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Gain-of-function mutations in FcγRI of NOD mice: implications for the evolution of the Ig superfamily

Amanda L.Gavin, Peck Szee Tan and P.Mark Hogarth[1]

The Austin Research Institute, Austin and Repatriation Medical Centre, Studley Road, Heidelberg, Victoria 3084, Australia

[1]Corresponding author
e-mail: pm.hogarth@ari.unimelb.edu.au

It has been postulated that, during evolution of the Ig superfamily, modifications of the function of individual receptors might occur by acquisition of exons and their subsequent modification, though evidence of this is lacking. Here we have analysed the interaction of mouse IgG subclasses with high-affinity FcγRI (CD64) which contains three Ig-like domains and is important in innate and adaptive immunity. This analysis has identified a mechanism by which the postulated modification of newly acquired exons provides gains in function. Thus, the most widely distributed FcγRI allele in mice (e.g. BALB/c), bound only a single IgG subclass, IgG2a, with high affinity. However, non-obese diabetic (NOD) mice expressed a unique allele that exhibits broader specificity and, in addition to binding IgG2a, FcγRI-NOD bound monomeric IgG3 and bound IgG2b with high affinity, an IgG subclass not bound by FcγRI of other mouse strains, either as monomer or multivalent immune complexes. Analysis of mutants of FcγRI wherein segments of the interdomain junctions were exchanged between FcγRI-BALB and FcγRI-NOD identified these regions as having major influence in 'gain-of-function' by the NOD form of FcγRI. Nucleotide sequence analysis of intron/exon boundaries encoding the interdomain junctions of the FcγRI alleles showed these to have arisen by mutation to alter existing or create new mRNA splice donor/acceptor sites, resulting in generation of modified junctions.

Keywords: diabetes/Fc receptor/IgG/Ig-superfamily/macrophage

Introduction

Most of the leukocyte Fc receptor (FcR) family are comprised of immunoglobulin (Ig)-like domains, and therefore belong to the Ig superfamily (Ig-SF) (Williams and Barclay, 1988). Structurally related to each other, and to their ligand, Ig, the FcR family represent examples of highly evolved structures that are able to function distinctly. Within the FcγR family, the two 'low-affinity' IgG receptor subfamily, FcγRII and FcγRIII, consist of two structurally conserved Ig-like domains (Hulett and Hogarth, 1994). These receptors exhibit relatively low affinity for IgG (i.e. $K_d < 10^{-7}$ M) and display a wide specificity for ligand, binding IgG2b, IgG2a and IgG1 immune complexes (Hulett and Hogarth, 1994).

FcγRI, the high-affinity receptor for IgG, however, is unique. Although the first two extracellular domains are structurally related to both FcγRII and FcγRIII, FcγRI contains a unique third Ig-like domain (Allen and Seed, 1989; Sears *et al.*, 1990). The most widely distributed allele of FcγRI in mice (e.g. BALB/c), binds only a single IgG subclass, IgG2a, with high affinity (FcγRI-BALB, $K_a = 1\times10^{-8}$ M), but will bind complexes of mouse IgG3 ($IC_{50} \approx 5\times10^{-7}$ M) (Gavin *et al.*, 1998). The interaction between the first two domains and the third domain is critical in determining the unique high affinity and restricted IgG specificity observed. Functional analysis of mouse FcγRI structure has revealed that when the third Ig-like domain is removed, the first two Ig-like domains are able to bind IgG with broad specificity, being able to interact with mouse IgG2a, IgG1 and IgG2b but only with a low affinity similar to the low-affinity Fc receptors; the binding of IgG3 was not tested (Hulett *et al.*, 1991).

Another allele of mouse FcγRI has been identified (Prins *et al.*, 1993). The non-obese diabetic (NOD) mouse has an allele (FcγRI-NOD) that differs from BALB/c strain (FcγRI-BALB) by 24 nucleotides in the coding region, 17 of which encode different amino acids, notably including a four-amino acid insertion between domains 2 and 3, and a frame shift that leads to the premature truncation of the cytoplasmic tail (Prins *et al.*, 1993). Ligand binding analysis of FcγRI-NOD revealed that this receptor bound mouse IgG2a with a 10-fold increase in affinity ($K_a \approx 1\times10^{-9}$ M) over that of FcγRI-BALB ($K_a \approx 1\times10^{-8}$ M), although less was expressed on the cell surface, and both the affinity and expression differences could be attributed to the amino acid differences in the extracellular domains (Gavin *et al.*, 1996).

This study identifies that, in addition to increased affinity for mouse IgG2a, the FcγRI-NOD molecule can also bind monomeric mouse IgG3, but most surprising is the absolute gain in function by binding IgG2b, which is unable to bind to other FcγRI allelomorphs. We have identified the extracellular domain interfaces as regions crucial to FcγRI-NOD function and expression. Moreover, the identification of these regions, together with an analysis of its gene, indicates that this IgG receptor system has implications for our understanding of function and evolution of one of the largest superfamilies, the Ig superfamily.

Results

NOD/Lt macrophages bind monomeric mouse IgG2b and IgG3

The binding of different IgG subclasses to NOD/Lt and BALB/c bone marrow macrophages (BMM) was determined by immunofluorescence (Table I). In two experiments, macrophages derived from BALB/c mice bound monomeric IgG2a but not IgG2b, IgG3 or IgG1. In

Table I. The binding of monomeric IgG by bone marrow macrophages of either BALB/c or NOD/Lt origin

Antibody	BALB/c	NOD/Lt
Experiment 1		
IgG2b	1.1[a]	21.6
IgG3	3.2	39.7
IgG2a	205.4	44.5
IgG1	1.2	3.1
Experiment 2		
IgG2b	1.6	13.8
IgG3	0.5	15.6
IgG2a	79.8	25.1
IgG1	0.1	0.2

[a]Data presented as mean fluorescence intensity after background subtracted.

contrast, NOD/Lt BMM bound IgG2b, IgG3 and IgG2a, but not IgG1. This finding was unexpected, as mouse FcγRI, the only IgG receptor that detectably binds monomeric IgG on macrophages, binds only IgG2a with high affinity. The observation of IgG2b and IgG3 binding by NOD/Lt macrophages suggests that either NOD/Lt macrophages express a novel Fc Receptor, or the FcγRI allele present on NOD/Lt macrophages displays unusual specificity for IgG.

