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High affinity IgG binding by FcγRI (CD64) is modulated by two distinct IgSF domains and the transmembrane domain of the receptor

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The high affinity IgG receptor, FcγRI, is comprised of three immunoglobulin superfamily (IgSF) domains (EC1, EC2 and EC3), a single transmembrane spanning region, and a short cytoplasmic tail. We have shown a role for three separate domains of FcγRI in the high affinity binding of IgG. Affinity measurements of chimeric FcγRs in which EC1 and EC2 of FcγRI have been replaced with the homologous EC1 and/or EC2 domains of the low affinity IgG receptor, FcγRII indicate that both EC2 and EC3 are essential for high affinity binding of monomeric IgG. Identification of EC3 from FcγRI as the binding site for the monoclonal antibody 10.1, which blocks IgG binding, provides further evidence for the role of this domain in binding. In addition, we have found that the affinity of FcγRI is increased threefold when co-expressed with its accessory molecule, γ-chain. Affinity measurements of further chimeras indicates that the transmembrane domain of FcγRI has a negative influence upon the affinity of the receptor. To account for these observations, we propose that receptor dimerization is required for maximal affinity of FcγRI. Dimerization may serve as the mechanism by which IgG binding triggers several FcγRI-mediated events.
Keywords: CD64/FcγRI/γ-chain/IgG/receptor chimeras

Introduction

Receptors for the Fc domain of IgG (FcγR) play a pivotal role in linking the humoral and the cellular arms of the immune system. Both low affinity (FcγRII) and high affinity (FcγRI) IgG receptors mediate a variety of biological responses, including endocytosis, phagocytosis, antibody-directed cellular cytotoxicity and cytokine release (for reviews see Ravetch and Kinet, 1991; van de Winkel and Capel, 1993; Ravetch, 1997; Daëron, 1997). The low affinity IgG receptor, FcγRII, is a type I integral membrane protein whose ligand binding extracellular region is comprised of two IgSF V-like domains (for review see Raghavan and Bjorkman, 1997). Analysis of the interaction between IgG and FcγRII has shown that the membrane proximal IgSF domain (EC2) is the principal site of interaction with IgG (Hogarth *et al*., 1992; Hulett and Hogarth, 1994; Hulett *et al*., 1994 and 1995). Moreover, a clinically relevant polymorphism that affects the binding of specific IgG isotypes is the result of a single amino acid change in this domain (Parren *et al*., 1992). In contrast, the high affinity IgG receptor, FcγRI, has an extracellular ligand binding region comprised of three IgSF V-like domains. The outer two domains (EC1 and EC2) of FcγRI show considerable homology to the respective domains of FcγRII, but the third domain (EC3) appears unique. It has been postulated that the third IgSF may confer upon FcγRI the ability to bind monomeric IgG (Allen and Seed, 1989; Sears *et al*., 1990). Previous studies have shown that EC3 is indeed necessary, but not sufficient, for high affinity IgG binding (Hulett *et al*., 1991; Porges *et al*., 1992). A more detailed understanding of how IgG binds FcγRI is particularly relevant in light of several recent reports showing that IgG binding to the receptor can elicit a biological response (Harrison *et al*., 1994a; Pfefferkorn *et al*., 1995); previous dogma was that FcγR-mediated events required receptor cross-linking and that binding of IgG to the receptors was functionally passive.

In this study we have constructed three FcγRI chimeric receptors in which EC1 and EC2 of FcγRI have been replaced with EC1 and/or EC2 of FcγRII; the homologous domains of the two receptors. These chimeras have been used to investigate the influence that each domain has on the capacity of the receptor to bind monomeric human IgG. Only the FcγRI-based chimera which retains EC2 and EC3 of FcγRI ($II_{EC1}.I_{EC2}.I_{EC3}$) binds monomeric IgG with high affinity. In contrast, the FcγRI-based chimera which has both EC1 and EC2 from FcγRII ($II_{EC1}.II_{EC2}.I_{EC3}$) can only bind IgG with low affinity. We have also investigated the influence of expression of γ-chain on the affinity of FcγRI for hIgG. The measured affinity of FcγRI for ligand increased nearly threefold when co-expressed with the γ-chain; the measured affinity of FcγRI co-expressed with γ-chain corresponds with the affinity of FcγRI for IgG observed in endogenously expressing cells. Affinity measurements of additional FcγRI-chimeras in which the transmembrane domain of FcγRI has been removed or replaced with TM domains of other receptors suggests a novel mechanism by which this occurs.

Materials and methods

Cells and cell culture

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. COS cells were plated at a density of approximately 4×10^7 cells per 100 mm plate one day before transfection.

Transient expression

The SV40-based expression vector CDM (Seed and Aruffo, 1987) was used for the transient expression of the cDNAs of all clones in COS-7 cells using the DEAE-dextran method (Allen and Seed, 1989). The cDNAs for FcγRI (p135; Allen and Seed, 1989), $FcγRIIa^{HR}$ (PC23, Stengelin *et al*., 1988) and CD2 (Seed and Aruffo, 1987) were in CDM. The cDNA for the γ-chain of FcεRI (Küster *et al*., 1990) was in the vector pSVL. All experiments were performed two or three days post transfection, when surface expression is maximal. Transfection

