

# Identification of amino acid residues involved in the interaction of canine IgE with canine and human FcεRIα

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## ABSTRACT

The interaction of immunoglobulin E (IgE) antibodies with the high-affinity receptor (FcεRI) is important in anti-parasitic immunity and plays a central role in allergic responses. It has been shown that the human Cε3 domains comprise the binding sites for FcεRIα and crystal structure determination has shown that amino acids in four sites contribute to the high affinity of the interaction. The role of homologous residues within canine IgE-Fc, i.e. amino acids located at Cε2–Cε3 interface (residues 332–337), loop BC (residues 362–365), loop DE (residues 393–396), and loop FG (residues 424–427) in canine Cε3 domain were targeted by site-specific mutagenesis. The functional consequences of the mutations to support (i) IgE-mediated, antigen-induced release of β-hexosaminidase from RBL cells transfected with canine or human FcεRIα and (ii) the affinity of the mutants for the soluble extracellular domain of the α-chain expressed in *Pichia pastoris* were determined by Surface Plasmon Resonance (SPR). Kinetic analysis supports the observed effects of IgE mutations on stimulus secretion coupling. Potential applications of this study, leading to the generation of an IgE variant with a disabled FcεRIα binding site, are discussed.

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## 1. Introduction

### 1.1. Allergic responses and IgE

Worldwide the number of people suffering from allergies is increasing. Domestic animals are also developing new allergies and experience an increase in severity and duration of existing ones. The dog is one of the main animal species that suffers from allergic diseases and falls prey to seasonal allergies involving pollen, plants, mold, etc., in addition to more common canine allergies to fleas, pet food, and occasionally allergic reactions to vaccinations (Derer et al., 1998). Canine allergies generally produce symptoms that affect the skin, exhibiting sore, irritated, red patches of skin, hot spots, patchy hair loss. Other symptoms of canine allergies involve sneezing, coughing, persistent head shaking and discharge from the eyes or nose. Asthma, a very common allergic disease in humans, is frequently observed in dogs (Sakaguchi et al., 2001), whose genome is nearly 90% identical to the human genome. Canine allergic responses have been studied for many years on a clinical level but there still is not much information regarding the canine allergic response mechanism in these diverse allergic

manifestations. Canine, like human antibodies of the IgE isotype, are expressed by B cells in response to environmental antigens, many of which share structural and functional homologies with proteins of parasitic origin (Machado et al., 1996). IgE is the least common serum immunoglobulin compared to other Ig isotypes. Very low levels are found in human sera from healthy individuals, but dog sera contain much higher levels of IgE. Canine (ca) IgE plays an essential role in IgE-mediated hypersensitivity reactions (Groeben et al., 1997). The incubation of canine mast cells with IgE increased expression of FcεRI and also an increase of TNF-α release (Brazis et al., 2002), previously observed in humans (MacGlashan et al., 1997). Ca IgE has been observed to passively sensitize human skin, but human (hu) IgE cannot sensitize canine skin (Lowenthal et al., 1993). We have previously shown that ca IgE can bind to hu FcεRIα, but hu IgE does not bind to ca FcεRIα (Hunter et al., 2008; Ye, University of Sheffield, PhD Thesis, 2010). IgE levels in healthy dogs are ~200 μg/ml, which is a thousand times higher than levels in human sera (Hämmerling and de Weck, 1998), and this has been attributed to the increased exposure of dogs to parasitic organisms (de Weck et al., 1997; Ermel et al., 1997; Racine et al., 1999).

IgE-Fc has 4 constant domains (Cε1–4), where the extra domain referred to as Cε2 is thought to replace the hinge region found in other immunoglobulin classes. Structure determination of hu IgE/FcεRIα co-crystals have shown that the Cε3 domain comprises the binding sites for the extracellular domain of FcεRIα and is the only domain necessary for interaction of hu and ca IgE with its

Abbreviations: Canine (ca), IgE/FcεRIα; Human (hu), IgE/FcεRIα; Soluble (s), FcεRIα.

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cognate receptor high affinity receptor (Nissim et al., 1991; Hunter et al., 2008).

FcεRIα contains the IgE binding sites, and this predominantly extracellular, glycosylated subunit is a type I integral membrane protein of 50–60 kDa and a member of the immunoglobulin superfamily called C2 (Williams and Barclay, 1988). Amino acid comparison for individual FcεRIα subunit shows that different species share 38% identity of their sequence, which is the lowest homology between the three FcεRI subunits. There are two distinct regions on the IgE high affinity receptor chain subunit, FcεRIα, for interaction with the paired Cε3 domains in IgE Fc (Garman et al., 2000). In hu IgE-Fc 4 distinct sequences including the Cε2–Cε3 linker (residues 334–336), the BC loop (362–365), the DE loop (393–396) and the FG loop (residue 424) are shown to interact with residues in the receptor (Garman et al., 2000).

The amino acid sequence identity between hu and ca IgE is 56% which is higher than rat (46%) and mouse (44%). Additionally, the amino acid sequence identity between hu and ca FcεRIα is 53%, which is also higher than rat (46%) and mouse (47%). The higher amino acid sequence homology between hu and ca IgE and FcεRIα suggests that a study of the functional and structural relationships between ca IgE and FcεRIα would be more relevant to the human situation than a rodent model (Hunter et al., 2008).