FcγRI-NOD binds monomeric mouse IgG3 and IgG2b

To address whether the unique FcγRI allele, FcγRI-NOD, was contributing the unusual IgG2b and IgG3 binding pattern observed of the NOD/Lt macrophages, the binding specificity of the two FcγRI alleles, FcγRI-NOD and FcγRI-BALB, was examined using transfected CHO-K1 cell lines expressing FcγRI-BALB (1N3-2) and FcγRI-NOD (2B5-13C) (Figure 1). Immunofluorescence analysis revealed that transfectants expressing FcγRI-NOD bound monomeric IgG2b, IgG3 and IgG2a, but not IgG1 (Figure 1A, B, C and D, respectively). In contrast, FcγRI-BALB-expressing cells bound only monomeric IgG2a (Figure 1G). Thus, the binding of monomeric mouse IgG2b and IgG3 by NOD/Lt macrophages is due to the unusual specificity of FcγRI-NOD expressed by these cells.

Mouse IgG2b and IgG3 compete with IgG2a for the FcγRI-NOD binding site

To determine whether the unique binding properties of FcγRI-NOD were either due to the creation of a new binding site, or to alteration of the existing IgG2a binding site, competition experiments were performed (Figure 2). Both monomeric IgG2b and IgG3 inhibited the binding of radiolabelled IgG2a to FcγRI-NOD at high concentrations, i.e. mIgG2b IC_{50} ≈13.5 μg/ml (or 8.6×10^{-8} M) and mIgG3 IC_{50}≈ 5.8 μg/ml (or 3.9×10^{-8} M). The implication of this experiment is that the IgG2b and IgG3 binding sites are identical or in proximity to the binding site for IgG2a. As expected, non-radiolabelled IgG2a inhibited radiolabelled IgG2a binding and gave an IC_{50} value that correlates with the known affinity of FcγRI-NOD for IgG2a (i.e. IC_{50}≈ 0.22 μg/ml or 1.4×10^{-9} M) (Gavin *et al.*, 1996). Mouse IgG1, however, was unable to inhibit the binding of IgG2a at concentrations up to

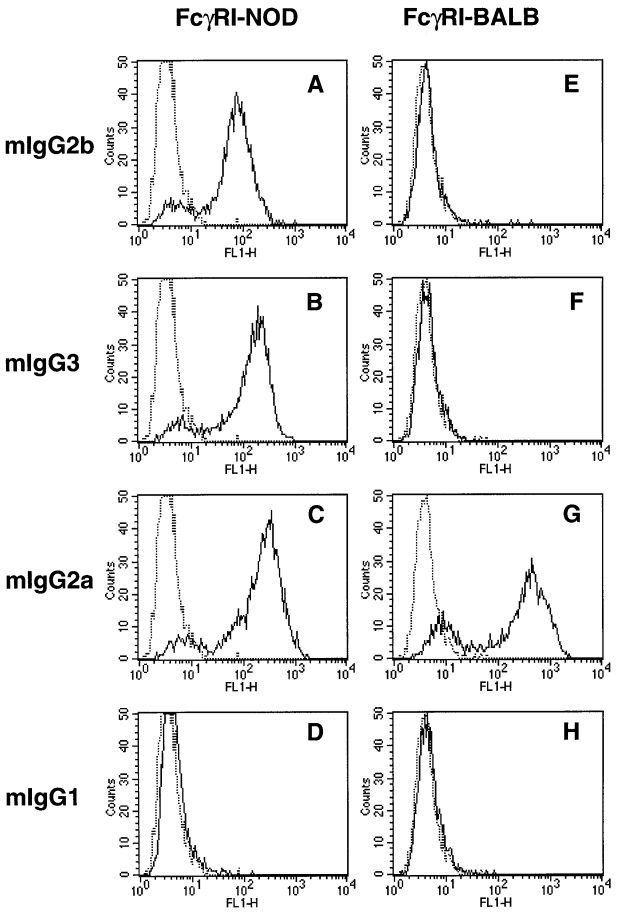

Fig. 1. FcγRI-NOD but not FcγRI-BALB binds mouse IgG2b and IgG3. FACScan analysis of IgG binding by CHO cells transfected with either FcγRI-NOD cDNA (2B5-13C) (**A**–**D**) or FcγRI-BALB cDNA (1N3-2) (**E**–**H**) was assessed. The binding of the mouse IgG isotypes IgG2b (A and E), IgG3 (B and F), IgG2a (C and G) or IgG1 (D and H) to FcγRI-transfected CHO cells was detected with FITC-conjugated sheep (Fab) anti-mouse Ig (solid line). Background fluorescence (dotted line) was determined by incubation with FITC-conjugated sheep (Fab) anti-mouse Ig only.

150 μg/ml, confirming that this IgG isotype binds poorly, if at all, to FcγRI-NOD. The surprising finding that FcγRI-NOD binds monomeric IgG2b and IgG3 represent clearly defined gains of function and the functional differences between FcγRI-NOD and FcγRI-BALB alleles provide useful tools for defining regions involved in ligand binding.