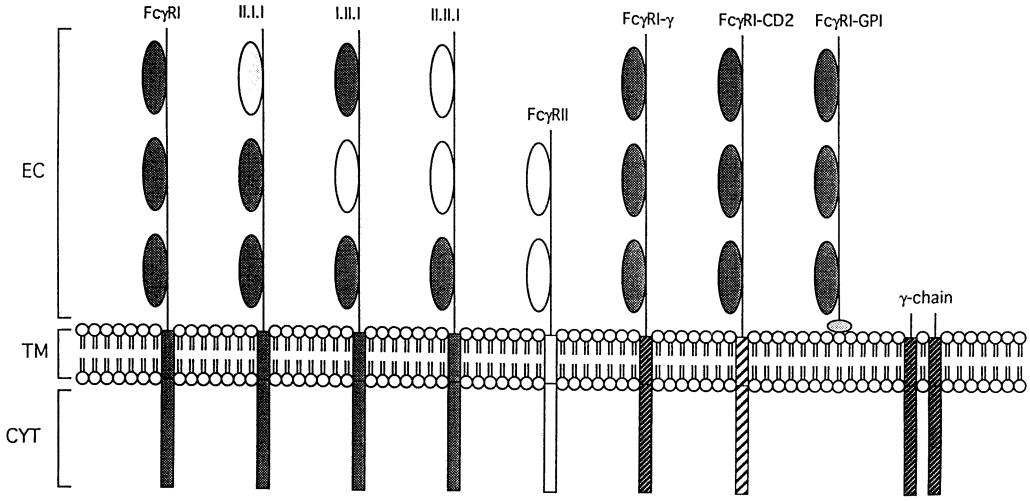

Fig. 1. Cartoon representation of FcγRI, FcγRII, the FcγRI-based chimeras and γ-chain. EC represents the extracellular domain, TM represents the transmembrane domain, and CYT represents the cytoplasmic domain.

efficiency was routinely of the order of 20–30% as assessed by immunofluorescence and FACS analysis (Figure 2).

Chimeric receptor construction

The FcγRI chimeras FcγRI-γ and FcγRI-GPI have been described elsewhere (Harrison *et al*., 1994b; Hutchinson *et al*., 1995). FcγRI-CD2 was constructed by PCR amplification of the transmembrane and cytoplasmic domains of CD2. The forward primer for the amplification of CD2 (5′-GAAAGGTCTAGATCTCTATCTC-3′) contained a *Bgl*II site. The reverse primer (5′-GCTAACTAGAGAACCCACTG-3′) hybridises to the CDM vector. The PCR fragment, which contains an internal *Not*I site was then subcloned between the *Bam*HI and *Not*I sites of FcγRI-GPI (Harrison *et al*., 1994b) to create a clone (FcγRI-CD2) comprising the extracellular domain of FcγRI fused to the transmembrane and cytoplasmic domains of CD2.

Domain swap chimeric receptor construction

All FcγRI:FcγRII domain swap chimeras were constructed in the vector pBluescript II SK^+ and then subcloned into CDM for expression. The domain boundaries were defined by the position of the exon boundaries in the gene sequence of FcγRI (van de Winkel *et al*., 1991). To construct the chimera $II_{EC1}.II_{EC2}.I_{EC3}$, a *Sac*I site was introduced into both FcγRI (using the oligonucleotide 5′-AGAGCTCTTTCCAGC-3′) and FcγRII (using the oligonucleotide 5′-ACTGTCAAA-GAGCTCAGCATG-3′) by site-directed mutagenesis (Künkel, 1985). The 647 bp *Hin*dIII–*Sac*I fragment that contained EC1 and EC2 of FcγRII was subcloned into the *Hin*dIII/*Sac*I sites in FcγRI. To construct the chimera $I_{EC1}.II_{EC2}.I_{EC3}$, a *Pst*I site was introduced into FcγRII (which already contained the new *Sac*I site) using the oligonucleotide 5′-CTGGTGCTGCA-GACC-3′ by site-directed mutagenesis. The 243 bp *Pst*I–*Sac*I fragment that contained EC2 of FcγRII was subcloned into the *Pst*I/*Sac*I sites in FcγRI (*Pst*I site already exists; *Sac*I site introduced by site-directed mutagenesis as above). To construct the chimera $II_{EC1}.I_{EC2}.I_{EC3}$, EC1 of FcγRII was amplified by PCR using a forward primer (5′-TTCCTTCACAAA-GATCCTCT-3′) that hybridises to the CDM vector and a reverse primer (5′-GGGGTATGCATCACCAGCC-3′) that hybridises to FcγRII and introduces an *Nsi*I site. The amplified product, which contains an internal *Hin*dIII site was subcloned into the *Hin*dIII/*Pst*I sites in FcγRI -MANX (Davis *et al*., 1995).

FACS analysis

The monoclonal antibody 10.1 (Dougherty *et al*., 1987) was a gift from Nancy Hogg (ICRF, London). All other antibodies were purchased from Sigma. Binding of mAb, 10.1, (10 nM) and human IgG (5 μM) was assessed by FACS analysis using a Becton Dickinson FACScan. Briefly, 48 h post-transfection, COS cells were harvested in phosphate-buffered saline/1 mM EDTA and incubated with human IgG (5 μM) or mAb (10 nM) for 30 min at 4°C. Binding of primary antibody was determined by using FITC-labelled goat anti-mouse IgG1 or goat anti-human IgG antibodies.