## 1.2. Aim of the investigation

The interaction of IgE antibodies with the high affinity IgE receptor, FcεRI, is a key step in the initiation of allergic responses. The IgE-mediated, allergen induced cross-linking of FcεRI on surface of target cells initiates an intracellular signal transduction cascade that triggers the release of mediators of the allergic response. Many research data relevant to the interaction between hu IgE and hu FcεRIα have been published, with relatively modest information about corresponding canine system although the allergic symptoms observed in dogs are very similar to those suffered by humans. An investigation into the mechanism of interaction between ca IgE and its ca FcεRI may therefore generate information relevant for the management of allergic responses in both species. The development of an in vitro ca IgE/FcεRIα model system is likely to promote our understanding of the molecular basis of both canine and human allergic responses and forms the basis of a test system to assess rational therapeutic intervention strategies in allergic disease independent of live animal experimentation. Furthermore, it is also likely to assist the development of IgE vectors to selectively activate the anti-cancer activity associated with the release of activated oxygen species from cells expressing the low affinity receptor such as monocytes and macrophages, as first proposed by Helm et al. (1989) through mutation of the sites of interaction between IgE and FcεRIα, generating an IgE variant that will selectively activate target cells expressing FcεRII without precipitating an anaphylactic response. We have already shown that mutation of Lys 352 in hu IgE leads to a variant with a disabled FcεRII binding site, which selectively recognizes cells expressing FcεRIα (Sayers et al., 2004).

## 1.3. Residues in ca IgE-Fc targeted

Amino acid substitutions have been introduced by site specific mutagenesis replacing residues known to contribute to hu IgE/FcεRIα in order to identify the “key” residues involved in the ca Cε3 binding region. Residues targeted in ca Cε3 include R334 (Cε2–Cε3 linker), D362 (loop BC), F392 (loop BC), H423 (loop FG), K426 (loop FG) with the objective to identify the amino acid residues in ca IgE that influence interaction with hu and ca FcεRIα.

## 2. Materials and methods

### 2.1. Cell lines

RBL-2H3.1 cells transfected with hu or ca FcεRIα. The Rat Basophilic Leukaemia RBL-2H3.1 cell line is a high mediator secreting variant of the RBL-2H3 cell line (Bingham, University of Sheffield, PhD Thesis, 1994). The method describing RBL-2H3.1 cell transfection has been described in several publications which show that the expression levels of the transfected hu or ca FcεRIα tend to drift toward lower levels during extended periods in culture requiring monitoring of expression levels by flow cytometry/FACS as described previously (Sayers et al., 2004; Hunter et al., 2008; Rashid et al., 2010, 2012). A good FACS signal is usually indicative of 50–100,000 receptors per cell. This goes down on passage and cells require resorting.

The transfected RBL-2H3.1 cell line with human/canine FcεRIα was used in the present study and the transfected cell line does express endogenous rat FcεRIα. The previous studies have described this cell line extensively and shown that the high secreting variant expresses ~450,000 rat FcεRIα (Bingham, University of Sheffield, PhD Thesis, 1994), human FcεRIα transfected RBL cells express 10–100,000 rat receptors (Wilson et al., 1993; Sayers et al., 1998; Rashid et al., 2010), and expression of canine FcεRIα transfected cells varies 20–100,000 rat receptors (Hunter et al., 2008; Rashid et al., 2012). Rodent IgE binds to receptors from higher species like dog, human, horse, while dog or human IgE does not engage rat FcεRIα (Wilson et al., 1993; Sayers et al., 1998; Hunter et al., 2008).

J558L: The J558L (Oi et al., 1983) cell line was utilized as host to transfect the pSV-V<sub>NP</sub>Cε plasmid encoding ca IgE heavy chain gene and mutants thereof (Neuberger et al., 1985; Brüggemann et al., 1987).

### 2.2. Generation of chimeric IgE gene constructs encoding ca Fc R334K, D362E, K426R, F392Y, and H423F

Oligonucleotide primers were designed to generate point mutations, to amplify gene fragments, and to sequence the gene in ca IgE-Fc or FcεRIα region with the aid of the PRIMER program. The primers for point mutations were based on a 18–22 bp sequence around the target codon, while multiple mutations required longer primers, as listed below, with the altered codon underlined (see Table 1).

### 2.3. Expression of canine and human FcεRIα in *Pichia pastoris*

#### 2.3.1. PCR of ca and hu FcεRIα fragment

The extracellular domain in ca and hu FcεRIα fragments were amplified using two DNA templates, including the plasmid of pIRES2-EGFP-Canine FcεRIα and pGEM-Human FcεRIα. The oligonucleotide primers for generation of two alpha chain constructs were designed containing both the restriction site EcoRI at the start of each fragment and the restriction site NotI at the end, while the start codon ATG and a stop codon TGA were also added to the start and end of each fragment, respectively. The start codon ATG exists automatically in ca FcεRIα gene sequence (see primer 5' ca FcεRIα). The engineered EcoRI and NotI were required for insertion into pPIC9K vector (see Table 2).

#### 2.3.2. Cloning of recombinant gene into pPIC9K

A standard PCR program was employed to amplify both alpha chain fragments, followed by their analysis on 1% agarose gels. The PCR product bands were excised from agarose gel and DNA was purified using Quantum Prep™ Freeze N Squeeze DNA gel extraction spin columns kit (Bio-Rad), followed by treatment with both

**Table 1**  
Sequence of oligonucleotide primers.

|          |                                |
|----------|--------------------------------|
| R334K    |                                |
| 5' R334K | 5' GACCCCAAGGCGTGACGAGC 3'     |
| 3' R334K | 5' GCTCGTCACGCCTTTGGGGTC 3'    |
| D362E    |                                |
| 5' D362E | 5' CCTGGTAGTGGAATGGGCC 3'      |
| 3' D362E | 5' GGCCAGTTCCACTACCAGG 3'      |
| F392Y    |                                |
| 5' F392Y | 5' AAGGATCACTACAATGGGACGATC 3' |
| 3' F392Y | 5' TCGTCCATTGTAGTGATCCT 3'     |
| H423F    |                                |
| 5' H423F | 5' ACCCACCCTTCTGCCCAAGGAC 3'   |
| 3' H423F | 5' CCTTGGGCAGGAACGGGTGGTC 3'   |
| K426R    |                                |
| 5' K426R | 5' GCACCTGCCAGGGACATC 3'       |
| 3' K426R | 5' GATGTCCTGGGACGGTGC 3'       |

