

Characterization and allelic polymorphisms of rhesus macaque (*Macaca mulatta*) IgG Fc receptor genes

Doan C. Nguyen · Franco Scinicariello ·
Roberta Attanasio

Received: 4 November 2010 / Accepted: 11 January 2011 / Published online: 16 February 2011
© Springer-Verlag 2011

Abstract Macaque models are invaluable for AIDS research. Indeed, initial development of HIV-1 vaccines relies heavily on simian immunodeficiency virus-infected rhesus macaques. Neutralizing antibodies, a major component of anti-HIV protective responses, ultimately interact with Fc receptors on phagocytic and natural killer cells to eliminate the pathogen. Despite the major role that Fc receptors play in protective responses, there is very limited information available on these molecules in rhesus macaques. Therefore, in this study, rhesus macaque CD32 (FcγRII) and CD64 (FcγRI) homologues were genetically characterized. In addition, presence of CD16 (FcγRIII), CD32, and CD64 allelic polymorphisms were determined in a group of nine animals. Results from this study show that the predicted structures of macaque CD32 and CD64 are highly similar to their human counterparts. Macaque and human CD32 and CD64 extracellular domains are 88–90% and 94–95% homologous, respectively. Although all cysteines are conserved between the two species, macaque CD32 exhibits two additional N-linked glycosylation sites, whereas CD64 lacks three of them when compared to humans. Five CD32, three CD64, and three CD16 distinct allelic sequences were identified in the nine animals examined, indicating a relatively high level of polymorphism in macaque Fcγ receptors. Together, these results

validate rhesus macaques as models for vaccine development and antibody responses, while at the same time, underscoring the need to take into account the high degree of genetic heterogeneity present in this species when designing experimental protocols.

Keywords Fc receptor · FcγR · CD16 · CD32 · CD64 · Allele · Polymorphism · Rhesus macaque

Introduction

Fc receptors (FcR) are membrane-bound glycoproteins that specifically recognize and bind the multifunctional Fc portion of immunoglobulins (IG). Binding of these cell-surface receptors to IG-opsonized particles (pathogens or antigens, including autoantigens and allergens) triggers cell signal transduction events, thus initiating various effector functions and ultimately resulting in activation or inhibition of immune-competent cells (Ravetch and Bolland 2001). FcR, therefore, form a bridge between humoral and cell-based immunity and couple the potency of cellular effector responses with the specificity of IG.

FcR are expressed on most of immunologically active and competent cells (Sondermann and Oosthuizen 2002). In humans, IgG Fc receptors (FcγR) are classified into three classes, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) (Jefferis and Lund 2002). Structurally, all FcγR are type I transmembrane glycoproteins, are members of the immunoglobulin superfamily (IgSF) and include a unique ligand-binding chain consisting of two or three C-like extracellular domains, followed by a connecting region (CO), a transmembrane region (TM) and a cytoplasmic region (CY) (Daëron 1997). A total of eight genes have been identified for the human FcγR, including three genes

D. C. Nguyen · R. Attanasio (✉)
Department of Biology, Georgia State University,
Atlanta, GA 30303, USA
e-mail: rattanasio@gsu.edu

F. Scinicariello
Division of Toxicology and Environmental Medicine,
Agency for Toxic Substances and Disease Registry,
Centers for Disease Control and Prevention,
Atlanta, GA 30333, USA

for the high-affinity IgG receptor Fc γ RI (Fc γ RIa, Fc γ RIb, and Fc γ RIc) as well as five genes for the low-affinity IgG receptors Fc γ RII (Fc γ RIIa, Fc γ RIIb, and Fc γ RIIc) and Fc γ RIII (Fc γ RIIIa and Fc γ RIIIb). All classes are encoded by a gene cluster located on chromosome 1. Specifically, the low-affinity *Fc γ R* genes and the genes for the Fc γ RI α and FcR common γ (FcR γ) subunits are clustered on chromosome 1q23, whereas the three human *Fc γ RI* genes map to chromosome 1q21 (Takai 2005). The low-affinity Fc γ R genomic locus is complex, as it contains regions of copy number variation that may alter receptor expression and leukocyte responses to IgG (Niederer et al. 2010).

Fc γ R genes share common overall intron–exon genetic organization consisting of five or six exons: two encoding the leader (L) region, two or three encoding the C-like domains ([D]), and one encoding the connecting transmembrane and cytoplasmic (CO-TM-CY) regions (van de Winkel and Capel 1993). CD32 and CD16 contain two conserved C-like domains, the N-terminal (membrane-distal) [D1] and C-terminal (membrane-proximal) [D2], whereas CD64 possesses a third domain, [D3] (Ravetch and Bolland 2001). The products of the eight human *Fc γ R* genes exhibit considerable genetic, structural, and functional diversity, and also differ significantly in both binding affinity and specificity for their ligands (IgG subclasses), as well as in cellular distribution pattern and effector functions (Jefferis and Lund 2002; Ravetch and Kinet 1991). Adding to the complexity of distinct expression patterns, recognition, and activation profiles of Fc γ R, is the presence of multiple allelic polymorphisms (Jefferis and Lund 2002; Takai 2005). Characterization of these allelic polymorphisms and their relations with two-dimensional and three-dimensional structures are important for the understanding of their physiological effects. In this context, IMGT Collier de Perles graphical two-dimensional representations may provide useful information for relating structure to potential function (Bertrand et al. 2004).

The correlation between allelic polymorphism and function has been shown, in particular, for R131 (CD32a) ([D2] R80, IMGT unique numbering), F158 (CD16a) ([D2] F108, IMGT unique numbering), and NA2 (CD16b), which exhibit higher affinity and functional activity compared to their corresponding alleles (H131, V158, and NA1, respectively) (Binstadt et al. 2003). As a result, these allelic variants have the ability to affect susceptibility and/or severity of certain autoimmune disorders, especially systemic lupus erythematosus, as well as inflammatory and infectious diseases (Binstadt et al. 2003; van Sorge et al. 2003; Takai 2005). Thus, even a single amino acid change in Fc γ R could result in significant differences in functional properties and, ultimately, establish clinical relevance.

Rhesus macaques are being increasingly used as animal models for studies involving a variety of human diseases, as

well as for assessing therapeutics and vaccines, especially for AIDS research (Hérodin et al. 2005; Staprans et al. 2010). The design and interpretation of experimental data related to these studies often require the evaluation of humoral responses and are typically based on the assumption that rhesus macaque antibody molecules and their interactions with Fc receptors mimic those present in humans. Although the information available on rhesus macaque antibody molecules and corresponding receptors is steadily increasing (Calvas et al. 1999; Scinicariello and Attanasio 2001; Scinicariello et al. 2004; Rogers et al. 2004, 2006a, 2008), a basic characterization is still lacking for the majority of Fc receptors. The only rhesus macaque *Fc γ R* gene currently characterized is Fc γ RIII. This gene shows 91.7% identity to the human Fc γ RIIIa (Rogers et al. 2006b). Further characterization of Fc γ R in macaques is necessary, especially in light of the finding that despite the extensive sequence identity and almost complete overlap of amino acids involved in antibody Fc binding, human and macaque CD16 molecules differ as it relates to number of isoforms (no CD16b has been identified in macaques) and cell-type expression patterns (as compared to human neutrophils, known to express CD16, macaque neutrophils do not appear to express this protein) (Rogers et al. 2006b). Additionally, rhesus macaques are known to exhibit a high level of antibody constant region polymorphism (Rogers et al. 2008; Scinicariello and Attanasio 2001; Scinicariello et al. 2004), thus possibly resulting in heterogenous interactions with Fc receptors. Indeed, a number of studies have demonstrated that individual rhesus macaques exhibit variable susceptibility to infectious diseases, particularly AIDS (Joag et al. 1994; Ling et al. 2002; Marthas et al. 2001; Reimann et al. 2005). Moreover, several macaque CD16 polymorphisms have been identified and appear responsible for affecting treatment with a monoclonal antibody (Miller et al. 2007). Together, these observations highlight the need to further characterize Fc receptor molecules in rhesus macaques.