Interdomain junctions influence allele-specific affinity and expression

Although the FcγRI-NOD and FcγRI-BALB alleles are 93% identical in the extracellular domains, they exhibit remarkable functional differences in Ig binding. The FcγRI-NOD phenotype is one of high affinity for multiple isotypes, and low surface expression, while the FcγRI-BALB phenotype is one of restricted high affinity for IgG2a, with surface expression that is relatively high. The major sequence differences between FcγRI-NOD and FcγRI-BALB occur in the interdomain junctions where the FcγRI-NOD allele contains a change (Asn88→Lys88) and an insertion (Glu89) between domains 1 and 2 (D1/D2) and a four-amino acid insertion (Ala173-Phe174-Pro175-Leu176) between domains 2 and 3 (D2/D3) (Prins

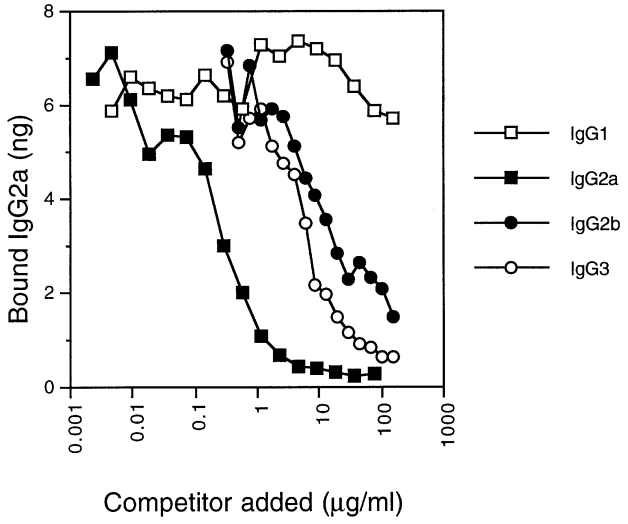

Fig. 2. IgG2a, IgG2b and IgG3 compete for similar binding sites on FcγRI-NOD. The inhibition of radiolabelled IgG2a (7-20.6/30) binding to FcγRI-NOD on 2B5-13C cells by mouse IgG1 (UM1, □), IgG2a (UM2a, ■) IgG2b (49.2, ●) or IgG3 (UM3, ○) was determined. The data are expressed as amount of ^{125}I-IgG2a bound (ng) against the concentration of competitor added (μg/ml).

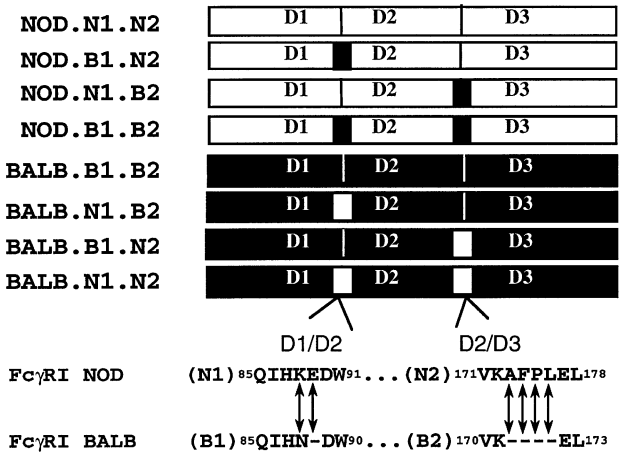

Fig. 3. Mutants at exon/intron boundaries map regions involved in Ig binding. Schematic representation of cDNAs used are shown. The regions of FcγRI are indicated as D1, D2, D3, extracellular domains 1, 2 and 3, respectively. The origin of the appropriate template sequence is indicated by various shading (NOD/Lt, white; BALB/c, black). The sequence involved in the exchanges are shown in detail between the domain junctions, with the mouse strain of origin shown (see Materials and methods for details). The mutant name indicates origin of template, either NOD or BALB, and then the origin of the interdomain region 1 (D1/D2) or region 2 (D2/D3) is indicated by either N (NOD/Lt) or B (BALB/c). The wild-type alleles of FcγRI-NOD and FcγRI-BALB are presented as NOD.N1.N2 and BALB.B1.B2, respectively.

et al., 1993; Gavin *et al.*, 1996). Utilizing the extensive homology of the two alleles, and their dramatic functional differences, mutant receptors were generated by exchanging interdomain regions between FcγRI-NOD and FcγRI-BALB to determine to what extent these regions contributed to the unique phenotype of each allele (Figure 3). Mutants were constructed wherein the sequence of FcγRI-NOD between D1/D2 (residues HKED termed N1) and D2/D3 (residues KAFPLE termed N2) were replaced with the corresponding regions of FcγRI-BALB (residues HND

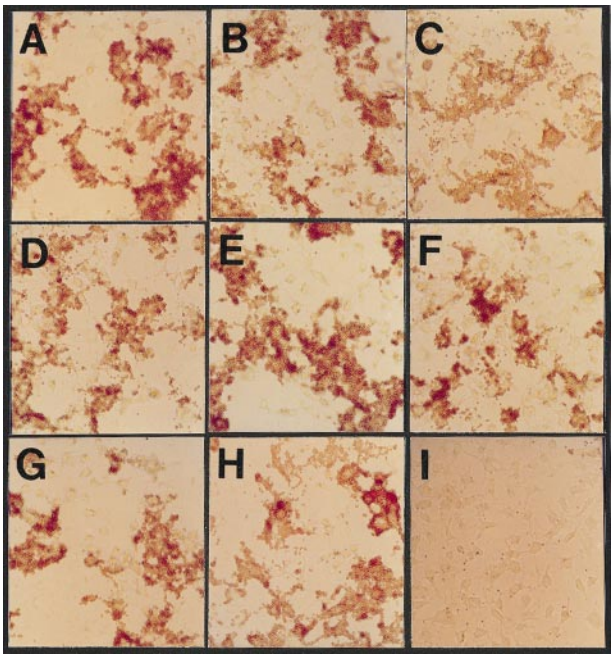

Fig. 4. Mutated FcγRI are expressed on the cell surface and are functional. Monolayers of COS cells transfected with either NOD.N1.N2 (**A**), NOD.B1.B2 (**B**), NOD.N1.B2 (**C**), NOD.B1.N2 (**D**), BALB.B1.B2 (**E**), BALB.N1.N2 (**F**), BALB.B1.N2 (**G**) and BALB.N1.B2 (**H**) cDNA (see Figure 3 for mutant FcR construction), or expression vector only (**I**) rosetted with IgG2a anti-TNP-sensitized sheep erythrocytes.