Site directed mutagenesis was employed to create R334K, D362E, F392Y, H423F and K426R mutations respectively in ca IgE-Fc region using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). The plasmid of pCRII-TOPO containing the inserted ca Fc gene sequence (Hunter et al., 2008), was used as template. Mutations were confirmed by gene sequencing (Core Genomic Facility, University of Sheffield). Selection of transfectants was carried out by established methods and Surface Plasmon Resonance was applied to assess protein expression (Sayers et al., 1998). The highest expressing clones were sent to the Antibody Resource Centre, University of Sheffield, for large scale expression followed by antibody purification on a NIP-HSA affinity column (Neuberger et al., 1985; Brüggemann et al., 1987; Sayers et al., 1998). The purified samples were stored at  $-20^{\circ}\text{C}$  after quantification by SPR.

EcoR I and Not I for insertion into pPIC9K. The expression vector pPIC9K was linearized by digestion with both EcoR I and Not I restriction enzymes at the same time for ligation of alpha chain PCR products, and the vector was then de-phosphorylated to prevent religation. Digested PCR products of ca and hu Fc $\epsilon$ RI $\alpha$  were ligated into pPIC9K vector into the EcoR I and Not I restriction sites. The ligation products were subsequently used to transform XL1-Blue competent cells and clones were cultured in a 5 ml LB media containing kanamycin for small scale DNA extraction using QIAprep miniprep kit (QIAGEN). The purified plasmid of pPIC9K-Fc $\epsilon$ RI $\alpha$  was analyzed by digestion with both EcoR I and Not I enzymes.

### 2.3.3. Transformation of alpha chain gene construct into *Pichia pastoris*

Gene transformation was effected by electroporation and the expression plasmids were linearized by digestion with Sac I restriction enzyme prior to transformation into *Pichia pastoris*. Sac I digest is for insertion of gene at AOX1 to generate Mut<sup>+</sup> phenotypic in GS115, alternate for insertion at HIS4, linearized with Sal I. 16–20  $\mu\text{g}$  of plasmid was digested with Sac I restriction enzyme for 2 h and an aliquot of the digest was resolved on a 1% agarose gel to check for completion of digestion. The linearized DNA including pPIC9K + caFc $\epsilon$ RI $\alpha$ , pPIC9K + huFc $\epsilon$ RI $\alpha$ , and pPIC9K with no insert

as a blank respectively, was used to transform into the wild type *Pichia* strain GS115, which had been prepared in a final volume of 2 ml in advance.

After transformation, the His<sup>+</sup> transformants were selected by plating the yeast strains on histidine negative plates (RDB). Approximately 1–10% of transformant clones on the RDB plates have multiple inserts and are commonly higher expressors of cloned proteins (Clare et al., 1991). In order to detect multiple copies of the alpha chain gene, all the clones obtained after initial selection of His<sup>+</sup> transformants were further screened using YPD plates that contain increasing concentrations of Geneticin<sup>®</sup> G-418. The screened G-418 resistant clones were isolated to assess their expressions using small scale culture (30 ml). Clones that appeared on the highest concentration of G-418 plates were selected.

G-418 resistant clones for canine alpha chain, human alpha chain, and pPIC9K as negative control were used to inoculate 30 ml BMGY employing a 250 ml baffled flask, followed by induction with 0.5% methanol in 30 ml BMMY, where methanol induction was performed between 2 and 6 days. Supernatant from this expression was assessed by SPR using ca IgE antibody. Affinity Chromatography was employed for purification using the immobilized ligands on CNBr-activated Sepharose 4B resin (GE Healthcare). Wild type ca IgE was chosen as the ligand to purify the ca alpha chain, while mouse IgE was the ligand for purification of human alpha chain.

### 2.4. $\beta$ -Hexosaminidase release assay

Ca IgE-Fc chimeric and mutant antibodies were assessed for their capability to sensitize hu/ca Fc $\epsilon$ RI $\alpha$  for antigen/antibody induced release of  $\beta$ -hexosaminidase from target cells after cell sensitization for 1 h or 16 h, the latter being the standard incubation period routinely employed (Siraganian, 2003; Wilson et al., 1993; Hunter et al., 2008).

#### 2.4.1. Antigens

NIP-HSA (4-hydroxy-3-iodo-5-nitrophenylacetyl coupled to human serum albumin) for ca/human/horse IgE-Fc chimeric antibodies; DNP-HSA (dinitrophenol coupled to human serum albumin) for mouse IgE (Wilson et al., 1993; Sayers et al., 1998; Hunter et al., 2008).

#### 2.4.2. Antibodies

Canine IgE-Fc chimeric variants. The spurious mutations K75E, T124A, and T337S, have no significant effects on IgE effector functions, as evidenced by their effect on  $\beta$ -hexosaminidase release and kinetic analysis (Ye, University of Sheffield, PhD Thesis, 2010).