Determination of binding affinity

Human IgG1 (Serotec) was labelled with $Na^{125}I$ (Amersham International) as described (Fraker and Speck, 1978). Forty-eight hours post-transfection, COS cells were harvested as above and incubated with tracer amounts of ^{125}I-human IgG1 (20 pM) in the presence of increasing concentrations of unlabelled human IgG1 (range 5×10^{-10} to 5×10^{-5} M) for 20 min in PBS/5% non-fat milk/0.2% sodium azide. Bound and free fractions were separated by centrifugation through a 3:2 mixture of dibutyl:dioctyl phthalates. Data were corrected for non-specific binding by subtraction of counts bound per minute in the presence of a large excess of IgG (1×10^{-5} M) and were normalized to 1.0 for the counts bound in the presence of tracer alone (absence of any displacing cold human IgG1). The IC_{50} was calculated, being the concentration of human IgG1 that displaces the radiolabelled tracer by 50%, for all the data points (four separate experiments performed in duplicate) for each assay condition. The mean value and standard deviation are shown in the tables.

Results

Design, construction and cell surface expression of domain swap chimeras

The three domain swap chimeras in which EC1 and EC2 of FcγRI were replaced with EC1 and/or EC2 of FcγRII (Figure 1) were designed using the exon boundaries (van de Winkel *et al*., 1991) as a guide to define the domain boundaries. Only one chimera, $I_{EC1}.II_{EC2}.I_{EC3}$, required an amino acid substitution to facilitate cloning; leucine 91 in EC1 of FcγRII was changed to another large hydrophobic amino acid, methionine. Surface

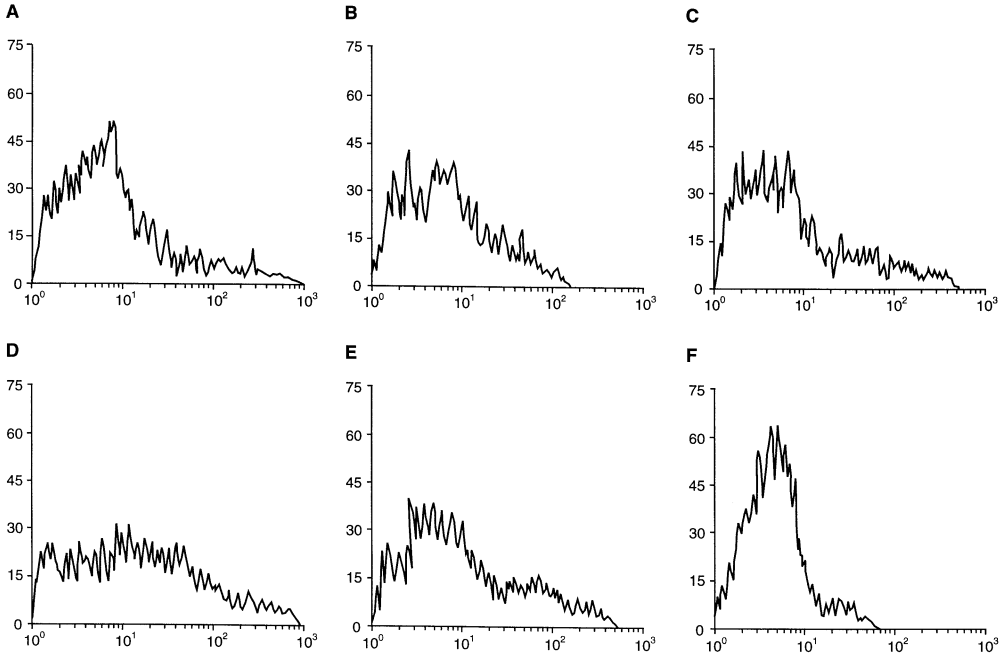

Fig. 2. FACS analysis of COS cells expressing FcγRs and chimeras incubated with polyclonal IgG and FITC-labelled GαH. (**A**) FcγRI; (**B**) FcγRII; (**C**) $II_{EC1}.II_{EC2}.I_{EC3}$; (**D**) $II_{EC1}.I_{EC2}.I_{EC3}$; (**E**) $I_{EC1}.II_{EC2}.I_{EC3}$; (**F**) mock transfected.

expression of the clones was assessed by their ability to bind human polyclonal IgG by FACS analysis (Figure 2). All three chimeras could bind IgG and were expressed at similar levels to wild type FcγRI and FcγRII, with approximately 20–30% of cells expressing FcγRs.

EC2 is required of FcγRI is required for high affinity binding of human IgG1

As shown above, all three domain swap chimeras can bind IgG, at least when present at high concentrations. We have determined the affinity of the various chimeric FcγRs for human monomeric IgG in a quantitative fashion using a displacement assay. The outer two IgSF domains of human and mouse FcγRI show considerable amino acid identity with the respective domains of the two low affinity IgG receptors, FcγRII and FcγRIII. Together with the fact that both these receptors only have two IgSF domains and bind IgG at low affinity, it has been suggested that the third domain of FcγRI confers to the receptor its feature of high affinity (Allen and Seed, 1989; Sears *et al*., 1990). To test whether the third domain was solely responsible for the high affinity characteristics, the affinity of the chimera $II_{EC1}.II_{EC2}.I_{EC3}$ [which comprises the two extracellular domains of hFcγRII fused to the innermost domain (EC3) of hFcγRI] was measured to assess whether it was possible to convert an FcγR with low affinity for monomeric IgG to an FcγR with high affinity by providing the third domain. Although this clone can bind polyclonal human IgG at high concentration when measured by FACS analysis (Figure 2), the affinity of this clone for monomeric IgG was too low to measure in the displacement assay. This suggests that features within the outer two domains of FcγRI, that are not shared with the FcγRII, contribute to the high affinity characteristics of the receptor.