termed B1) and (residues KE termed B2), respectively (Figure 3). Reciprocal mutants were also generated where the D1/D2 and D2/D3 regions of FcγRI-BALB were replaced with the appropriate amino acids of FcγRI-NOD (Figure 3). The wild-type alleles of FcγRI-NOD and FcγRI-BALB were presented as NOD.N1.N2 and BALB.B1.B2, respectively. All of the mutant FcR generated could be translated *in vitro* and when transiently expressed by COS cells, all bound erythrocytes sensitized with mouse IgG2a (EA-IgG2a), indicating that they were expressed on the cell surface (Figure 4A–H). These cells did not bind unsensitized erythrocytes (not shown) and mock-transfected COS cells did not bind EA-IgG2a (Figure 4I), indicating that the immune complex binding by the mutant FcR was specific. Following transfection of COS cells with the mutant cDNA, cells were assayed for the capacity to bind monomeric IgG2a, and IgG3 (Figure 5). A summary of the mutant receptors' affinity for IgG2a and IgG3, and also IgG2b, and the levels of functional surface expression determined by IgG2a binding (B_{max} values presented as sites/cell) are presented in Table II.

FcγRI-NOD-transfected cells (termed NOD.N1.N2) bound IgG2a, IgG2b and IgG3 and expressed ~12×10^3 sites on the surface (Figure 5A and Table II) while FcγRI-BALB-transfected cells (termed BALB.B1.B2) bound IgG2a with approximately one-tenth the affinity of FcγRI-NOD while being expressed at higher levels on the cell surface (5×10^5 sites/cell) (Figure 5E and Table II).

The analysis of the mutant receptors revealed that modification of the interdomain junctions can have allele-specific effects on receptor function. Replacement of FcγRI-NOD D1/D2 and D2/D3 regions (N1 and N2 respectively) with sequence of FcγRI-BALB (B1 and B2)

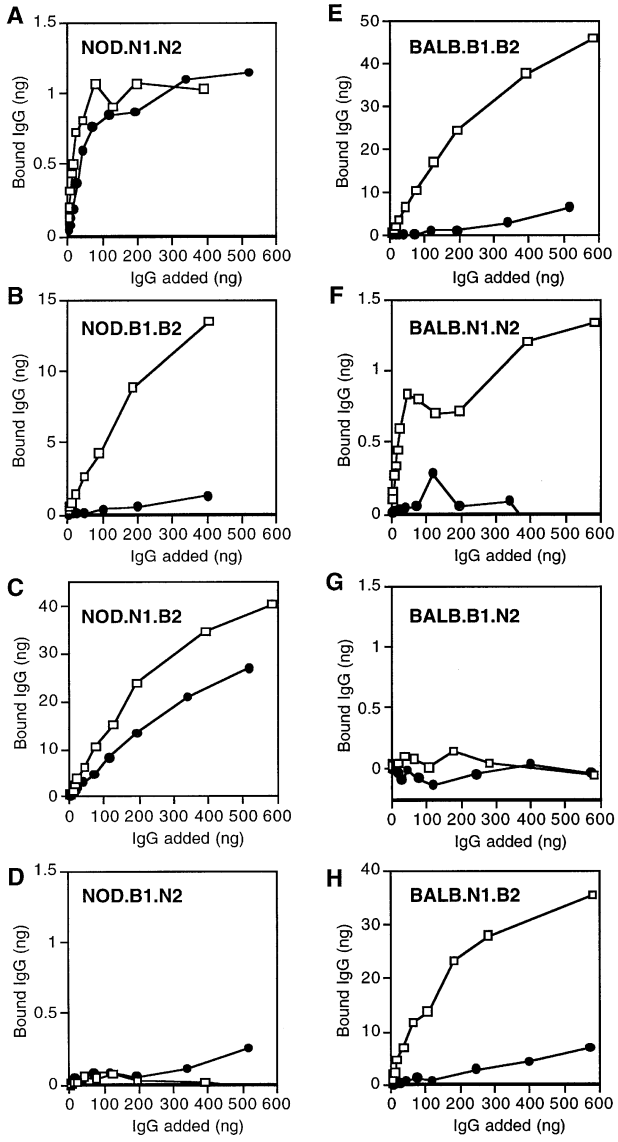

Fig. 5. Titration of radiolabelled monomeric mouse IgG2a and IgG3 binding to mutant FcγRI. Shown is the binding of mouse IgG2a (□) and mouse IgG3 (●) by COS cells expressing NOD.N1.N2 (**A**), NOD.B1.B2 (**B**), NOD.N1.B2 (**C**), NOD.B1.N2 (**D**), BALB.B1.B2 (**E**), BALB.N1.N2 (**F**), BALB.B1.N2 (**G**) and BALB.N1.B2 (**H**) (see Figure 3 for mutant FcR construction).

produces a receptor (NOD.B1.B2) with a 'BALB-like' phenotype; this mutant only detectably binds IgG2a (not IgG2b or IgG3) with affinity similar to that of FcγRI-BALB (18.4×10^{-9} M versus 20.9×10^{-9} M) and has relatively high surface expression (15-fold increase over NOD) (Figure 5B and Table II).