### 2.5. IgE protein quantification and kinetic analysis by SPR

The Binding Analysis Wizard BiaCore software was used to identify the cell clones that express IgE, or to quantify the purified IgE

**Table 2**  
Oligo primers for generation of ca and hu Fc $\epsilon$ RI $\alpha$ .

| Primers                         | Restriction sites | Complementary sequences                                |
|---------------------------------|-------------------|--------------------------------------------------------|
| 5' ca Fc $\epsilon$ RI $\alpha$ | $\square$         | 5' GGCCGG <u>GAATTC</u> ATG TCATCAGATACCTTGAAAC 3'     |
|                                 | EcoR I            | Start codon                                            |
| 3' ca Fc $\epsilon$ RI $\alpha$ | $\square$         | 5' ATTATA <u>GCGGCCGC</u> TCA TAGCCCGGAGTACTTGTTTGC 3' |
|                                 | Not I             | Stop codon                                             |
| 5' hu Fc $\epsilon$ RI $\alpha$ | $\square$         | 5' GCTTAT <u>GAATTC</u> ATG GTCCCTCAGAAACCTAAG 3'      |
|                                 | EcoR I            | Start codon                                            |
| 3' hu Fc $\epsilon$ RI $\alpha$ | $\square$         | 5' GTACTT <u>GCGGCCGC</u> TCA AGCTTTTATTACAG 3'        |
|                                 | Not I             | Stop codon                                             |

protein based on a standard graph calibrated with a known IgE standard. IgE samples were usually diluted 1/10 in HBS-EP prior to test.

The kinetic Analysis Wizard BiaCore software was used to determine kinetic constants for target IgE and soluble Fc $\epsilon$ R1 $\alpha$ . IgE in HBS-EP was passed over the sensor chip to reach an approximate R.U. of 1000 where IgE was captured by the NIP-HSA antigen on the surface of the chip, and subsequently increasing concentrations of Fc $\epsilon$ R1 $\alpha$  in HBS-EP were passed over the chip surface to bind to immobilized IgE antibody followed by a dissociation period. For each experiment, the binding signal from association and dissociation traces was obtained at duplicate five different analyte concentrations. Kinetic constants were calculated automatically by kinetic analysis wizard within the BiaCore operating software and analyzed by the distribution model (Schuck and Zhao, 2010).

### 3. Results

#### 3.1. Expression of ca IgE variants

Transfection of J558L cells was performed using electroporation to express ca Fc IgE variants, as described previously (Sayers et al., 1998). The plasmid of pSV-V<sub>NP</sub>C $\epsilon$  with constructs in the correct orientation, encoding R334K, D362E, F392Y, H423F and K426R respectively, was used to transfect into J558L cells. Each transfection was carried out in parallel with a negative reference control using the pSV-V<sub>NP</sub> vector without insert. Transfectants started to appear after 3 weeks, while most negative control cells were dying. Transfected cells expressing IgE variants were identified by Surface Plasmon Resonance (SPR) after 4–5 weeks in culture. Supernatants from each clone were maintained for 3–5 days without changing media. Two high expressing clones were selected for each mutant. The highest expressing clones of R334K, D362E, F392Y, H423F and K426R were transferred to fresh selection media for a large scale production.

#### 3.2. $\beta$ -Hexosaminidase release assays

To assess the ability of the expressed ca IgE variants to bind to and initiate downstream signaling from the receptor complex,  $\beta$ -hexosaminidase release assays were performed using RBL-2H3.1 cell line transfected with ca and hu Fc $\epsilon$ R1 $\alpha$  and release assays were carried out where the cells were incubated with each ca IgE variant for either 16 h or 1 h prior to stimulation with increasing concentrations of the antigen NIP-HSA. A control experiment was included in each set of experiments using parental RBL-2H3.1 cells expressing endogenous rat receptors which are not recognized by ca or hu IgE (see Fig. 1).

#### 3.3. Expression of ca and hu alpha chain in *Pichia pastoris*

Expression of a stable soluble high affinity receptor chain for canine and human (ca Fc $\epsilon$ R1 $\alpha$  and hu Fc $\epsilon$ R1 $\alpha$ ) facilitates kinetic analysis of the IgE-Fc $\epsilon$ R1 $\alpha$  interaction by surface plasmon resonance (SPR) to determine the changes in binding affinity that occur as a result of the changes in structure introduced by site directed mutagenesis into the expressed ca Fc IgE chimeric antibody (Ye, University of Sheffield, PhD Thesis, 2010).

#### 3.4. Kinetic analysis

The rates of association ( $k_a$ ) and dissociation ( $k_{off}$ ), can be determined from the sensorgram, in which the response increases if binding occurs as protein sample pass over a prepared BiaCore sensor chip surface and subsequently a constant signal will be seen if equilibrium is reached. The values of  $k_a$  and  $k_{off}$  are generated

by fitting the data to interaction using BiaCore evaluation software. To measure the change in affinity when the ca IgE variants bind to hu/ca sFc $\epsilon$ R1 $\alpha$ , the application to a kinetic sensorgram was performed using the hapten NIP-HSA immobilized sensor chip in BiaCore. The experiments were carried out over a range of concentrations for ca sFc $\epsilon$ R1 $\alpha$  (1–20  $\mu$ g/ml) to bind to each the IgE variant. As IgE variants are captured by their hapten NIP-HSA via their antigen binding variable regions, the receptor binding sites on IgE heavy chain domains (C $\epsilon$ 3) are exposed for binding to the Fc $\epsilon$ R1 $\alpha$  (see Fig. 2 and Table 3).