Materials and methods

Blood samples Heparinized blood samples were collected from nine healthy rhesus macaque (*Macaca mulatta*) of different ages housed at the Language Research Center of Georgia State University, Atlanta, GA. Animal blood collection was done according to local and federal guidelines.

Total RNA isolation, reverse transcription, and cDNA synthesis of *Fc γ R* genes Total RNA was extracted from whole blood using QiaAmp RNA Blood Mini kit (Qiagen Inc., Valencia, CA). Total RNA (~1–3 μ g) was then reverse

transcribed into single-stranded cDNA using either oligo (dT)₁₅ or random primers, followed by primer extension with the AMV reverse transcriptase (Roche Diagnostics North America, Indianapolis, IN), in a total reaction volume of 15–30 μ l. Next, the resulting first-strand cDNA products were serially diluted and PCR-amplified with Expand High Fidelity polymerase (Roche Diagnostics North America, Indianapolis, IN) using appropriate oligonucleotide primer pairs (see below) in a total of 50 or 100 μ l reaction volume. PCR conditions were as follows: after initial denaturation at 95°C for 10 min, the cDNAs were amplified for 40 cycles, with each cycle consisting of 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min and 30 s. To ensure complete extension, a final step at 72°C for 10 min was applied. At least two independent RT-PCR reactions using the same total RNA were performed, and from 10 to 15 independent cDNA clones per individual macaque and from each of two separate PCR reactions were analyzed. These steps were performed to rule out that the putative allelic polymorphisms were due to a PCR amplification artifact.

Design of primers Primers for amplification of macaque *Fc γ RII* (CD32) and *Fc γ RI* (CD64) genes were designed from the corresponding human sequences (assuming conserved homology between primates). The terminal sense and antisense *Fc γ RII*/*Fc γ RI* primers are located within the start of the leader region (exon 1) and within the end of the cytoplasmic region (*Fc γ RII* exon 5 and *Fc γ RI* exon 6), respectively, of the cDNAs shared by multiple human *Fc γ RIIa*/*Fc γ RIa* sequences (Table 1). Internal primers for each of these two receptors were situated within exon 4, which encodes the [D2] domain of CD32, or exon 5, which encodes the [D3] domain of CD64. Using these primer sets,

the PCR amplification product should span most of the *Fc γ RII* or *Fc γ RI*-targeted cDNAs (covering from exon 1 to the most 3' exon). Specifically, the CD32.1 and CD32.2 primer pair was used to generate a 566-bp product spanning the first part of *Fc γ RII*, beginning from the leader region. CD32.3 and CD32.8 primer pair would amplify a 502-bp fragment that had a 191-bp overlapping sequence with the fragment produced by CD32.1 and CD32.2, and that spanned the second part of *Fc γ RII*, including the CO-TM-CY region. Similarly, two *Fc γ RI* fragments that had a 194-bp overlapping sequence were amplified by the use of CD64.1 and CD64.4 (yielding an 800-bp fragment) and CD64.9 and CD64.10 (generating a 424-bp fragment) primer pairs. For *Fc γ RIII*, primers included FCG3aF (located within the start of the leader region) and FCG3aR (located within the end of the cytoplasmic region) (Table 1). All primers were designed to generate *Fc γ RIIa*, *Fc γ RIa*, and *Fc γ RIIIa* homologue products, and were used for each individual animal of the nine macaques examined.

Cloning and sequencing of amplified *Fc γ R* cDNAs

Appropriate volume of PCR reactions were run on a 2% agarose gel and the bands of interest (showing expected size) were excised and purified using QIAquick Gel Extraction kit (Qiagen Inc., Valencia, CA) using a microcentrifuge. The purified PCR products were then cloned into the TOPO TA pCR2.1 cloning vector (Invitrogen, Carlsbad, CA) for sequencing analysis. The plasmids were transformed into Top10 *Escherichia coli* chemically competent cells using TOPO TA Cloning and One Shot Chemical Transformation following the manufacturer's protocols (Invitrogen, Carlsbad, CA). Subsequently, 10–15 inserted, positive colonies from each sample were picked and incubated

Table 1 Primers used for amplification of rhesus macaque *Fc γ R*^a cDNAs

Primer name	Sequence (5'-3')	Product size (bp)	Position	Location	Accession number ^c
FCG3aF (fw) ^b	ATGTGGCAGCTGCTCCTCCCA	765	(-51)–(-31)	L-encoded region	DQ423379.1
FCG3aR (rv)	TCATTGTCTTGAGGGTCCTT		(714)–(694)	CY-encoded region	DQ423379.1
CD32.1 (fw)	TTTTGCTGCTGCTGGCTTCTGC	556	(-32)–(-11)	L-encoded region	BC020823.1
CD32.2 (rv)	ATGCTGGCACTTGACAGTGATG		(527)–(504)	[D2]-encoded exon 4	BC020823.1
CD32.3 (fw)	AAGGACAAGCCTCTGATCAAGGTC	502	(337)–(360)	[D2]-encoded exon 4	XM_001118066.2
CD32.8 (rv)	TGACATGGTCGTTGGGAGGAAG		(838)–(817)	CY-encoded region	BC020823.1
CD64.1 (fw)	GACAGCTCTGCTCCTTGGGTTTC	800	(-40)–(-18)	L-encoded region	NM_000566.3
CD64.4 (rv)	AGGACATTTCCGTCTTCTGTGG		(758)–(737)	[D3]-encoded exon 5	NM_000566.3
CD64.9 (fw)	AATCTGGTACCCTGAGCTGTG	424	(565)–(586)	[D3]-encoded exon 5	NM_000566.3
CD64.10 (rv)	AATGTCTGTCTTCTGAAGGCTGG		(988)–(965)	CY-encoded region	NM_000566.3

^a Abbreviation and full gene designations are the following: *Fc γ RI*: IgG Fc high-affinity I receptor (CD64); *Fc γ RII*: IgG Fc low-affinity II receptor (CD32)

^b fw, forward direction; rv, reverse direction

^c GenBank accession number for the reference sequences used for designing primers

overnight in LB culture tubes at 37°C in a shaking rotary incubator (200–230 rpm). The minipreps DNA Purification System kit (Promega Corporation, Madison, WI) was used to purify plasmid DNA, and the plasmid DNA was then screened on a 1% agarose gel after digestion with EcoRI to confirm the inserted fragment size. All DNA sequences were determined using the ABI Prism Dye Termination Cycle Sequencing kit and on an ABI model 3100 automated sequencing apparatus (PerkinElmer, Wellesley, MA). Sequencing primers were those used for PCR amplifications and the forward and reverse M13 primers.

Sequence analysis MacVector software (Accelrys Inc., San Diego, CA) and BioEdit program (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) were used to identify overlapping regions and edit sequences. Sequences were aligned with each other within the same receptor group and with other known counterparts using the CLUSTAL function of the MEGALIGN, part of the LASERGENE software package (DNASTAR Inc., Madison, WI). Construction of the phylogenetic tree was also based on the use of the LASERGENE's MEGALIGN part. Prediction of potential glycosylation site was performed online using EnsembleGly website (<http://turing.cs.iastate.edu/EnsembleGly/>; Caragea et al. 2007). Standardized nomenclature, labels and numbering of IMGT®, the international ImMunoGeneTics information system® (<http://www.imgt.org>) (Lefranc et al. 2009) were used to display and discuss sequencing data based on human reference sequences. Allelic polymorphisms and characteristic positions of the C-like domains were described using the IMGT unique numbering for C domain (C-DOMAIN of IG and T cell receptors (TR) and C-LIKE-DOMAIN of IgSF other than IG and TR) (Lefranc et al. 2005).