Mutant receptors, where only single interdomain junctions were modified, were analysed to dissect the effects individual junctions have on receptor function. The D2/D3 junction has a major impact on FcγRI-NOD expression and function. Cell surface expression of FcγRI-NOD is modified by the D2/D3 region, as replacement of this junction (N2) with sequence of BALB (B2) produces a mutant FcγRI (NOD.N1.B2) which is expressed at levels 30-fold higher than NOD (370×10^3 sites/cell versus 12×10^3 sites/cell). It is clear that this region also contributes to the unique affinity and specificity of NOD, as

Table II. Exchange of amino acids at the interdomain junctions of FcγRI modify both affinity for IgG and cellular expression

Mouse FcγRI	K_d ($\times10^{-9}$ M) IgG2a	IgG2b	IgG3	B_{max} IgG2a ($\times10^3$ sites/cell)
NOD.N1.N2	1.3	20.6	11.4	12.5
NOD.B1.B2	18.4	–[a]	–[a]	180.6
NOD.N1.B2	16.5	16.1	119.3	373.4
NOD.B1.N2	–[a]	–[a]	–[a]	ND
BALB.B1.B2	20.9	–[a]	–[a]	502.3
BALB.N1.N2	3.3	–[a]	–[a]	4.7
BALB.B1.N2	–[a]	–[a]	–[a]	ND
BALB.N1.B2	18.5	–[a]	–[a]	421.6

[a]K_d value could not be determined (i.e. affinity was $<1\times10^{-7}$ M). ND, B_{max} value could not be determined due to lack of monomeric IgG2a binding.

replacement of the N2 sequence in the NOD.N1.B2 receptor leads to a loss in receptor affinity for both IgG2a and IgG3 with the K_ds for this interaction increased ~10-fold (Figure 5C and Table II). The affinity of NOD.N1.B2 for IgG2a closely matches that described for the BALB/c allele (16.5×10^{-9} M versus 20.9×10^{-9} M, respectively). It is interesting that the decrease in affinity caused by change of the D2/D3 region in the receptor is not observed for IgG2b (Table II). This would indicate that the binding interaction of FcγRI-NOD with IgG2b is distinct from that of IgG2a and IgG3, although all compete with each other, indicating that they all bind in close proximity but the focus of the interaction is different. Interestingly, although the affinity of FcγRI-NOD for IgG2b is not affected by the replacement of the D2/D3 region, IgG2b binding is markedly affected by the origin of the D1/D2 junction (compare NOD.N1.B2 with NOD.B1.B2).

The D1/D2 junction has a distinct role in the function of FcγRI-NOD. Its replacement (NOD.B1.N2) results in complete loss of monomeric Ig binding by this mutant receptor (Figure 5D and Table II). The lack of monomeric Ig binding by NOD.B1.N2 implies that the D1/D2 region either directly interacts with IgG at the binding site, or this region contributes indirectly by interacting with other residues to influence binding.

Analysis of the role the interdomain junctions have on BALB allele function revealed similar effects. The inclusion of both D1/D2 and D2/D3 sequence of NOD into the BALB sequence produces a mutant receptor (BALB.N1.N2) able to bind IgG2a with a 7-fold increase in affinity, and this molecule exhibits very low surface expression (100-fold decrease when compared with BALB) (Figure 5F and Table II). This mutant confirms that the interdomain regions have the capacity to influence markedly ligand binding and surface expression. The BALB.N1.N2 mutant displays a partial NOD phenotype, i.e. the affinity for IgG2a is remarkably enhanced, while the cell surface expression is dramatically reduced (compare Figure 5F and A). In contrast to the NOD allele, however, the inclusion of the NOD interdomain regions in FcγRI-BALB did not create the capacity to bind IgG2b and IgG3, implying that other regions distinct in NOD must be additionally involved in this unique interaction.

The roles that the individual junctions have on FcγRI-

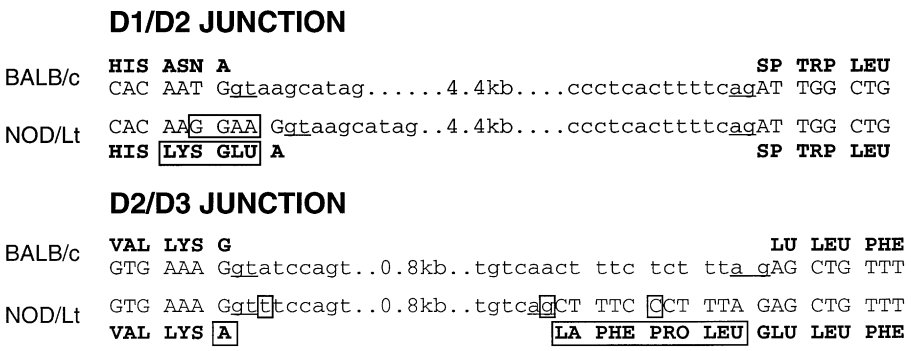

Fig. 6. Alteration of intron/exon boundaries alters amino acid content of FcγRI-NOD. Comparison of the sequence and encoded amino acids at the intron/exon junctions encoding the extracellular domains (D1,D2 and D3) of FcγRI-NOD and FcγRI-BALB are illustrated. Coding region sequences are shown in upper case and intron sequences in lower case. Splice donor and acceptor sites are underlined. Regions of sequence alteration or insertion in the FcγRI-NOD gene are indicated by boxes.

BALB expression and function were also examined. Modification of the D2/D3 junction of FcγRI-BALB however, is not well tolerated, as the insertion of the NOD-derived AFPL residues (N2) into FcγRI-BALB to generate the BALB.B1.N2 mutant results in complete loss of monomeric Ig binding (Figure 5G and Table II). This result indicates that the presence of the AFPL sequence markedly reduces affinity for ligand when presented in the context of the BALB protein. In contrast to the dramatic effects of the D2/D3 junction on BALB function, the introduction of NOD-derived sequence to the interface between the D1/D2 of the FcγRI-BALB (BALB.N1.B2) did not alter the affinity for IgG2a, IgG2b or IgG3, nor did it alter the levels of receptor expression (Figure 5H and Table II). By comparing BALB.N1.N2 with BALB.B1.N2, it is clear that the presence of NOD-derived D1/D2 sequence is required for high-affinity IgG2a binding. This observation implies that: (i) each interdomain region is dependent upon the presence of the other to maintain a functional high-affinity receptor; and (ii) each can contribute different aspects of function, e.g. N1 is essential for IgG2b binding (compare NOD.B1.B2 with NOD.N1.B2).