### 4. Discussion and conclusion

The role of the specific amino acids residues in ca IgE, homologous to those identified in the co-crystal structure between hu IgE-Fc and its Fc $\epsilon$ R1 $\alpha$ , including R334 and T337S in C $\epsilon$ 2–C $\epsilon$ 3 interface, D362 and F392 in loop BC of C $\epsilon$ 3, H423 and K426 in loop FG of C $\epsilon$ 3, was assessed (i) with regard to IgE binding to RBL cells transfected with either hu or ca sFc $\epsilon$ R1 $\alpha$  and assessing IgE-mediated, antigen induced  $\beta$ -hexosaminidase release after a 1 h and 16 h sensitization period, or (ii) by binding of ca IgE and its variants to the corresponding soluble extracellular domain of ca and hu Fc $\epsilon$ R1 $\alpha$  using surface plasmon resonance for determination of the kinetic constants for the association and dissociation between the native and mutated ligand.

#### 4.1. The effect of the site directed mutagenesis on the ca IgE – ca sFc $\epsilon$ R1 $\alpha$ interaction monitored by SPR

##### 4.1.1. R334 and T337 in C $\epsilon$ 2–C $\epsilon$ 3 interface for IgE-Fc $\epsilon$ R1 $\alpha$ interaction

It is well established that the high-affinity of IgE for its mast cell/basophil receptor is due to a rapid association and slow dissociation of the ligand. Standard  $\beta$ -hexosaminidase release assay from RBL-2H3 cell line, as described by Siraganian (2003), are based on a 16 h cell sensitization period, but we have previously shown that domain swapping can have a profound effect on the kinetics of interaction of IgE with this receptor (Hunter et al., 2008) and in this study we evaluated the effect of mutations predicted to involve residues in the receptor binding regions of IgE. Our observations showed that wild type ca IgE supported a maximal release of 49% following a 16-h sensitization period and 35.2% following a 1-h incubation with IgE, respectively. In contrast, when ca Fc $\epsilon$ R1 $\alpha$  transfected cells were sensitized with the ca IgE mutant R334K were challenged with an increasing concentration of NIP-HSA, cells exhibited a maximal release of 2.2% above background after a 16-h cell sensitization assay while a maximal release of 5.1% following a 1-h cell sensitization period was observed compared to the wild type ca IgE which supported almost 50% mediator release. The outcome of the release assays suggest that ca IgE binds transiently to its receptor alpha chain when replacing arginine 334 by lysine, followed by a very rapid dissociation from the receptor since mediator release was lower after 16 h compared to 1 h sensitization period.

The results are supported by the outcome of the kinetic analyses of ca IgE R334K variant – ca sFc $\epsilon$ R1 $\alpha$  interaction, where the mutation of R334K in ca IgE showed a significant decrease in binding activity compared to the wild type. The mutant still engages the alpha chain, but this substitution is associated with a rapid dissociation as evidenced by the  $K_D$ . The current studies indicate that this residue also plays an important role in the binding of ca IgE to its cognate Fc $\epsilon$ R1 $\alpha$ , and furthermore, this also demonstrates that the linker region of C $\epsilon$ 2–C $\epsilon$ 3 plays a direct role in the interaction of IgE-Fc $\epsilon$ R1 $\alpha$  in both species. Although K is highly homologous to R it is smaller in size and this may allow the entry of water into the interactive site between ligand and receptor and thus effect a