Results

We identified macaque FcγR at the cDNA level and examined whether allelic polymorphisms were present by analyzing PCR-amplified cDNA sequences. These sequences were obtained from reverse transcription of whole blood total RNA extracts, followed by PCR amplification using primers complementary to human sequences located within the beginning L and the ending CO-TM-CY exons. All PCR amplifications were successful in each of the nine animals examined. The introduction of PCR artifacts was minimized by the use of a high-fidelity polymerase with proofreading ability, and by carrying out separate PCRs and multiple clone selections for each individual animal. The cDNA sequences determined in the present study are available as GenBank accession numbers HQ423386-

HQ423388 (FcγRIII), HQ423389-HQ423393 (FcγRII), and HQ423394-HQ423396 (FcγRI).

Macaque FcγRII and FcγRI cDNA clones exhibit the typical mRNA primary structure of their human counterpart

Using the primer sets which were designed based on human *FcγRII* gene sequences, we identified an almost full-length macaque FcγRII homologue cDNA. The molecule contains 865 bp, encoding a 288-amino acid protein (Fig. 1; shown is a representative sequence). The structure of the deduced amino acid sequence was inferred by analogy to published human sequences. It was found extensively similar to that described for human FcγRIIa mRNA structure, typical of type I membrane IgSF C-like domain proteins. The first 12-amino acid stretch, which formed the partial putative N-terminal leader (L) region, is followed by a predicted mature protein (Fig. 1). The mature protein is composed of an extracellular portion of 176 amino acids, which, as previously described for the human counterpart receptor, contains two extracellular domains, namely [D1] and [D2]. As expected, four cysteines (C26, C68, C107, and C151), that have potential to form the characteristic C-like intradomain disulfide bonds, are conserved in the extracellular portion (1st-CYS 23 and 2nd-CYS 104 in the IMGT unique numbering; Lefranc et al. 2005). The conserved C-like domain structure suggests that disulfide bonds are formed between C26 and C68 and between C107 and C151 in [D1] and [D2], respectively.

The conserved C-like extracellular portion is followed by a relatively short segment of 32 amino acids (AA) that, by analogy to human CD32a, represents the connecting region (CO) of 10 AA and the transmembrane region (TM) of 22 AA. The remaining amino acids of the deduced sequence form the partial C-terminal cytoplasmic (CY) region. Three potential N-linked glycosylation sites are conserved between humans and macaques, two of which, [D1] N97 and [D2] N95 (IMGT unique numbering; Lefranc et al. 2005) are present in the extracellular portion, whereas the third one, [CY] N10, located in the CY region, has probably no functional significance (Table 2). Macaques carry two additional N-linked glycosylation sites [D2] N77 and N88 (resulting from amino acid changes) compared to humans (Table 2 and Fig. 2). However, these two positions are polymorphic: the first one, [D2] 77, in rhesus macaques, the N glycosylation site being or not present (Fig. 2), whereas the second one, [D2] 88, is polymorphic in humans, and may have an N-glycosylated site as shown in the '2fcb' 3D structure (IMGT/3Dstructure-DB; Ehrenmann et al. 2010). As in humans, two YxxL signatures, separated by 11 amino acids and representing a typical immunoreceptor

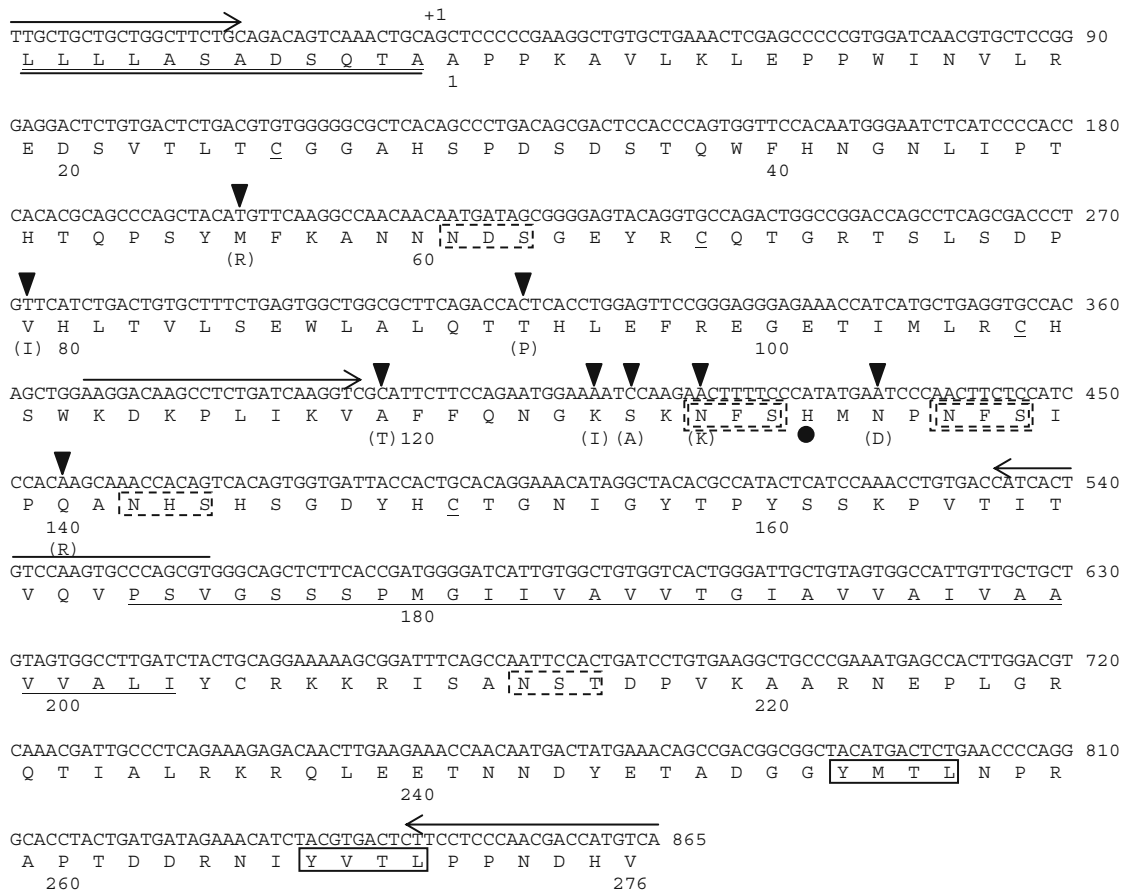


Fig. 1 Nucleotide and deduced amino acid sequences of rhesus macaque Fc γ receptor II (Fc γ RII, CD32) (GenBank accession number: HQ423393). Nucleotides are numbered at right and amino acids are numbered throughout. The amino acid numbering is in accordance with the IMGT unique numbering system for Ig C-like domains (Lefranc et al. 2005). Following the N-terminal signal sequence (a 12 amino acid part is shown *doubly underlined*) is the extracellular portion of ~180 amino acids in length. The predicted start site of the mature protein is indicated by the number “1” below the amino acid A. +1, above the nucleotide sequence, indicates the start of the extracellular chain. *Underlined Cs* ($n=4$) indicates cysteines that have the potential to form intra-chain disulfide bonds. Potential N-linked glycosylation sites ($n=5$; Caragea et al. 2007) (N-x-S/T; x denotes any amino acid) are represented by broken boxes, of which those with *broken double-line* ($n=2$) denote potential N-linked glycosylation sites not recognized in humans. The underlined

sequence corresponds to the CO-TM region. Closed boxes illustrate the two YxxL signatures, separated by 11 amino acids in the intracellular cytoplasmic tail, which represent a typical immunoreceptor tyrosine-based activation motif (ITAM; YxxLx(7–12)YxxL) in the molecule. The two *over-line arrows* at the beginning and the end of the nucleotide sequence represent the binding regions and directions for the designed CD32.1 (forward) and CD32.8 (reverse) primers, respectively. The other two *over-line arrows* in the middle of the sequence denote internal primers (CD32.3 (forward) and CD32.2 (reverse), in that order). *Arrowheads* indicate the allelic positions at which the polymorphic amino acids (represented by amino acids displayed below and in parentheses) identified in this work are present. The *closed black circle* indicates a functional polymorphic amino acid in human CD32 (131 H/R), known to define the specificity of human CD32 for IgG2

tyrosine-based activation motif (ITAM; YxxLx(7–12)YxxL), are present in the CY region.