To investigate the role of the outer two domains of FcγRI in the high affinity binding of IgG, we have constructed two additional chimeras. The first chimera, $I_{EC1}.II_{EC2}.I_{EC3}$, has EC2 of FcγRI replaced with EC2 of FcγRII. This clone will also

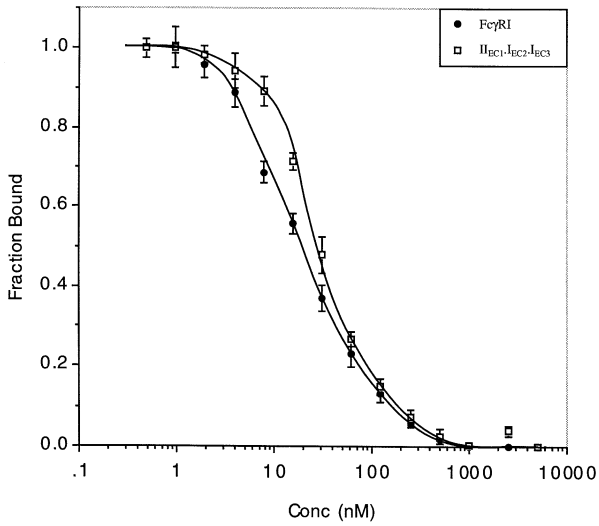

Fig. 3. Typical binding curves from displacement assay of FcγRs binding IgG. IC_{50} values were calculated from binding curves. The chimera $II_{EC1}.I_{EC2}.I_{EC3}$ binds IgG with high affinity (IC_{50} 2.8×10^{-8} M) compared with FcγRI (IC_{50} = 1.8×10^{-8} M). Results shown are mean of four experiments performed in duplicate.

bind human polyclonal IgG at high concentrations when measured by FACS (Figure 2). Again, the affinity was too low to measure in the displacement assay (data not shown), indicating that $I_{EC1}.II_{EC2}.I_{EC3}$ is also a low affinity receptor. The other chimera constructed was $II_{EC1}.I_{EC2}.I_{EC3}$ in which EC1 of FcγRI is replaced by EC1 of FcγRII. This chimera binds IgG by FACS analysis (Figure 2). More importantly, it has a very similar displacement profile to that for FcγRI expressed in COS cells (Figure 3). The calculated IC_{50} for $II_{EC1}.I_{EC2}.I_{EC3}$ was 2.8×10^{-8} M, compared with an IC_{50} of 1.8×10^{-8} M for FcγRI (Table I). Thus, the second domain of FcγRI (EC2) appears essential in mediating the high affinity characteristics.

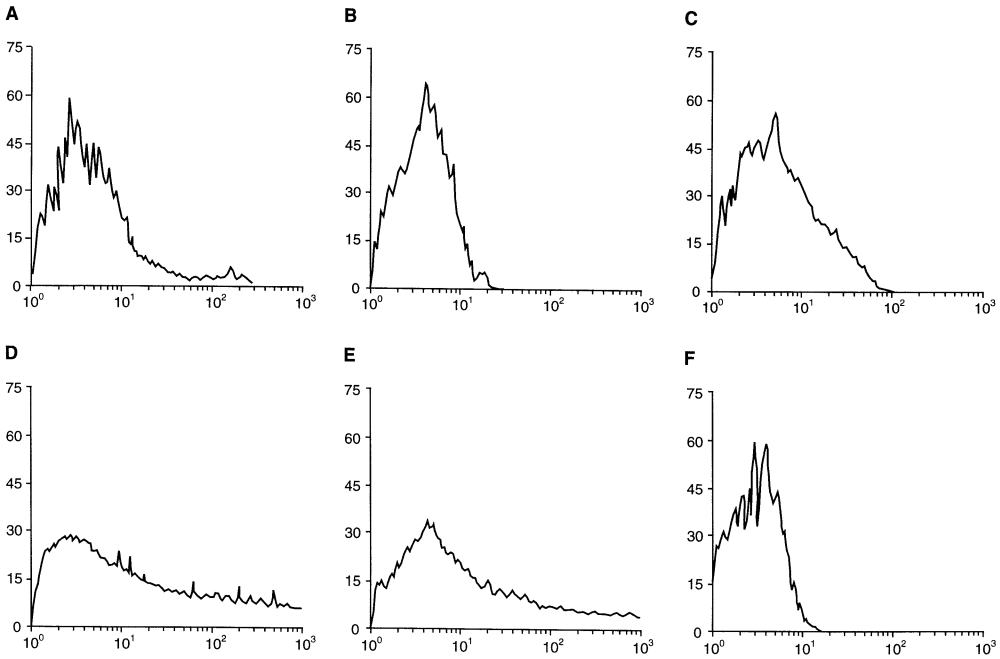

Fig. 4. FACS analysis of COS cells expressing FcγRs and chimeras incubated with mAb 10.1 and FITC-labelled GαM. (**A**) FcγRI; (**B**) FcγRII; (**C**) $II_{EC1}.II_{EC2}.I_{EC3}$; (**D**) $II_{EC1}.I_{EC2}.I_{EC3}$; (**E**) $I_{EC1}.II_{EC2}.I_{EC3}$; (**F**) mock transfected.

Table I. IC_{50} values of human FcγRI, FcγRI-based chimeras and FcγRI co-expressed with γ-chain as measured by displacement assay. Data was calculated from four to eight experiments and is expressed as the average IC_{50} ± standard deviation. Data from previous reports is shown for comparison purposes only.