Intron/exon boundaries affect receptor function

Analysis of the intron/exon boundaries of the FcγRI genes from NOD/Lt and BALB/c mice revealed that several nucleotide changes have occurred in the FcγRI-NOD gene to alter exons and splice acceptor sites at the intron/exon boundaries (Figure 6). Within the D1/D2 junction, one nucleotide change (T→G) and a three-nucleotide insertion at the 3′ end of the exon encoding domain 1, leads to the amino acid change Asn→Lys88, and the insertion of Glu89 in the NOD/Lt allele. Three nucleotide changes were identified proximal to the D2/D3 junction, most notably an A→G change creates a new splice acceptor site 12 nucleotides 5′ of the BALB/c acceptor site, leading to the translation of four extra hydrophobic residues (Ala173-Phe174-Pro175-Leu176) in the NOD/Lt allele. Also, an A→T change 3′ of the donor site, and a T→C change six nucleotides downstream from the newly created splice acceptor site were observed. The analysis of genomic sequence of FcγRI has revealed that both the insertion and alteration of nucleotides at intron/exon junctions has occurred to encode the amino acids changed within the interdomain regions, and in light of the mutant receptor-binding data, these alterations lead to dramatic changes in function and expression of the surface IgG receptor.

Discussion

The novel finding that NOD/Lt bone marrow macrophages can bind monomeric IgG2b, IgG3, as well as IgG2a, led us to identify gains in function of the FcγRI-NOD allele. In terms of the biological function of FcγRI, the gains in affinity for IgG isotypes identified herein suggest that FcγRI-NOD would be able to sample a broad range of IgG isotypes and participate in triggering effector functions at lower IgG concentrations than mice displaying the FcγRI-BALB allele. Moreover, as IgG3 is a T-cell-independent antibody isotype (Perlmutter *et al.*, 1978; Slack *et al.*, 1980; Mongini *et al.*, 1981), then FcγRI-NOD would provide cellular effector function in the developing phases of adaptive immunity (Gavin *et al.*, 1998). Although FcγRI-NOD can associate with the FcR signalling molecule, the FcεRI- γ subunit, the cytoplasmic tail of FcγRI-NOD is truncated and the signalling potential of FcγRI-NOD has not been determined (Gavin *et al.*, 1996). The pathological significance of NOD/Lt macrophages binding monomeric IgG3, an isotype known to form pathogenic complexes (Abdelmoula *et al.*, 1989; Lemoine *et al.*, 1992), and IgG2b, an isotype present at high levels in NOD/Lt mice and known to be specific for autoantigens including GAD65 and insulin (Serreze *et al.*, 1988; Hanson *et al.*, 1996; Luan *et al.*, 1996), remains unclear. Recent congenic mapping studies in mice, however, have demonstrated that although FcγRI-NOD is genetically linked to a diabetes susceptibility locus, it is clearly distinct (Podolin *et al.*, 1997).

The comparison of two highly related alleles of FcγRI displaying different specificities for IgG led to the identification of regions that contribute to the function of mouse FcγRI. Previous functional studies have demonstrated that in the absence of domain 3 (D3), the first two domains of mouse FcγRI can bind IgG2a, IgG2b and IgG1, but with a dramatic loss in affinity (IgG3 was not tested) (Hulett *et al.*, 1991). A similar decrease in affinity for IgG is observed in human FcγRIb2, a two-Ig domain splice variant (Porges *et al.*, 1992). Furthermore, exchange

of D1 of mouse FcγRI with D1 of mouse FcγRII, does not alter the specificity of Ig binding, or have a major influence on the high-affinity binding of IgG2a (M.Hulett and P.M.Hogarth, in preparation). These studies provide evidence that D3 interacts with D2 to mask the low-affinity IgG2b and IgG1 binding site(s) and forms a high-affinity site for IgG2a, although the presence of D1 is required to maintain the structural conformation of the receptor.

The experiments presented herein demonstrate that the affinity and specificity of FcγRI can also be modified by alteration of the domain junctions. Clearly, the D2/D3 junction is crucial for high-affinity binding as replacement of this region in both FcγRI-NOD and FcγRI-BALB leads to a decrease in affinity for IgG. As both NOD.N1.B2 and BALB.B1.N2 mutants can still bind IgG (BALB.B1.N2 binds IgG2a immune complexes only), then the D2/D3 junction itself is probably not the principal binding site. What becomes clear is that in the presence of BALB/c-derived D2/D3 sequence (B2), the affinity for IgG2a is ~16–20$\times10^{-9}$ M, when present in either a NOD/Lt- or BALB/c-derived scaffold. However, the presence of NOD/Lt-derived D2/D3 sequence (N2) can, in different FcγRI mutants, produce two contrasting effects. If N2 is co-expressed with N1, then very high affinity for IgG2a is achieved (1–3$\times10^{-9}$ M, see NOD.N1.N2 and BALB.N1.N2). If N2 is mismatched with BALB/c-derived sequence (B1), then a dramatic loss of monomeric Ig binding results. These data demonstrate that D1/D2 and D2/D3 junctions must interact or cooperate to generate high-affinity IgG binding, in the NOD/Lt allele at least. Moreover, the presence of the AFPL (N2) sequence at the D2/D3 junction can only be functionally tolerated in the presence of the appropriate D1/D2 junction sequence (compare BALB.B1.N2 and BALB.N1.N2).

The role of the D1/D2 junction in affinity is difficult to evaluate in isolation. When B1 is expressed with B2 then moderate affinity for IgG2a is achieved. In contrast, and as mentioned above, the presence of B1 with N2 results in a dramatic loss of affinity, implying a functional mismatch. It is clear, however, that the NOD/Lt-derived D1/D2 sequence can influence the specificity of the FcγRI-NOD allele. By comparing NOD.N1.B2 with NOD.B1.B2, it becomes clear that the N1 sequence must contribute to the broad specificity (IgG2b and IgG3 binding) exhibited by the FcγRI-NOD allele. However, inclusion of the FcγRI-NOD D1/D2 junction in the FcγRI-BALB (BALB.N1.B2) does not dramatically alter the affinity for IgG2a, IgG2b or IgG3. This implies that N1 must interact with other residues novel in the FcγRI-NOD allele to achieve the IgG2b and IgG3 binding pattern observed. It is interesting to note that one unique difference between FcγRI-NOD and FcγRI-BALB is an Asp135→Gly136 alteration that corresponds to an Ig interactive region defined for other FcR, namely FcγRII and FcεRI (Warmerdam *et al.*, 1991; Tate *et al.*, 1992; Hulett *et al.*, 1993, 1995).