Similar to Fc γ RII, macaque Fc γ RI cDNA clones also show a typical human Fc γ RI mRNA organization. The obtained 1,027-bp-encoded 342-amino acid polypeptide starts with a putatively partial leader region of 13 residues, followed by the extracellular region consisting of three domains [D1], [D2], and [D3] and is 267 amino acids in length, ending with a CO region (7AA), a TM region (23AA) and a partial CY region (Fig. 3). As in humans, six

cysteines (C26 and C68, C107 and C151, as well as C195 and C243), each pair of which (1st-CYS 23 and 2nd-CYS 104 in the IMGT unique numbering; Lefranc et al. 2005) forms disulfide bonds of the [D1], [D2] and [D3] domain, respectively, are conserved in macaques. However, only four potential N-linked glycosylation sites, [D1] N44, [D2] N95, [D3] N6 and N80 (Table 2), are present in macaques as compared to the seven present in humans (Fig. 4).

Together, these results show that rhesus macaque Fc γ RII and Fc γ RI cDNA clones exhibit the typical mRNA

Table 2 Correspondence between sequence positions and IMGT unique numbering for potential N-linked glycosylation sites in rhesus macaque FcγRs^a

Position	CD32 domains					CD64 domains				
	[D1]		[D2]	[CY]		[D1]	[D2]	[D3]		
In sequences	61	128	135	142	213	42	142	178	223	
In IMGT	97	77	88	95	10	44	95	6	80	
Compared to human	Conserved	Polymorphic	Polymorphic	Conserved	Conserved	Conserved	Conserved	Conserved	Conserved	

^a Abbreviation and full gene designations are the following: FcγRI: IgG Fc high-affinity I receptor (CD64); FcγRII: IgG Fc low-affinity II receptor (CD32)

primary structure/organization of their corresponding human receptor.

The macaque CD32 and CD64 extracellular C-like, ligand-binding portion shares high homology with that of human CD32 and, to a greater degree, CD64

We compared the FcγRII and FcγRI deduced amino acid sequences obtained from all nine macaques examined with the corresponding sequences in humans. Figures 2 and 4 show that macaque proteins display totally conserved intron–exon boundaries and exhibit high homology in sequence compared to their human counterparts. The IMGT Collier de Perles representation of CD32 and CD64 [D1] and [D2] domains are shown in Fig. 5. This representation allows to locate amino acids within a topological context by bridging the gap between linear amino acid sequences and 3D structures (Kaas et al. 2007). For the extracellular portion

alone, amino acid comparisons between these two primate species revealed a high level of similarity, which were ~88–90% for CD32 (18–22 changes in the entire 176-amino-acid extracellular region) and ~94–95% for CD64 (14–16 changes in the entire 267-amino-acid extracellular region) (percentage ranged depending on the alleles considered in comparison). For macaque CD32, these amino acid differences are distributed equally ($n=8$ to 11) between [D1] and [D2] domains (Fig. 2). However, for CD64, while each of the two first domains [D1] and [D2] show relatively equivalent number ($n=6$ to 8) of amino acid differences (compared to humans), its third domain [D3] displays the least change ($n=2$) (Fig. 4). The TM region in macaque CD32 and CD64 share ~83% (number of amino acid differences, $n=5$) and 93% ($n=2$) identity with that in humans, respectively. Thus, the number of macaque/human amino acid differences in these two receptors is substantially lower in the TM region than in the C-like domains. No attempt

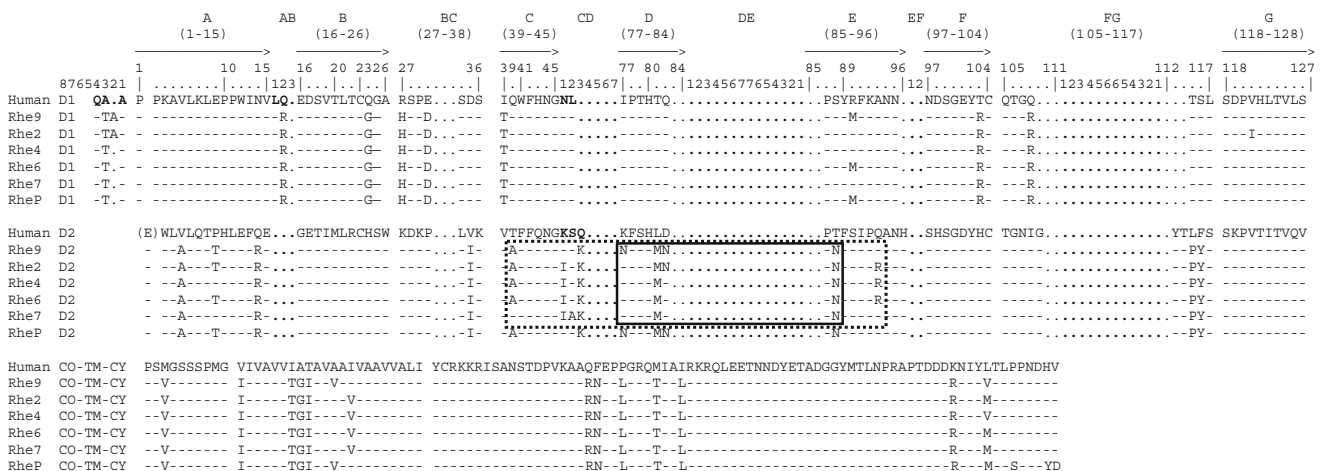


Fig. 2 Alignment of the deduced amino acid sequences of rhesus macaque CD32 (FcγRII) (GenBank accession numbers: HQ423389–HQ423393) obtained by cloning and sequencing whole blood cDNAs with a human CD32a representative (shown as “Human”; GenBank accession number: BC020823.1). The deduced amino acid sequences of macaque CD32 are compared and amino acids that differ from the human CD32a sequence are shown. Included are only macaque sequences carrying independent allelic polymorphic amino acids ($n=5$), in which each allele has a single polymorphic site or a unique

combination of polymorphic sites. A computationally predicted macaque CD32 (“RhesusP”; GenBank accession number (updated 6/1/2010): XM_001118066.2) was also counted in. *Hyphens* indicate identical residues and *dots* represent the IMGT gaps (missing amino acids) inserted to maximize alignment. The *broken-line box* represents the region where macaque polymorphic sites are clustered. The *unbroken-line box* indicates the eight-amino acid stretch bearing multiple N substitutions (see text for details)

