chimeric receptors of FcγRIIa/FcεRI was shown to be successful to map the specific epitopes of IV.3, 8.7 and 7.30. Furthermore the mapping of specific epitopes of IV.3, 8.7, and 7.30 validated these mAbs as possible targets for designing FcγRIIa inhibitors, which can be pursued further by designing small molecules to mimic the mAb antigen binding site and thus inhibit receptor function.

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