Clone expressed in COS cells	Ligand	IC_{50} ($\times 10^{-8}$) M	Fold increase relative to FcγRI
FcγRI	hIgG1	1.8 ± 0.25	–
FcγRI	hIgG1	1.8 [a]	–
$II_{EC1}.II_{EC2}.I_{EC3}$	hIgG1	–	–
$I_{EC1}.II_{EC2}.I_{EC3}$	hIgG1	–	–
$II_{EC1}.I_{EC2}.I_{EC3}$	hIgG1	2.8 ± 0.4	0.65

[a]Data from Allen and Seed (1989).

Mapping the IgSF domain recognized by the 10.1 monoclonal antibody: an important role for EC3 in the high affinity binding of monomeric IgG

Previous studies have shown that mAb 10.1 is able to block binding of human IgG3 and murine IgG2a to FcγRI, suggesting that it binds to an epitope of FcγRI at or near to the binding site for the Fc region of IgG (Dougherty *et al*., 1987). By FACS analysis, mAb 10.1 was shown to bind FcγRI and all three chimeras but, as expected, not FcγRII (Figure 4). Since the only IgSF domain shared between these chimeras is EC3, this data indicates that 10.1 binds to an epitope in this domain. Thus, it follows that EC3 of FcγRI constitutes part of, or is near to, the binding site for IgG.

Role of the γ-chain in FcγRI ligand binding affinity

To explore the possible role of the γ-chain in modulating the binding of IgG to FcγRI, the affinity of IgG binding was measured in COS cells expressing FcγRI, in the presence and absence of co-transfected γ-chain, using the displacement assay. The IC_{50} for FcγRI expressed alone in COS cells was 1.8×10^{-8} M, whereas FcγRI co-expressed with γ-chain shows an increased affinity for IgG ($IC_{50} = 0.7\times10^{-8}$ M; Figure 5A). We also measured the affinity of murine FcγRI expressed in COS cells for human IgG ($IC_{50} = 5.4\times10^{-8}$ M; Figure 5B); this was slightly lower than that of hFcγRI. However, the affinity was similarly increased in cells where murine FcγRI was co-expressed with γ-chain ($IC_{50} = 1.9\times10^{-8}$ M; Figure 5B). In both cases, for human (Table II) and murine FcγRI (Table III), the fold increase in affinity observed in cells co-expressing γ-chain was very similar.

The TM domain of FcγRI has a negative effect on the affinity of ligand binding

The mechanism by which γ-chain increases the affinity of FcγRI is unclear, but it does not bind ligand directly (data not shown). However, since FcγRI and γ-chain associate via their respective TM domains (Davis *et al*., 1995; Harrison *et al*., 1995), a conformational change may be induced upon this interaction to facilitate the extracellular domain recognition of ligand. To explore this possibility further, we measured the affinity of a chimeric receptor (FcγRI-γ) in which the TM and cytoplasmic domains of FcγRI were replaced by the equivalent domains of γ-chain (Hutchinson *et al*., 1995). This chimera showed an increased affinity for IgG ($IC_{50} = 0.8\times10^{-8}$ M), relative to FcγRI (Figure 6A); this is a similar increase to that seen for FcγRI co-expressed with γ-chain. This suggests that replacement of the FcγRI TM domain with that of γ-chain may induce a similar conformational change as when FcγRI associates with γ-chain. To determine if this effect was γ-chain specific, a second FcγRI chimera (FcγRI-CD2) was constructed in which the TM and cytoplasmic domains of FcγRI were replaced by the equivalent domains of the adhesion molecule CD2. The increase in affinity displayed by the chimera FcγRI-CD2 ($IC_{50} = 0.7\times10^{-8}$ M; Figure 6B) compared with FcγRI was similar to that observed for FcγRI co-expressed with γ-chain and the chimera FcγRI-γ (Table II).

The affinity of a GPI-anchored version of FcγRI (FcγRI-GPI; Harrison *et al*., 1994b) was also measured using the