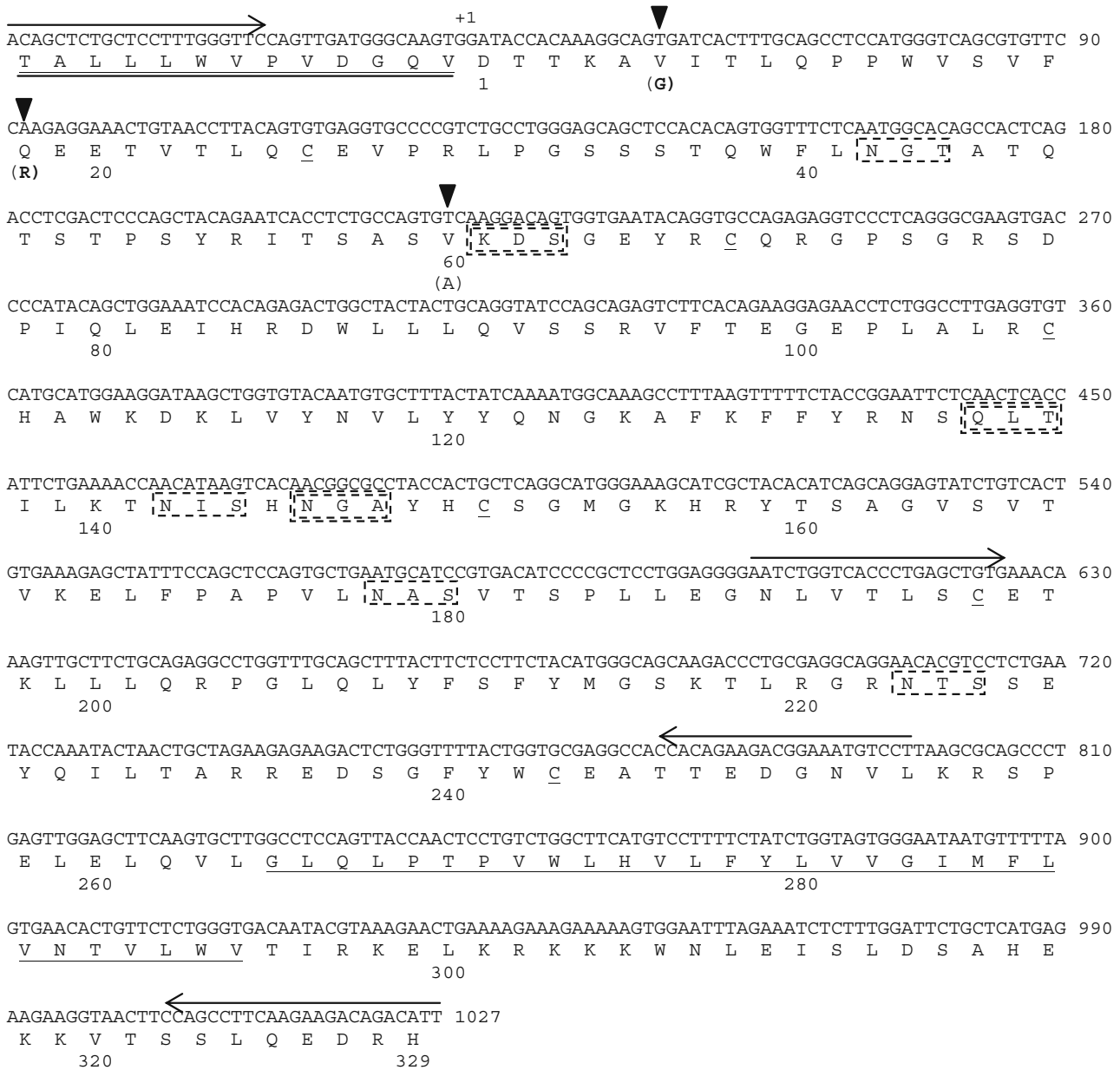


Fig. 3 Nucleotide and deduced amino acid sequences of rhesus macaque Fcγ receptor I (FcγRI, CD64) (GenBank accession number: HQ423396). Nucleotides are numbered at right and amino acids are numbered throughout. The amino acid numbering is in accordance with the IMGT unique numbering system for Ig C-like domains (Lefranc et al. 2005). Following the N-terminal signal sequence (a 13 amino acid part is shown *doubly underlined*) is the extracellular portion of ~270 amino acids in length. The predicted start site of the mature protein is indicated by the number “1” below the amino acid A. +1, above the nucleotide sequence, indicates the start of the extracellular chain. *Underlined Cs* indicates cysteines ($n=6$) that have potential to form intra-chain disulfide bonds. Potential N-linked

glycosylation sites ($n=4$; Caragea et al. 2007) (N-x-S/T) are represented by *single-line broken boxes*. *Boxes with broken double-line* ($n=3$) denote potential N-linked glycosylation sites recognized in humans only. The *underlined sequence* represents the CO-TM region. The two *over-line arrows* at the beginning and the end of the nucleotide sequence represent the binding regions and directions for the designed CD64.1 (forward) and CD64.10 (reverse) primers, respectively. The other two *over-line arrows* in the middle of the sequence denote internal primers (CD64.9 (forward) and CD64.4 (reverse), in that order). *Arrowheads* indicate the allelic positions at which the polymorphic residues (represented by amino acids displayed below and in parentheses) identified in this work are present

was made for comparing the L or CY region sequences, as when designing primer sets, we did not aim to confirm the full length of the receptors (several residues at L and CY terminals were not present in the final cloning products).

As mentioned above, two additional potentially glycosylated N are observed in macaque CD32 (Fig. 2). In contrast, two residues of this type are replaced by other amino acids in CD64 (Fig. 4). Furthermore, replacement

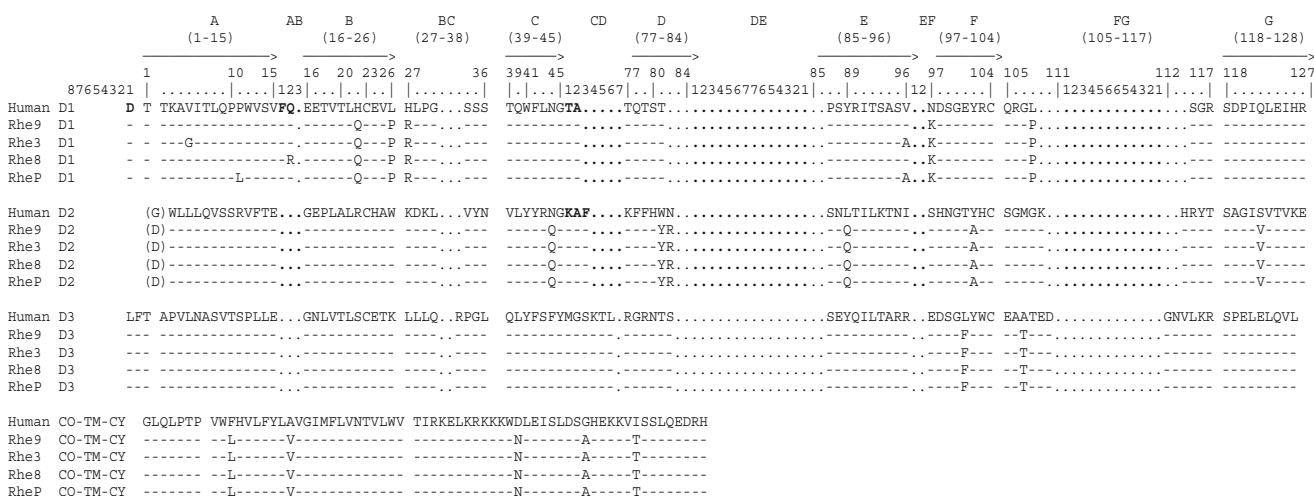


Fig. 4 Alignment of the deduced amino acid sequences of rhesus macaque CD64 (FcγRI) (GenBank accession numbers: HQ423394-HQ423396) (obtained by cloning and sequencing whole blood cDNAs) with a human CD64a representative (shown as “Human”; GenBank accession number: AK291451.1). The deduced amino acid sequences of the macaque CD64 are compared and residues that differ from the human CD64a sequence are shown. Included are only

macaque sequences carrying independent allelic polymorphic amino acids ($n=3$), in which each allele has a single polymorphic site or a unique combination of polymorphic sites. A computationally predicted macaque CD64-“like” (“RhesusP”; GenBank accession number (updated 6/1/2010): XR_013029) was also counted in. *Hyphens* illustrate identical residues and *dots* represent the IMGT gaps (missing amino acids) inserted to maximize alignment

of human [D2] T101 (148) in CD64 with A in macaques results in breaking of the potential N-linked glycosylation motif of [D2] N99 in the receptor. These amino acid changes are present in all animals examined, with the exception of the polymorphic amino acid CD32 [D2] 77 (128). All but one of the human CD32a amino acids known to be crucial for IgG binding (Maxwell et al. 1999) are conserved in macaque CD32. These residues include: [D2] K29 and P30 (BC loop), S79 and D82 (D strand), and Y113 (FG loop) (113, 114, 130, 133, and 157 in sequence), which presumably directly contact the IgG Fc ligand; [D2] L36, P87 and I108 (115, 134, and 155), which presumably have the ability to affect conformation of the loops; [D2] H80 (131) (D strand), the established low-responder polymorphic allele (Fig. 1). The only exception is at position [D2] 81 (132): it is L in humans but M in macaques. [D2] M81 is found in all nine macaques examined. It is noteworthy that all nine examined macaques display [D2] H80 (131) (Fig. 2), the well known IgG2 low-responder polymorphic allele in humans (Warmerdam et al. 1991). Because no human CD64a crystal structure has been solved to date (Ellsworth et al. 2010), and thus no specific residues crucial for ligand binding have been established, it is not possible to identify potential conserved IgG-binding amino acids in the macaque CD64 sequence. However, at least three binding interface residues in [D2], W3, W26, and K77 (87, 110, 128) found to be totally invariant among all human FcγRs (Radaev et al. 2001), are completely conserved in all nine macaque CD64 proteins (Fig. 4).