Studies of the binding sites of other FcR family members, namely FcγRII, FcγRIII and FcεRI, have identified loop and strand regions within the extracellular domains (principally domain 2) as being involved in direct Ig binding (Hulett *et al.*, 1993, 1994, 1995; Mallamaci *et al.*, 1993; Hibbs *et al.*, 1994; McDonnell *et al.*, 1996). This may be true for FcγRI also; however, this paper demonstrates that changes to the interdomain regions, can dramatically alter function of FcRs, perhaps by altering the structural presentation of the Ig binding domains. Based on homology with other Ig-like structures including CD2 (Jones *et al.*, 1992), CD4 (Wang *et al.*, 1990), KIR (Fan *et al.*, 1997) and FcγRII (M.Powell and P.M.Hogarth, in preparation), it is interesting to note that the D1/D2 interface would be spatially close to the FG loop in D2, a region known to play a key role in ligand binding in FcγRII and FcεRI (Hulett *et al.*, 1993, 1994). In the FcγRI-NOD allele, the presence of two highly charged residues (Lys88, Glu89) in this region may impinge on the FG loop, thereby influencing binding. The spatial orientation of the unique third Ig-like domain of FcγRI to D1 and D2 has not been determined and it is difficult to speculate how alteration to the D2/D3 junction may influence the binding site(s) contained in the first two Ig-like domains. The consequences of the interdomain alterations, in the case of the FcγRI-NOD allele at least, led to dramatic gains or diversity of function.

The Ig-superfamily is comprised of a vast array of members exhibiting diverse functions including cell–cell recognition and adhesion, soluble ligand binding and antigen recognition, all of which depend on the versatility of the shared common Ig homology unit. Williams, Barclay and colleagues postulated that during the evolution of the Ig superfamily, modifications of the function of individual receptors might occur by the acquisition of exons and their subsequent modification (Williams and Barclay, 1988). Analysis of the high-affinity receptor for IgG (FcγRI) from strains of mice described herein identifies a mechanism by which the postulated modification of newly acquired exons could take place to provide gains in function.

Sequence analysis of the FcγRI-NOD gene demonstrated that the change of A→G and subsequent generation of a new splice acceptor site encoding the D2/D3 junction of FcγRI-NOD leads to the translation of four extra residues (Ala173-Phe174-Pro175-Leu176). As demonstrated by mutant BALB.B1.N2, this single event would result in a receptor with dramatically reduced affinity for IgG. A second event at the D1/D2 junction leading to the amino acid change Asn→Lys88, and insertion of Glu89, of itself does not appear to influence function (see BALB.N1.B2); however, in combination both events produce a receptor with enhanced affinity for IgG2a and low cell surface expression (see BALB.N1.N2). Finally, it is the combination of these events, and their interaction with other sequence alterations in the FcγRI-NOD allele, that create an entirely new binding interaction with IgG2b and produce the broad specificity, high-affinity receptor that is encoded by the FcγRI-NOD allele.

Analysis of the Ig-superfamily reveals many mechanisms for the diversification of function within this family. In contrast to BCR and TCR, FcR must rely solely on evolutionarily acquired variation. Previous studies by our laboratory have demonstrated that the third Ig-like domain unique to FcγRI contributes markedly to the particular ligand affinity and specificity exhibited by this receptor class (Hulett *et al.*, 1991). As the related low-affinity receptors have only two Ig-like domains, the evolutionary acquisition of the extra exon encoding domain 3 (either by duplication or acquisition) supports the theory of exon acquisition as a means of acquiring new or diverse

functions. The characterization of two alleles of mouse FcγRI further develop our ideas about the mechanisms of diversification of function. Indeed, these alleles provide examples where modification at intron/exon junctions can markedly alter function of Ig homology domains.

Materials and methods

Antibody reagents

Antibodies used as either tissue culture supernatant or protein A-purified IgG from culture supernatant included UM1 (anti-TNP, mIgG1), UM2a (anti-TNP, mIgG2a) and UM3 (anti-TNP, mIgG3) (Tsujimura *et al.*, 1990), 7-20.6/30 (anti-Ly1.1, mIgG2a) (Hogarth *et al.*, 1980). The antibody 49.2 (anti-TNP, IgG2b) was purchased from PharMingen (San Diego, CA, USA). The isotype of each antibody was confirmed by ELISA using specific anti-mouse Ig isotype antibodies (PharMingen). The monoclonal antibodies that were used in FACS analysis were detected with FITC-conjugated sheep anti-mouse Ig (Amrad Pharmacia Biotech, Boronia, Australia). Purified antibodies were stored at –80°C and thawed once only for use. Analytical gel filtration (Superdex-200, Pharmacia, Uppsala, Sweden) routinely showed that the antibody preparations used were 90–95% monomeric. UM1, UM2a and UM3 were kind gifts from Dr Tohru Masuda (Kyoto University).

Transfection of FcγR cDNA constructs

CHO-K1 cells stably expressing either FcγRI of BALB/c (1N3-2) or NOD/Lt (2B5-13C) origin were generated by calcium phosphate transfection of the expression vector pCDNA3 (Invitrogen, Carlsbad, CA, USA) containing either mouse FcγRI cDNA (Sears *et al.*, 1990; Gavin *et al.*, 1996). Because of initial low surface expression of FcγRI-NOD, the stable line 2B5 was sorted twice for clones with high expression (2B5-13C). BALB/c, NOD/Lt and mutant FcγRI were also transiently expressed by COS cells using Lipofectamine™ according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD, USA). Binding assays were performed on transfected cells 48 h post transfection and the efficiency of transient transfection and expression was ~50%.