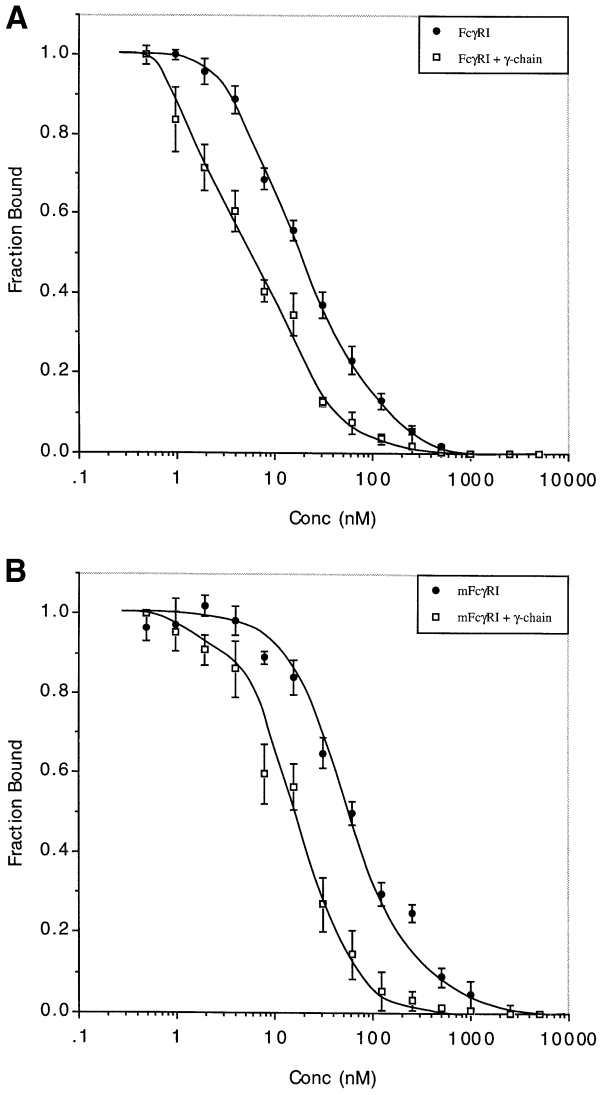

Fig. 5. Typical binding curves from displacement assay of FcγRs binding IgG. IC_{50} values were calculated from binding curves. (**A**) Co-expression of human FcγRI with human γ-chain increases the affinity of the receptor 2.6-fold (FcγRI + γ-chain = 0.7×10^{-8} M). (**B**) Co-expression of murine FcγRI with human γ-chain increases the affinity of the receptor 2.8-fold (mFcγRI, $IC_{50} = 5.4\times10^{-8}$ M; mFcγRI + γ-chain, $IC_{50} = 1.9\times10^{-8}$ M). Results shown are mean of four experiments performed in duplicate.

Table II. IC_{50} values of human FcγRI-based chimeras as measured by displacement assay. Data was calculated from six to eight experiments and is expressed as the average $IC_{50}\pm$standard deviation.

Clone expressed in COS cells	Ligand	IC_{50} ($\times10^{-8}$) M	Fold increase relative to FcγRI
FcγRI	hIgG1	1.8 ± 0.25	–
FcγRI-GPI	hIgG1	0.7 ± 0.05	2.6
FcγRI-γ	hIgG1	0.8 ± 0.1	2.3
FcγRI-CD2	hIgG1	0.7 ± 0.14	2.6
FcγRI + γ-chain	hIgG1	0.7 ± 0.05	2.6
FcγRI (U937 cells)	hIgG1	0.4 ± 0.5[a]	4.5

[a]Note: K_a values from previous work has been expressed as K_d to give a meaningful comparison to IC_{50} values. Values range from 0.1 to 1.0 (Anderson and Abraham, 1980; Fries *et al*., 1982; Kurlander and Batker, 1982).

Table III. IC_{50} values of murine FcγRI in the presence and absence of γ-chain as measured by displacement assay. Data was calculated from six independent experiments and is expressed as the average $IC_{50}\pm$standard deviation. Data from previous reports is shown for comparison purposes only.

Clone expressed in COS cells	Ligand	IC_{50} ($\times10^{-8}$) M	Fold increase relative to FcγRI
mFcγRI	hIgG1	5.4 ± 0.6	–
mFcγRI + γ-chain	hIgG1	1.9 ± 0.5	2.8
mFcγRI	mIgG2a	2.0[a,b]	–
mFcγRI ($P388D_1$ cells)	mIgG2a	0.8[a,c]	2.5

[a]Note: K_a values from previous work has been expressed as K_d to give a meaningful comparison to IC_{50} values.
[b]Data from Sears *et al*. (1990).
[c]Data from Unkeless and Eisen (1975).

displacement assay (Figure 6C). This receptor binds IgG and also shows a similar increase in affinity for IgG ($IC_{50} = 0.7\times10^{-8}$ M) to that observed for FcγRI co-expressed with γ-chain (Table II). Together, these observations indicate that the TM domain of FcγRI also contributes to the affinity of the receptor for ligand, but in a 'negative' manner, and that this negative influence can be offset by co-expression of the γ-chain.

Discussion

Analysis of the molecular basis of the interaction between Fc receptor and immunoglobulins has already revealed much about the biological and physiological functions of this family of cell surface receptors. However, the interactions between FcγRI and its ligand are still largely unexplored, and could lead to a better understanding of how the binding of monomeric IgG1 to the receptor leads to its dissociation from actin binding protein (Ohta *et al*., 1991), triggers receptor mediated endocytosis (Harrison *et al*., 1994a) and primes cells for superoxide release (Pfefferkorn *et al*., 1995).

It was originally predicted that the high affinity binding of FcγRI was attributable to the presence of EC3 (Allen and Seed, 1989; Sears *et al*., 1990). Indeed, removal of EC3 from murine FcγRI reduces its affinity (Hulett *et al*., 1991) and a naturally occurring splice variant of human FcγRI that lacks EC3 has been found to bind IgG with low affinity (Ernst *et al*., 1992; Porges *et al*., 1992). However, further studies have shown that the situation is more complex. Hulett *et al*. (1991) have shown that EC3 alone cannot confer upon a low affinity mouse Fc receptor the ability to bind IgG with high affinity. We have found that this is also the case for human Fc receptors. In fact, it turns out that both EC2 and EC3 of human FcγRI are required for high affinity IgG binding. Studies of FcγRII (Hulett *et al*., 1994), FcγRIII (Tamm *et al*., 1996) and FcεRI (Hulett *et al*., 1994) have all implicated EC2 as the major contact domain of each receptor with its cognate immunoglobulin (for review see Raghavan and Bjorkman, 1997). Since EC2 of FcγRI has significant sequence similarity with EC2 from these other Fc receptors (see Table IV), it seems likely that EC2 of FcγRI also plays a direct role in ligand binding. In support of this we have identified two single amino acid substitutions which significantly reduce IgG binding in this domain (unpublished data), one of which lies in a highly conserved region of the FcRs (Arg_{175} to Gly_{180} in FcγRI). This region has previously been speculated to play a role in IgG binding by FcγRI (Sears *et al*., 1990; Symons and Clarkson, 1992) and overlaps with the major IgG binding site of FcγRII