These amino acids are also conserved in most macaque CD32 proteins examined (Fig. 2).

Together, these data show that the extracellular C-like, ligand-binding portion of rhesus macaque CD32 and CD64 shares extensive homology with that of human CD32 and, to a greater degree, CD64.

A relatively high level of intra-species polymorphism is present in macaque FcγR sequences

Nine (M55R, V79I, T93P, A119T, K125I, S126A, N128K, N133D, and Q142R) or three (V6G, Q18R, and V60A) allelic polymorphic residues were identified from all examined macaque FcγRII or FcγRI alleles, respectively (Table 3). In CD64, all variable residues occur in membrane-distal [D1], whereas in CD32, most (seven out of nine) allelic polymorphic sites are clustered on a short segment in the membrane-proximal [D2] domain (Table 3 and Fig. 2). Unlike FcγRII and FcγRI, the macaque FcγRIII sequence and a number of its allelic polymorphisms have recently been described (Miller et al. 2007; Rogers et al. 2006b). In the present study, two previously unreported CD16 allelic variants at the amino acid level were identified from the nine macaques examined: most macaques carry [D1] L117 (75) and [D2] A8 (92), whereas one of the animals exhibits [D1] P117 and two animals exhibit [D2] T8 in their CD16 domains (Table 3).

Among the nine macaques examined, five (CD32) and three (CD64 and CD16) distinct allelic sequences, differing

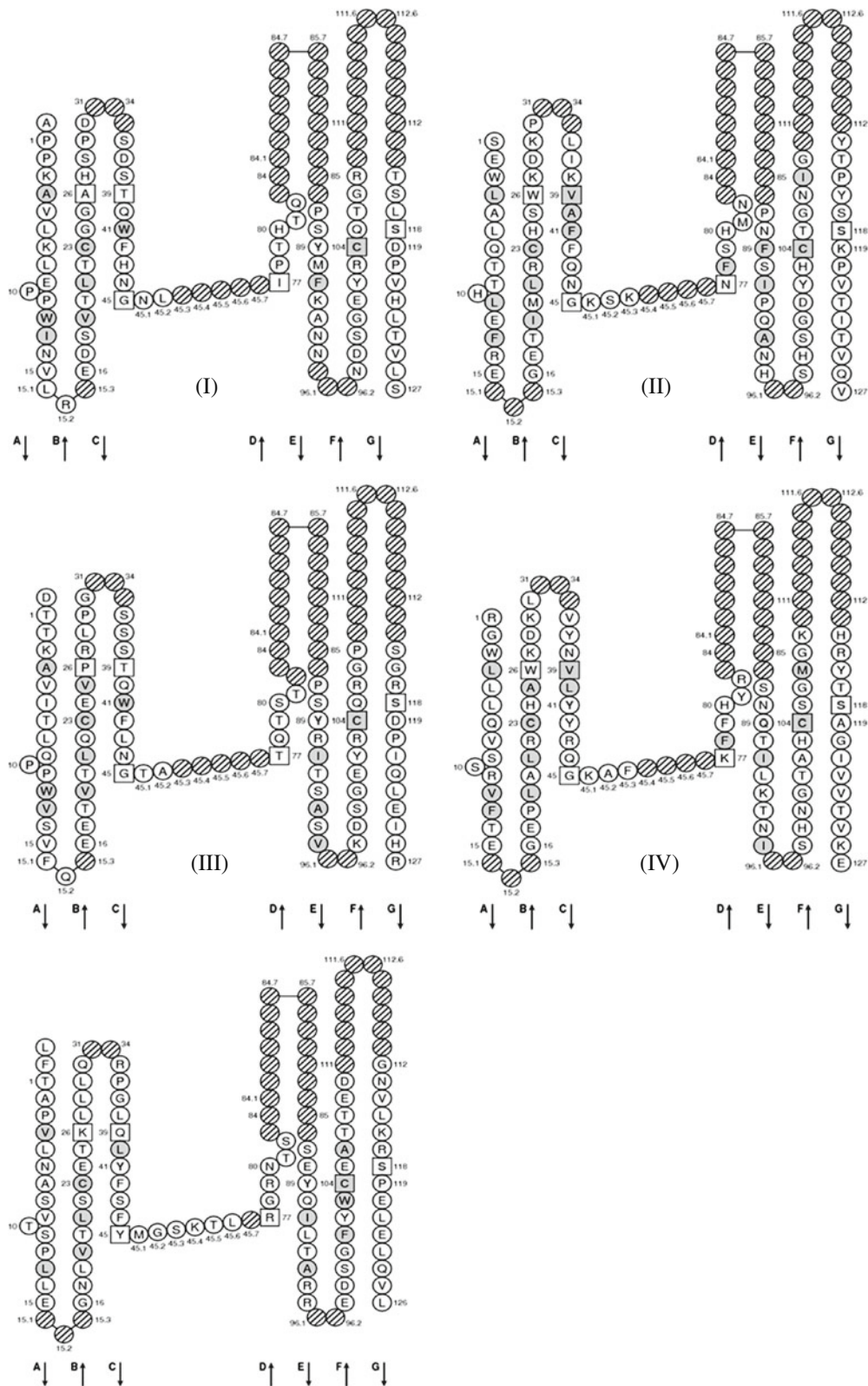


Fig. 5 IMGT Colliers de Perles of rhesus macaque CD32 (FcγRII) C-like [D1] (I), and [D2] (II) as well as CD64 (FcγRI) C-like [D1] (III), [D2] (IV) and [D3] (V) domains (on one layer; obtained with the IMGT/Collier-de-Perles tool in the IMGT/DomainGapAlign) (Ehrenmann et al. 2010). The conserved C (position 23), and C (position 104), are indicated. Shaded circles or squares represent positions at which

hydrophobic amino acids (I, V, L, F, C, M, A) and W are found in more than 50% of analyzed IgSF sequences. The BC loop, CD transversal strand, and FG loop are limited by amino acids displayed in squares. Hatched circles indicate missing positions according to the IMGT unique numbering for C-DOMAIN and C-LIKE-DOMAIN (Lefranc et al. 2005). Arrows indicate the beta-strand direction

Table 3 Location and type of allelic polymorphism present in rhesus macaque Fc γ Rs^a

Fc γ R (total novel polymorphism) ^c	[D1] amino acid positions (number of animals) ^b						[D2] amino acid positions (number of animals)							
	6 (5) ^d	18 (15.2)	55 (90)	60 (96)	75 (117)	79 (121)	92 (8)	93 (9)	119 (40)	125 (46)	126 (47)	128 (77)	133 (82)	140 (93)
CD16 (2)	–	–	–	–	L/P (1)	–	A/T (2)	–	–	–	–	–	–	–
CD32 (9)	–	–	M/R (4)	–	–	V/I (1)	–	T/P (3)	A/T (1)	K/I (5)	S/A (1)	N/K (5)	N/D (4)	Q/R (4)
CD64 (3)	V/G (1)	Q/R (1)	–	V/A (1)	–	–	–	–	–	–	–	–	–	–

^a Abbreviation and full gene designations are the following: Fc γ RI: IgG Fc high-affinity I receptor (CD64); Fc γ RII: IgG Fc low-affinity II receptor (CD32)

^b Number of individual animals (among of the nine animals examined) whose sequences exhibit polymorphism

^c Number of novel allelic polymorphic amino acids that were identified in this work

^d Numbers in parentheses indicate the IMGT positions per domain (Lefranc et al. 2005)

by at least one amino acid, were recognized (Figs. 2 and 4, and data not shown).