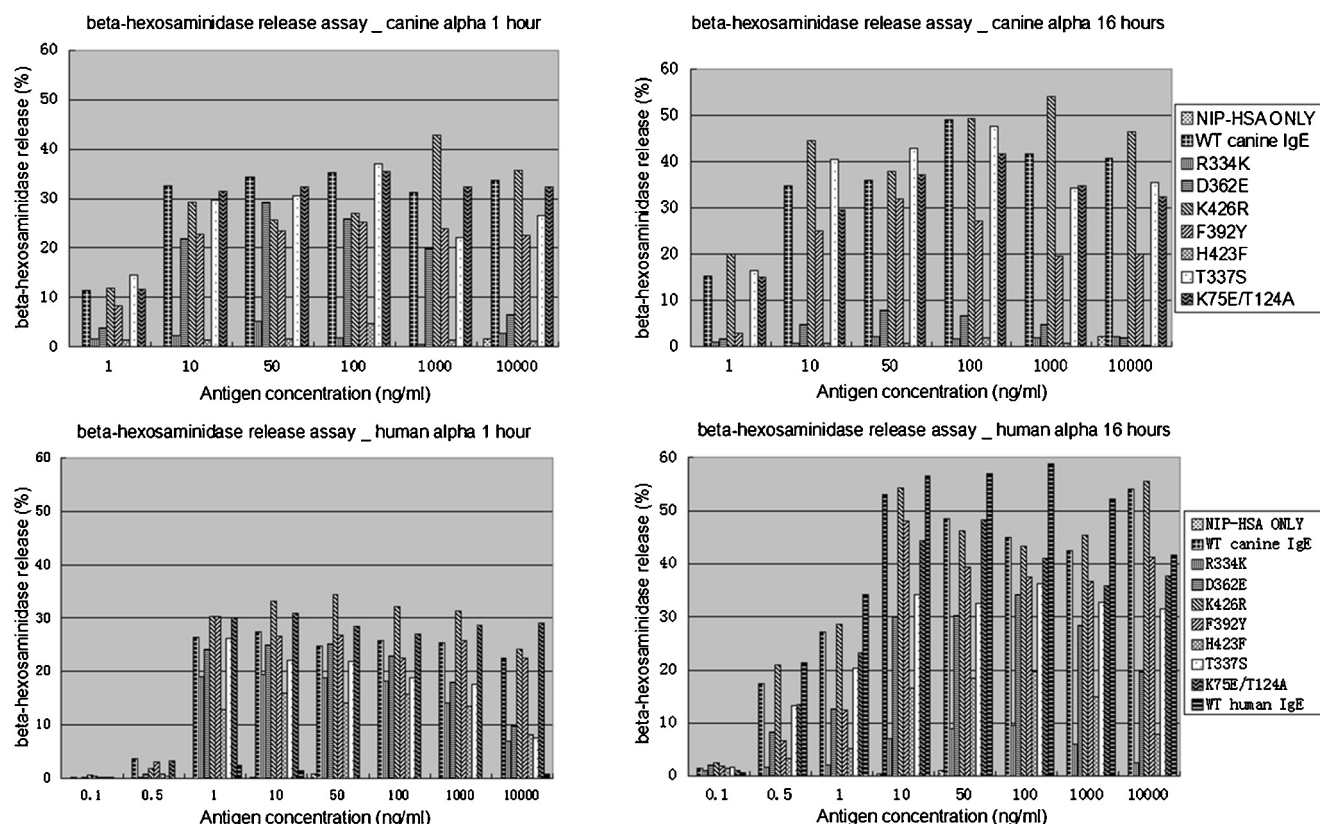


**Fig. 1.**  $\beta$ -Hexosaminidase release assays for assessing the effector functions of ca IgE variants to ca/hu Fc $\epsilon$ R1 $\alpha$ . RBL-2H3.1 cells transfected with ca Fc $\epsilon$ R1 $\alpha$  or hu Fc $\epsilon$ R1 $\alpha$  were incubated with 1  $\mu$ g/ml each IgE variant for 16 h or 1 h, followed by 30 min stimulation with increasing concentrations of NIP-HSA (0.1, 0.5, 1, 10, 50, 100, 1000, and 10,000 ng/ml). The cells incubated with NIP-HSA in the absence of IgE, act as a negative control.  $\beta$ -Hexosaminidase release values IgE antibodies are presented as the proportion of the total enzyme obtained from cell lysis using 1% Triton X-100. The values are represented as the mean of >3 experiments performed in triplicate. The variants K75E/T124A and T337S, act as controls for the spurious mutations introduced during cloning, but their effector functions are indistinguishable from native ca IgE. Wild type human IgE was applied in the release assay of human alpha chain as a positive control. The results of canine alpha panels do not show 0.1 and 0.5 ng/ml because the current concentration period 1–10,000 ng/ml is already enough to indicate the trend of data.

dramatic increase in the dissociation. It was previously shown that the replacement of R334 at the N-terminal end of strand A in C $\epsilon$ 3 by the non homologous residue S greatly increases the rate of dissociation of hu IgE from sFc $\epsilon$ R1 $\alpha$ , computed to result in a 120 fold decrease in the affinity of hu IgE for hu Fc $\epsilon$ R1 $\alpha$  (Henry et al., 1997).

The T337S mutation essentially has maintained the association and dissociation rate constants of its wild type counterpart, with a negligible change in  $K_D$  ( $2.80 \times 10^{-8} \text{ M}^{-1}$ ) compared to the wild type  $K_D$  ( $2.10 \times 10^{-8} \text{ M}^{-1}$ ). In  $\beta$ -hexosaminidase release assay, the mutant T337S supported, within limits of experimental error, identical release levels. Kinetic analyses for T337S also display similar

association and dissociation rates compared to the wild type, which is further substantiated by the T337S results in release assays indicating that this residue plays no significant role in the binding of IgE to Fc $\epsilon$ R1 $\alpha$ .

#### 4.1.2. D362 and F392 in loop BC and loop DE of C $\epsilon$ 3 domain

The mutation of D362E induces a profound change in the binding of the mutant ligand to ca sFc $\epsilon$ R1 $\alpha$ , as evidenced by the binding curves and the dissociation/association rate constant, with a  $K_D$  value of  $5.90 \times 10^{-7} \text{ M}^{-1}$ , indicating that aspartic acid 362 in loop BC plays an important role in IgE-Fc $\epsilon$ R1 $\alpha$  interaction.

**Table 3**

Kinetic constants of ca IgE and its variants for ca/hu Fc $\epsilon$ R1 $\alpha$  and Comparison kinetic constants for ca IgE variants binding to hu/ca sFc $\epsilon$ R1 $\alpha$ .