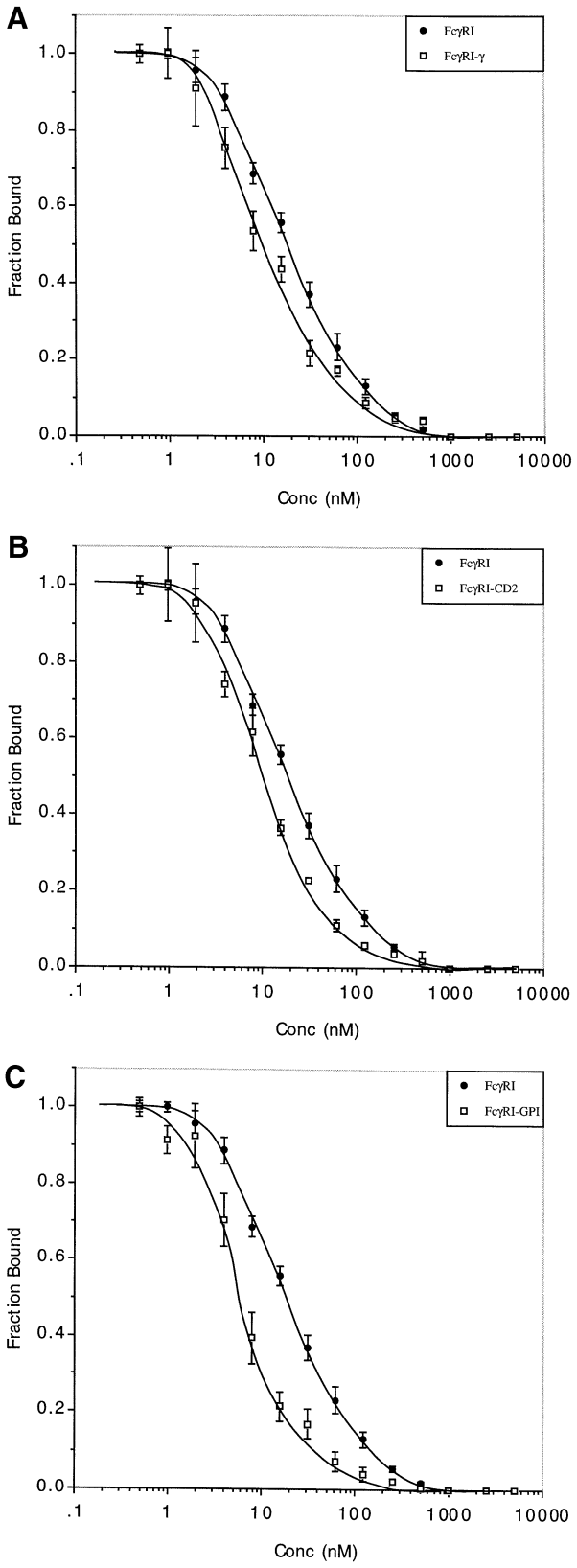

Fig. 6. Typical binding curves from displacement assay of FcγRs binding IgG. IC_{50} values were calculated from binding curves from six to eight experiments. (**A**) The chimera FcγRI-γ binds IgG with higher an affinity 2.3-fold higher (IC_{50} 0.8×10^{-8} M) than FcγRI. (**B**) The chimera FcγRI-CD2 binds IgG with higher an affinity 2.6-fold higher (IC_{50} 0.7×10^{-8} M) than FcγRI. (**C**) The chimera FcγRI-GPI binds IgG with higher an affinity 2.6-fold higher (IC_{50} 0.8×10^{-8} M) than FcγRI. Results shown are mean of four experiments performed in duplicate.

Table IV. Table of amino acid identity (shown in bold) and similarity between EC2 domains of FcγRI, FcγRII, FcγRIII and FcεRI. Values are calculated from the sequence alignment of Raghavan and Bjorkman (1997).

	FcγRI	FcγRII	FcγRIII	FcεRI
FcγRI	–	65.9% (54/82)	63.4% (52/82)	65.9% (54/82)
FcγRII	41.5% (34/82)	–	61.0% (50/82)	53.7% (44/82)
FcγRIII	40.2% (33/82)	46.3% (38/82)	–	58.5% (48/82)
FcεRI	42.7% (35/82)	34.1% (28/82)	42.7% (82/82)	–

and a second IgE binding site of FcεRI (Hulett *et al*., 1994). Since EC2 of FcγRI almost certainly binds ligand directly, what then is the role for EC3? We have shown that 10.1, a monoclonal antibody previously shown to bind at or near the binding site for IgG (Dougherty *et al*., 1987), recognizes this third domain. However, this does not tell us whether EC3 binds ligand directly and/or plays a modulatory role in IgG binding (see below).