Thus, these results suggest a relatively high level of intra-species heterogeneity of CD32 and, to a lesser extent, CD64 and CD16, in rhesus macaques.

Discussion

The well-recognized protective nature of antibody responses in HIV-infection (Mascola and Montefiori 2010), along with the need to test candidate vaccines in the SIV-infected rhesus macaque model, underscores the importance of characterizing Fc receptors in this species. Indeed, recent findings indicate the importance of exploiting antibody responses that depend on the interactions with Fc receptors to increase vaccine efficacy. Well-characterized anti-HIV neutralizing monoclonal antibodies and even some non-neutralizing antibodies show markedly increased viral inhibitory activity when operating through an Fc γ R-mediated mechanism (Forthal and Moog 2009). Using a different host-pathogen model system, it has been shown that antigen-antibody complexes enhance antiviral cytotoxic T cell responses through Fc γ R-mediated binding to dendritic cells (Michaud et al. 2010). Clearly, the breadth of Fc γ R functions appears more extensive than what initially assumed. Therefore, the characterization of Fc receptors in rhesus macaques is necessary to correctly and fully utilize this major animal model.

Although the Fc γ RII and Fc γ RI-“like” sequences have recently (June 2010) been predicted computationally, results from our study provide the first analysis of Fc γ RII and Fc γ RI macaque homologues along with their intra-species allelic polymorphisms. The predicted cDNA structure of the macaque CD32 and CD64 represents the typical primary structure of their human counterparts, suggesting similar nature interactions with IgG molecules. Macaque Fc γ R exhibit characteristic Fc γ R structural

features. In particular, the length of a domain and the number of amino acids present between cysteine pairs, both of which are responsible for the folding pattern, is shorter in Fc γ R (~88 and 42–44 residues, respectively) as compared to IG proteins (~110 and no fewer than 80 residues, respectively) (Maxwell et al. 1999; Williams and Barclay 1988).

The high amino acid sequence identity of the Fc γ R domains between humans and macaques is consistent with the highly conserved nature of the individual C-like Fc γ R binding domains. Nevertheless, not all amino acids from the Fc binding region and the interdomain region packing are completely conserved in the macaque CD32 or CD64 sequences. One example is the addition of two predicted N-linked glycosylation sites at [D2] 77 and 88 in macaque CD32, and the lack of three of these sites at [D1] 97, [D2] 88 and 99 in CD64, as compared to their human counterparts. It is noteworthy that all these amino acid changes are recognized in the computer-predicted sequences, as well as in every individual animal examined, except for the polymorphic [D2] 77. Given that N-glycosylation sites have the ability to affect structure and ligand-binding properties of Fc γ R (van Sorge et al. 2003), it might be predicted that these inter-species variations in glycosylation pattern might have a functional consequence on macaque CD32 and CD64 proteins.

The majority of amino acids known to be crucial for CD32 ligand binding in humans (Maxwell et al. 1999) are conserved in macaques. However, the human [D2] L81 is substituted by M81 in all nine animals. As L and M are both amino acids with a hydrophobic side chain and both favor the formation and stabilization of domain structures, it is reasonable to speculate that no major alteration in the hydrophilic/hydrophobic profile results from this change. Nevertheless, it is noteworthy that this position is located downstream next to the functionally polymorphic amino acid [D2] H/R80 (131). This residue defines the specificity of CD32 for IgG in humans (Warmerdam et al. 1991).

Interestingly, all nine macaques investigated display H131, the IgG2 low-responder polymorphic allele. It remains to be seen, however, if this substitution affects IgG-binding affinity and/or specificity of the receptor.

Because the C-terminal membrane-proximal [D2] domain is the principal IgG-binding domain, its polymorphisms may influence binding properties. The N-terminal membrane-distal [D1] domain, on the other hand, mainly plays a supporting role in domain–domain interaction; thus, its variations might only affect the orientation of the spatial domain (Radaev and Sun 2002). In macaque CD64 proteins, all four (including RhesusP) polymorphic amino acids occur in [D1] (three in A strand and AB turn and the fourth one at position 93) (at amino acid 6–60; Table 3 and Figs. 3 and 4), whereas in CD32 proteins, most (seven of nine) allelic polymorphic sites are clustered on a short segment in the middle of D2 (at amino acid 120–140 segment; Table 3 and Figs. 1 and 2). Thus, these regions might be capable of mutating easily, indicating the impact of the evolutionary forces and the active functions of the receptors. In CD32 molecules, this short segment represents the type of small-region, broad-variation occurrence that might render the CD32 amino acid 120–140 segment a mutation hot spot. Even more interesting is the presence of a shorter stretch of eight amino acids located within another segment (residues 128 to 135): four of the eight residues are polymorphic, three of these four polymorphic residues are N, and two of these three N residues are potential glycosylated ones, thus indicating that this segment/stretch represents the most actively functional and evolutionarily instable area of the molecule.

Although the polymorphic variations identified in the present study, including the two novel CD16 variants, are entirely located in the ligand-binding regions, no single, specific variation is at a location known to be crucial for IgG binding in the corresponding human molecules. However, even a single amino acid change may significantly alter the ligand-binding affinity of Fc γ R. Fc γ R–IgG interactions are complex and multi-facial, and therefore, until binding studies are carried out, it is difficult to predict the functional implications of these allelic variations.

Under natural evolutionary selection, polymorphisms, when occurring co-locally in both Fc γ R molecules and their native Fc ligand within the binding areas, have the potential to optimize functional activity and increase the binding fitness of both interacting players (Kim et al. 2001). Recent work from our group demonstrates the existence in macaques of a high level of IgA and IgG intra-species polymorphisms, particularly in the IgG2 and IgG4 subclasses (Rogers et al. 2008; Scinicariello and Attanasio 2001; Scinicariello et al. 2004). The amino acid changes are primarily located at the hinge region, including the lower

hinge where all Fc γ Rs bind (Radaev and Sun 2002). Therefore, as both Fc γ R and IgG are highly polymorphic in macaques, the binding fitness and effector functions associated with these molecules might vary from individual to individual and might differ from those present in humans. Additionally, since particular allelic variations can be associated with disease susceptibility and/or severity and may influence the outcome of treatment with monoclonal antibodies (Cartron et al. 2002; van Sorge et al. 2003), this high heterogeneity must be considered when designing studies and interpreting data obtained using macaques for assessing immunogenicity and protective efficacy of HIV vaccines or vaccines for other infectious diseases, the genetic basis of IgG-mediated human diseases, and antibody or Fc γ R-derived immunotherapeutic approaches.

Acknowledgments This work was supported in part by NIH grant R21 AI078855, by the Research Program Enhancement from the GSU Office of Research and Sponsored Programs and by the Georgia Research Alliance. The authors thank the Language Research Center of Georgia State University, Dr. Michael Hart and Matthew Davis for providing and collecting all rhesus macaque blood samples used in this study.

Disclaimer The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers of Disease Control and Prevention/Agency for Toxic Substances and Disease Registry.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Bertrand G, Duprat E, Lefranc MP, Marti J, Coste J (2004) Characterization of human FCGR3B*02 (HNA-1b, NA2) cDNAs and IMGT standardized description of FCGR3B alleles. *Tissue Antigens* 64:119–131
- Binstadt BA, Geha RS, Bonilla FA (2003) IgG Fc receptor polymorphisms in human disease: implications for intravenous immunoglobulin therapy. *J Allergy Clin Immunol* 111:697–703
- Calvas P, Apoil P, Fortenfant F, Roubinet F, Andris J, Capra D, Blancher A (1999) Characterization of the three immunoglobulin G subclasses of macaques. *Scand J Immunol* 49:595–610
- Caragea C, Sinapov J, Silvescu A, Dobbs D, Honavar V (2007) Glycosylation site prediction using ensembles of Support Vector Machine classifiers. *BMC Bioinform* 8:438
- Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, Watier H (2002) Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor Fc γ 3 gene. *Blood* 99:754–758
- Daëron M (1997) Fc receptor biology. *Annu Rev Immunol* 15:203–234
- Ehrenmann F, Kaas Q, Lefranc MP (2010) IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobulins or antibodies, T cell receptors, MHC, IgSF and MhcSF. *Nucleic Acids Res* 38:D301–D307
- Ellsworth JL, Hamacher N, Harder B, Maurer M, Bukowski TR, Lantry M, Noriega C, Rixon MW, Fox B, Lewis K, Meengs B, Rollins E, Greeff K, Meyer J, Birks C (2010) Generation of a high-affinity Fc γ 3 receptor by Ig-domain swapping between human CD64A and CD16A. *Protein Eng Des Sel* 23:299–309