IgG isotype binding and competition assays

In the FACS analysis, the binding of monoclonal antibodies UM1 (anti-TNP, mIgG1), UM2a (anti-TNP, mIgG2a), 49.2 (anti-TNP, IgG2b) UM3 (anti-TNP, mIgG3) to transfected CHO cells (1N3-2 and 2B5-13C) were detected using FITC-conjugated sheep anti-mouse Ig (Silenus, Melbourne, Australia). In the competition assays, various concentrations of unlabelled mouse IgG1 (UM1), IgG2a (UM2a), IgG2b (49.2) and IgG3 (UM3) were incubated with 2B5-13C cells at 1×10^6 cells/ml for 15 min on ice prior to the addition of ^{125}I-labelled IgG2a (2 μg/ml). After further incubation on ice for 2 h, the amount of bound ^{125}I-labelled IgG2a was determined as described (Gavin *et al.*, 1996). The IC_{50} value was determined to be the concentration of IgG at which half of the maximum level of radiolabelled IgG2a remained bound. The binding of radiolabelled monomeric IgG2a (7-20.6/30) and IgG3 (UM3) and IgG2b (49.2) to various mouse FcγRI-transfected COS cells was assayed and K_d and B_{max} values determined by Scatchard plot analysis. The B_{max} value presented is the amount of IgG2a binding sites per cell. Non-specific Ig binding was determined by either the amount of ^{125}I-labelled IgG bound in the presence of 100-fold excess of non-radiolabelled IgG, or by non-specific binding to mock-transfected COS cells and the counts subtracted from total binding to give specific IgG bound.

Erythrocyte antibody rosetting

The expression of FcγRI mutants was tested using erythrocyte antibodies (EAs), wherein sheep erythrocytes were coated with TNP before sensitization with IgG2a anti-TNP (UM2a) as described (Hulett *et al.*, 1991). Monolayers of cells were directly tested for the expression of functional FcγRI by incubation with 2% EA for 10 min at 37°C, then place on ice for 30 min, after which unbound erythrocytes were washed away and the monolayers observed. A cell was scored as binding positively when greater than six red blood cells bound to its surface.

Generation of mutant FcγRI cDNA expression constructs

Mutant FcRs were generated using splice overlap extension PCR (SOE–PCR) (Horton *et al.*, 1989) using either FcγRI-NOD or FcγRI-BALB cDNA. FcγRI-NOD cDNA was used as template for the generation of NOD.B1.N2 and NOD.N1.B2 mutants. Briefly, NOD.B1.N2 was generated using oligonucleotide pairs MDH3 + NM1 and NM2 + TISM6 to generate two fragments which were spliced together using oligonucleotides MDH3 + TISM6. For NOD.N1.B2, oligonucleotide pairs MDH3 + NM3 and NM4 and TISM5, followed by MDH3 + TISM6 were used. Full-length mutant cDNAs (1.2kb) were ligated into the expression vector pCDNA3 (Invitrogen, Carlsbad, CA, USA). The NOD.B1.B2 mutant was generated by restriction enzyme digest of NOD.B1.N2 (*Kpn*I and *Bst*BI) and NOD.N1.B2 (*Bst*BI and *Apa*I), and subsequent ligation of purified fragments into pCDNA3 digested with *Kpn*I and *Apa*I. FcγRI-BALB cDNA was used as a template to generate BALB.N1.B2 and BALB.B1.N2 mutants. Briefly, the generation of BALB.N1.B2 used the oligonucleotide pairs MDH3 + NOD-1 and NOD-2 + TISM6 to generate two fragments which were spliced together using the oligonucleotides MDH3 and TISM6. Similarly, BALB.B1.N2 was generated using MDH3 + NOD-7 and NOD-6 and TISM6, followed by MDH3 + TISM6. Following subcloning into the expression vector pCDNA3 (Invitrogen) the mutant BALB.N1.N2 was generated by ligating purified restriction enzyme fragments (*Bbs*I) of BALB.N1.B2 and BALB.B1.N2. All constructs were confirmed by ABI Prism Dye terminator cycle sequencing, analysed by ABI PRISM 377 (Perkin-Elmer).

Sequencing intron/exon boundaries

Genomic DNA isolated from either NOD/Lt or BALB/c mice was used as template in PCR using Expand™ High Fidelity enzyme according to the manufacturer's recommendations (Boehringer Mannheim, Germany). Oligonucleotides EX1 and EX8, and EX5 and EX6 were used to generate fragments spanning exons encoding domains 1 and 2, and domains 2 and 3 respectively, which were purified and sequenced using ABI PRISM 377.

Oligonucleotides are listed:

NM1: 5′-CCAATCATTGTGGATTTGCAACTG-3′;
NM2: 5′-ATCCACAATGATTGGCTGCTACTC-3′;
NM3: 5′-AACAGCTCTTTCACCGTGATGGA-3′;
NM4: 5′-ACGGTGAAAGAGCTGTTTACCAC-3′;
NOD-1: 5′-CCAATCTTCCTTGTGGATTTGC-3′;
NOD-2: 5′-CCACAAGGAAGATTGGCTGCTA-3′;
NOD-6: 5′-GAAAGCTTTCCCTTTAGAGCTGTTTACCACGC-3′;
NOD-7: 5′-CTCTAAAGGGAAAGCTTTCACCGTGATGGA-3′;
MDH3: 5′-TTTGTCGACATGATTCTTACCAGCTT-3′;
TISM6: 5′-TTTGAATTCCAGTCTGTATATTTGC-3′;
EX1: 5′-caccagtggcttaggttctgc-3′;
EX8: 5′-ggatgcttttgtagtggctcttc-3′;
EX5: 5′-AAGAATAAACTGGTGTACAATGTGG-3′;
EX6: 5′-AAGGAGAAGTGAAGCTGTAAGCC-3′.

The oligonucleotide name is in bold with coding sequence in upper case; the intronic sequence is presented in lower case.

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