We have also shown that co-expression of either human or mouse FcγRI with their accessory molecule, γ-chain, increases the affinity of both receptors for human IgG1 by approximately threefold. Interestingly, the higher affinity of FcγRI when co-expressed with γ-chain in COS cells (relative to FcγRI expressed alone in COS cells) is remarkably similar to the affinity of FcγRI when endogenously expressed in macrophages and monocytes where the γ-chain is constitutively expressed and physically associates with FcγRI (Ernst *et al*., 1993). A similar enhancement in binding affinity for murine IgG2a has been shown to be conferred on both human and mouse FcγRI by co-expression of the γ-chain in COS cells (Miller *et al*., 1996). Recruitment of the γ-chain by FcγRI is necessary for the activation of tyrosine kinases such as syk following receptor aggregation to initiate intracellular signalling cascades (for review see Daëron, 1997). A corollary of the observation that an association with γ-chain is necessary for maximal FcγRI affinity for ligand means that receptor molecules that are 'pre-coupled' to the accessory, effector molecule will bind IgG more readily than the free receptor, thus facilitating the induction of signal transduction pathways involved in macrophage activation. The finding that γ-chain can modulate the affinity of FcγRI for ligand contrasts with the situation observed for FcεRI, which also uses γ-chain as a signalling molecule (for review see Sutton and Gould, 1993), where it has been shown that only the α-chain is necessary for high affinity binding (Hakim *et al*., 1990). However, since FcεRI requires co-expression of γ-chain for surface expression (whereas FcγRI does not), these experiments were performed using a chimeric version of FcεRI in which its TM and cytoplasmic domains were replaced by the corresponding domains of the IL-2R (an unrelated type I membrane protein). Since this chimeric receptor had the same affinity as FcεRI co-expressed with γ-chain, it was proposed that γ-chain did not have an effect on binding (Hakim *et al*., 1990). However, in the light of our observation that FcγRI chimeras in which the TM domain is replaced have higher affinity than the wild type receptor, this interpretation needs to be treated with caution.

FcγRI interacts with γ-chain via its TM domain (Indik *et al*., 1994; Davis *et al*., 1995; Harrison *et al*., 1995; Harrison, 1996). In an attempt to understand the mechanism by which the association between γ-chain and FcγRI leads to an increased affinity for IgG, we measured the affinity of several additional FcγRI-chimeras. Surprisingly, when the TM domain (and

cytoplasmic tail) of FcγRI were replaced by the TM domain (and cytoplasmic tail) of γ-chain, a similar increase in affinity for IgG, relative to wild type FcγRI, was observed. This effect was not specific to γ-chain, as an almost identical increase in affinity was seen when the TM domain (and cytoplasmic tail) of FcγRI was replaced by the TM domain (and cytoplasmic tail) of CD2. Taken together, these observations strongly suggest that the TM domain of FcγRI has a negative effect on the affinity of the receptor. What is the basis of the negative effect of the TM domain? One clue comes from the observation that a GPI-anchored version of FcγRI also increases the affinity for IgG by a factor of three. This is of particular interest, since the GPI-anchored version of FcγRIII has a lower affinity than its counterpart that is co-expressed with γ-chain (van de Winkel and Capel, 1993; Miller *et al*., 1996). Since a GPI-anchored version of FcγRI has the same affinity as FcγRI co-expressed with γ-chain, but the GPI-anchored version of FcγRIII has a significantly lower affinity than FcγRIII co-expressed with γ-chain, this implies that there is a novel feature of FcγRI that explains this difference.

We propose the following model to account for the latter observation concerning the TM domain of FcγRI. Since sequences within the TM domain have a negative effect on affinity, this suggests that a conformational change of some kind is induced upon interaction with the γ-chain, or when the TM domain is removed/replaced. Since FcγRI is a type I membrane protein with a single TM spanning domain, one possible way to envisage the TM inducing such a conformational change is by dimerization of the receptor. Thus, if the TM domain of FcγRI (in the absence of γ-chain) is oriented such that dimerization is not favoured, the presence of the γ-chain could cause a re-orientation facilitating dimer formation. To account for the observation that the GPI-anchored version of FcγRI, and the chimeras FcγRI-CD2 and FcγRI-γ, also have increased affinity, dimerization should be mediated through the extracellular domain; the obvious candidate is EC3 which is unique to FcγRI.

Previous studies have investigated the stoichiometry of the FcγRI:IgG interaction. Koolwijk *et al*. (1989) tried to determine the number of FcγRI molecules that a single IgG molecule could bind using hybrid mouse antibodies. They showed that a hybrid IgG consisting of one mIgG2a Fc and one mIgG1 Fc bound with ~3.7-fold lower affinity than a wild type antibody comprising two mIgG2a Fc domains. On the basis that FcγRI can bind mIgG2a, but not mIgG1, they suggest that the interaction of a single Fc is sufficient for high affinity binding, thus apparently ruling out receptor dimerization. However, since our model proposes that a 3-fold increase in affinity occurs upon dimerization, their data actually supports our model.

Dimerization of FcγRI may, at least in part, explain how the mAb 10.1 displaces IgG from FcγRI, even though 100% displacement does not occur (Dougherty *et al*., 1987). Dimerization could also explain how binding of monomeric IgG to FcγRI triggers endocytosis (Harrison *et al*., 1994a) and primes cells for superoxide formation (Pfefferkorn *et al*., 1995). Without dimerization, it is difficult to conceive how a single spanning TM domain could convey any conformational changes induced upon ligand binding across the lipid bilayer. In contrast, it is relatively simple to envisage how this might happen if the receptor where to dimerize. Indeed, dimerization is a widely used mechanism used by cell surface receptors for signal transduction.

In summary, we have shown a role for three separate domains of FcγRI in the high affinity binding of IgG. EC2 and EC3 are both required for high affinity binding of monomeric IgG. EC2 is likely to have a direct interaction with IgG, whereas EC3, which is the site of binding of the mAb 10.1, appears to play a role in receptor dimerization. Maximal affinity of the receptor is only achieved when it interacts with the γ-chain. Careful interpretation of these findings leads us to the conclusion that two molecules of FcγRI can bind a single IgG molecule, thereby explaining how binding of IgG triggers at least two FcγRI-mediated responses.

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