| IgE variants            | Canine sFc $\epsilon$ R1 $\alpha$        |                                                |                               | Human sFc $\epsilon$ R1 $\alpha$         |                           |                               |
|-------------------------|------------------------------------------|------------------------------------------------|-------------------------------|------------------------------------------|---------------------------|-------------------------------|
|                         | $k_a$ ( $\text{M}^{-1} \text{ s}^{-1}$ ) | $K_D$ ( $\text{M}^{-1}$ )                      | $k_{off}$ ( $\text{s}^{-1}$ ) | $k_a$ ( $\text{M}^{-1} \text{ s}^{-1}$ ) | $K_D$ ( $\text{M}^{-1}$ ) | $k_{off}$ ( $\text{s}^{-1}$ ) |
| WT Ca IgE               | $6.30 \times 10^4$                       | $2.10 \times 10^{-8}$                          | $1.30 \times 10^{-3}$         | $1.50 \times 10^4$                       | $9.20 \times 10^{-9}$     | $1.30 \times 10^{-4}$         |
| R334K <sup>a</sup>      | $1.20 \times 10^3$                       | $7.40 \times 10^{-6}$                          | $9.20 \times 10^{-3}$         | $1.40 \times 10^4$                       | $1.60 \times 10^{-7}$     | $2.20 \times 10^{-3}$         |
| D362E <sup>a</sup>      | 18                                       | $5.90 \times 10^{-7}$                          | $1.10 \times 10^{-5}$         | $1.20 \times 10^4$                       | $1.20 \times 10^{-7}$     | $1.50 \times 10^{-3}$         |
| K426R <sup>a</sup>      | $4.50 \times 10^4$                       | $3.30 \times 10^{-8}$                          | $1.50 \times 10^{-3}$         | $1.30 \times 10^4$                       | $1.30 \times 10^{-8}$     | $1.70 \times 10^{-4}$         |
| F392Y                   | $2.80 \times 10^4$                       | $8.10 \times 10^{-8}$                          | $2.20 \times 10^{-3}$         | $7.90 \times 10^3$                       | $4.40 \times 10^{-8}$     | $3.50 \times 10^{-4}$         |
| H423F                   | 96                                       | $8.20 \times 10^{-6}$                          | $7.90 \times 10^{-4}$         | $1.40 \times 10^3$                       | $4.00 \times 10^{-7}$     | $5.60 \times 10^{-4}$         |
| T337S <sup>a</sup>      | $4.60 \times 10^4$                       | $2.80 \times 10^{-8}$                          | $1.30 \times 10^{-3}$         | $9.20 \times 10^3$                       | $2.70 \times 10^{-8}$     | $2.50 \times 10^{-4}$         |
| K75E/T124A <sup>b</sup> | $4.40 \times 10^4$                       | $4.60 \times 10^{-8}$                          | $2.00 \times 10^{-3}$         | $1.30 \times 10^4$                       | $1.60 \times 10^{-8}$     | $2.10 \times 10^{-4}$         |
| WT Hu IgE               | No affinity to canine alpha              | Stedman et al. (2001) and Hunter et al. (2008) |                               | $7.40 \times 10^4$                       | $2.78 \times 10^{-10}$    | $2.70 \times 10^{-6}$         |
|                         |                                          |                                                |                               | Sayers et al. (1998)                     | Sayers et al. (1998)      | Sayers et al. (1998)          |

<sup>a</sup> The spurious mutations in the following parentheses R334K (K75E, T124A), D362E (T337S, K75E, T124A), K426R (T337S, K75E, T124A) and T337S (K75E, T124A) have no discernible effect on the kinetics of ca IgE and ca and hu Fc $\epsilon$ R1 $\alpha$  interaction according to the current data in kinetic constants.

<sup>b</sup> The variant of K75E/T124A means that there are two mutants K75E and T124A involved in this IgE variant.