- Forthal DN, Moog C (2009) Fc receptor-mediated antiviral antibodies. *Curr Opin HIV AIDS* 4:388–393
- Hérodin F, Thullier P, Garin D, Drouet M (2005) Nonhuman primates are relevant models for research in hematology, immunology and virology. *Eur Cytokine Netw* 16:104–116
- Jefferis R, Lund J (2002) Interaction sites on human IgG-Fc for Fcγ₃R: current models. *Immunol Lett* 82:57–65
- Joag SV, Adams RJ, Foresman L, Galbreath D, Zink MC, Pinson DM, McClure H, Narayan O (1994) Pathogenesis of SIV_{mac} infection in Chinese and Indian rhesus macaques: effects of splenectomy on virus burden. *Virology* 200:436–446
- Kaas Q, Ehrenmann F, Lefranc MP (2007) IG, TR and IgSF, MHC and MhcSF: what do we learn from the IMGT Colliers de Perles? *Brief Funct Genomic Proteomic* 6:253–264
- Kim TD, Cho SE, Yang CH, Kim J (2001) Analysis of Fc gammaRIII and IgG Fc polymorphism reveals functional and evolutionary implications of protein-protein interaction. *J Mol Evol* 53:1–9
- Lefranc MP, Pommié C, Kaas Q, Duprat E, Bosc N, Guiraudou D, Jean C, Ruiz M, Da Piédade I, Rouard M, Foulquier E, Thouvenin V, Lefranc G (2005) IMGT unique numbering for immunoglobulin and T cell receptor constant domains and Ig superfamily C-like domains. *Dev Comp Immunol* 29:185–203
- Lefranc MP, Giudicelli V, Ginestoux C, Jabado-Michaloud J, Folch G, Bellahcene F, Wu Y, Gemrot E, Brochet X, Lane J, Regnier L, Ehrenmann F, Lefranc G, Duroux P (2009) IMGT, the international ImMunoGeneTics information system. *Nucleic Acids Res* 37:D1006–D1012
- Ling B, Veazey RS, Luckay A, Penedo C, Xu K, Lifson JD, Marx PA (2002) SIV_{mac} pathogenesis in rhesus macaques of Chinese and Indian origin compared with primary HIV infections in humans. *AIDS* 16:1489–1496
- Marthas ML, Lu D, Penedo MC, Hendrickx AG, Miller CJ (2001) Titration of a SIV_{mac}251 stock by vaginal inoculation of Indian and Chinese origin rhesus macaques: transmission efficiency, viral loads, and antibody responses. *AIDS Res Hum Retroviruses* 17:1455–1466
- Mascola JR, Montefiori DC (2010) The role of antibodies in HIV vaccines. *Annu Rev Immunol* 28:413–444
- Maxwell KF, Powell MS, Hulett MD, Barton PA, McKenzie IF, Garrett TP, Hogarth PM (1999) Crystal structure of the human leukocyte Fc receptor, Fcγ₃RIIa. *Nat Struct Biol* 6:437–442
- Michaud HA, Gomard T, Gros L, Thiolon K, Nasser R, Jacquet C, Hernandez J, Piechaczyk M, Pelegrin M (2010) A crucial role for infected-cell/antibody immune complexes in the enhancement of endogenous antiviral immunity by short passive immunotherapy. *PLoS Pathog* 6:e1000948
- Miller CJ, Genescà M, Abel K, Montefiori D, Forthal D, Bost K, Li J, Favre D, McCune JM (2007) Antiviral antibodies are necessary for control of simian immunodeficiency virus replication. *J Virol* 81:5024–5035
- Niederer HA, Willcocks LC, Rayner TF, Yang W, Lau YL, Williams TN, Scott JA, Urban BC, Peshu N, Dunstan SJ, Hien TT, Phu NH, Padyukov L, Gunnarsson I, Svenungsson E, Savage CO, Watts RA, Lyons PA, Clayton DG, Smith KG (2010) Copy number, linkage disequilibrium and disease association in the FCGR locus. *Hum Mol Genet* 19:3282–3294
- Radaev S, Sun P (2002) Recognition of immunoglobulins by Fcγ₃ receptors. *Mol Immunol* 38:1073–1083
- Radaev S, Motyka S, Fridman WH, Sautes-Fridman C, Sun PD (2001) The structure of a human type III Fcγ₃ receptor in complex with Fc. *J Biol Chem* 276:16469–16477
- Ravetch JV, Bolland S (2001) IgG Fc receptors. *Annu Rev Immunol* 19:275–290
- Ravetch JV, Kinet JP (1991) Fc receptors. *Annu Rev Immunol* 9:457–492
- Reimann KA, Parker PA, Seaman MS, Beaudry K, Beddall M, Peterson L, Williams KC, Veazey RS, Montefiori DC, Mascola JR, Nabel GJ, Letvin NL (2005) Pathogenicity of simian-human immunodeficiency virus SHIV-89.6P and SIV_{mac} is attenuated in cynomolgus macaques and associated with early T-lymphocyte responses. *J Virol* 79:8878–8885
- Rogers KA, Scinicariello F, Attanasio R (2004) Identification and characterization of macaque CD89 (immunoglobulin A Fc receptor). *Immunology* 113:178–186
- Rogers KA, Richardson JP, Scinicariello F, Attanasio R (2006a) Molecular characterization of immunoglobulin D in mammals: immunoglobulin heavy constant delta genes in dogs, chimpanzees and four old world monkey species. *Immunology* 118:88–100
- Rogers KA, Scinicariello F, Attanasio R (2006b) IgG Fc receptor III homologues in nonhuman primate species: genetic characterization and ligand interactions. *J Immunol* 177:3848–3856
- Rogers KA, Jayashankar L, Scinicariello F, Attanasio R (2008) Nonhuman primate IgA: genetic heterogeneity and interactions with CD89. *J Immunol* 180:4816–4824
- Scinicariello F, Attanasio R (2001) Intraspecies heterogeneity of immunoglobulin alpha-chain constant region genes in rhesus macaques. *Immunology* 103:441–448
- Scinicariello F, Engleman CN, Jayashankar L, McClure HM, Attanasio R (2004) Rhesus macaque antibody molecules: sequences and heterogeneity of alpha and gamma constant regions. *Immunology* 111:66–74
- Sondermann P, Oosthuizen V (2002) The structure of Fc receptor/Ig complexes: considerations on stoichiometry and potential inhibitors. *Immunol Lett* 82:51–56
- Staprans SI, Feinberg MB, Shiver JW, Casimiro DR (2010) Role of nonhuman primates in the evaluation of candidate AIDS vaccines: an industry perspective. *Curr Opin HIV AIDS* 5:377–385
- Takai T (2005) Fc receptors and their role in immune regulation and autoimmunity. *J Clin Immunol* 25:1–18
- van de Winkel JG, Capel PJ (1993) Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. *Immunol Today* 14:215–221
- van Sorge NM, van der Pol WL, van de Winkel JG (2003) Fcγ₃R polymorphisms: Implications for function, disease susceptibility and immunotherapy. *Tissue Antigens* 61:189–202
- Warmerdam PAM, Van de Winkel JGJ, Vluga A, Westerdal NAC, Capel PJA (1991) A single amino acid in the second Ig-like domain of the human Fcγ₃ receptor II is critical for human IgG2 binding. *J Immunol* 147:1338–1343
- Williams AF, Barclay AN (1988) The immunoglobulin superfamily—domains for cell surface recognition. *Annu Rev Immunol* 6:381–405