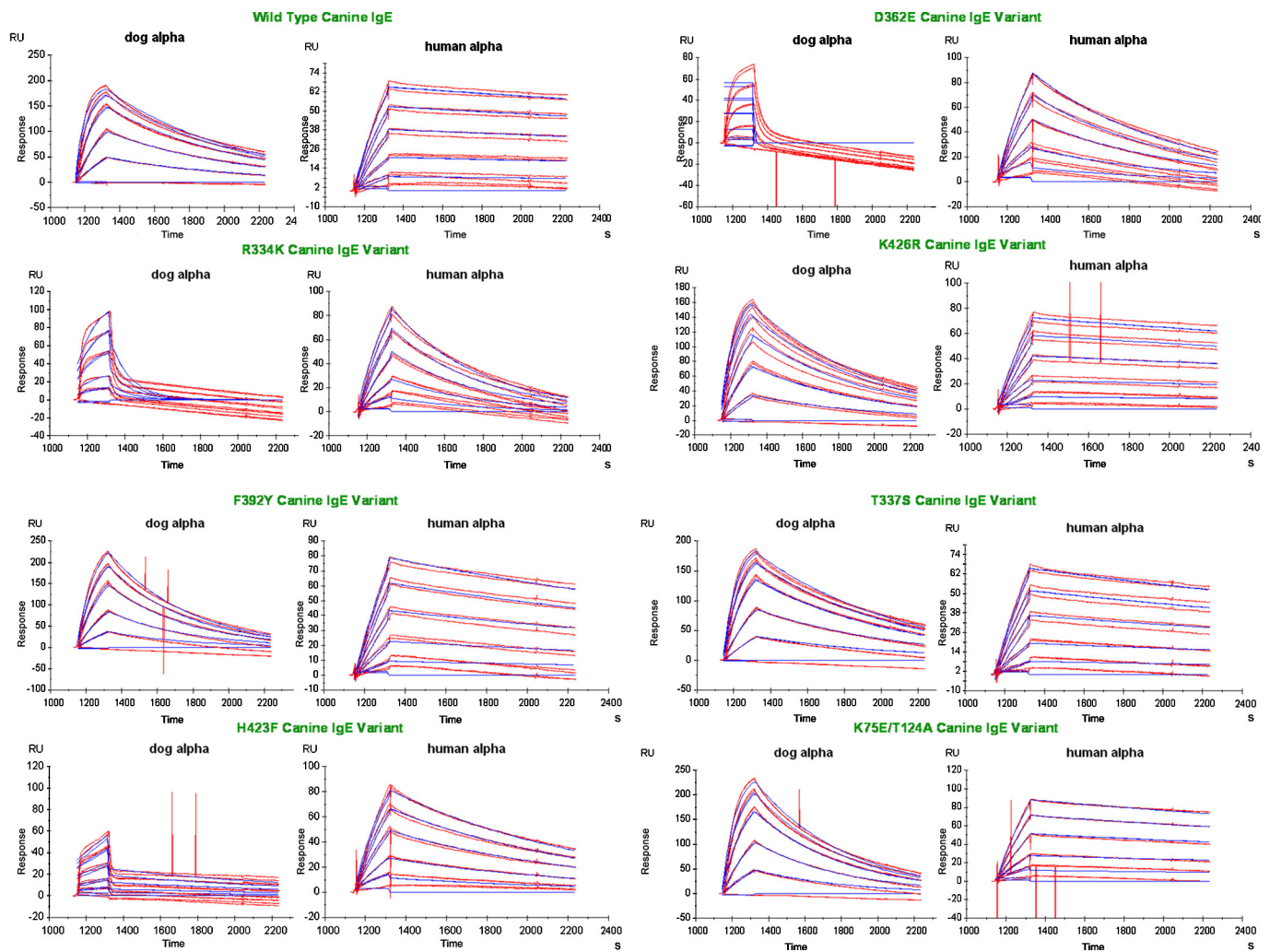


**Fig. 2.** Comparative analysis of ca IgE mutants – ca/hu sFcεRIα interaction, which is a comparison of kinetic analysis between hu and ca FcεRIα for ca IgE variants. All the curves here were generated when kinetic analysis package Wizard simplified the detail procedure. 1:1 interaction of IgE with FcεRIα is represented. To obtain a reliable kinetic analysis this experiment was carried out using at least 5 varying concentrations and a replicate process for each IgE variant. A kinetic constant was calculated automatically by kinetic analysis wizard within the BiaCore operating software.

β-Hexosaminidase assay exhibited a maximal release of 29.15% and 8.01% in 16-h and 1-h cell sensitization assays respectively for the ca IgE mutant when the Asp 362 was replaced by Glu. Compared to the wild type, D362E substitution has a profound effect on reducing mediator release after an extended incubation period indicating an increase in the dissociation of the mutant ligand from the receptor, an observation which is confirmed by the kinetic analyses when D362E variant binds to and dissociates from FcεRIα. Therefore, both release assay and kinetic constants illustrate that Asp 362 plays an important role in the interaction of IgE with its receptor.

The mutation of F392Y gives rise to association/dissociation kinetics similar to those observed for wild type ca IgE, and a negligible increase in its  $K_D$  ( $8.10 \times 10^{-8} \text{ M}^{-1}$ ) value was observed. Although it has been suggested that phenylalanine 392 in loop BC plays a role during the interaction between hu IgE and its alpha chain (Garman et al., 2000), its replacement by Try does not significantly influence the interaction between the IgE variant and the receptor.

The mutant F392Y supported a maximal release of 32.1% (after 16 h sensitization) and 25.3% (after 1 h sensitization) in β-hexosaminidase release assays and this represents an approximately 10–17% reduction compared to the wild type IgE, an observation which is further substantiated by kinetic analyses that

show a four fold decrease on association rate and a four fold increase on dissociation rate compared to the wild type kinetic constants.

#### 4.1.3. H423 and K426 in loop FG of Cε3 domain

The binding of IgE H423F displays a similar reduction in affinity when compared to R334K and D362E, with a very low  $k_a$  value of  $96 \text{ M}^{-1} \text{ s}^{-1}$  only, showing a very slow association with the alpha chain and subsequently a significant increased dissociation value of  $K_D$   $8.20 \times 10^{-6} \text{ M}^{-1}$  compared to the wild type IgE antibody, demonstrating that this mutation significantly reduces the affinity of the ligand for its receptor, proving that His 423 in loop FG is one of the key effector residues involved in the docking of IgE to FcεRIα. In contrast, the replacement of K426R by arginine does not cause a significant change in its kinetic constants compared to the wild type and its association and dissociation curves closely resemble wild type kinetics. This suggests that the substitution of lysine 426 by arginine does not affect the actual docking, although in future experiments the replacement by a non-homologous residue like serine may change the kinetics of interaction since this residue is located in the binding loop close to the effector determinant residue H423.

In β-hexosaminidase assay, the mutant H423F gives rise to a maximal release of 2% following a 16 h sensitization period

compared to 4.8% after 1 h sensitization, indicating a slow association phase, observations that were confirmed by the determination of the kinetic constants.  $\beta$ -Hexosaminidase release assays obtained for the mutant K426R show an almost identical percentage release value when compared to the wild type IgE for both 16 and 1 h sensitization periods, mirrored by a minute change in association and dissociation kinetics. In conclusion, the replacement of histidine 423 by the structural homologue phenylalanine has a profound effect on the binding of the mutant ligand to its receptor, and in contrast, the replacement of lysine 426 by its larger homologue arginine has a negligible effect on receptor recognition.

#### 4.2. The effect of site directed mutagenesis on the ca IgE – hu Fc $\epsilon$ R $\alpha$ interaction monitored by SPR

Since ca IgE engages the hu high affinity IgE receptor (Hunter et al., 2008), the effect on binding of the mutations introduced into ca IgE were also assessed for binding to hu Fc $\epsilon$ R $\alpha$ . Compared to their effect on ca IgE – ca Fc $\epsilon$ R $\alpha$  interaction, not all the mutations when assessed for binding to the hu Fc $\epsilon$ R $\alpha$  displayed the same changes. The kinetics for binding to the hu  $\alpha$ -chain show a similar association rate relative to the ca receptor but there is evidence of a considerable reduction in response units, and the dissociation phase is slower than the corresponding time observed for the ca receptor. All the ca IgE mutants associated with the hu  $\alpha$  chain from which they dissociated are more slowly than from the ca receptor.

R334K and D362E have an increased period of hu  $\alpha$ -chain occupancy which is due to the slow dissociation compared to the ca receptor, while H423F has similar  $k_a$  and  $K_D$  values to the wild type IgE when it binds to the hu receptor. A comparative kinetic analysis of the effect of these mutations on the interaction with hu Fc $\epsilon$ R $\alpha$  shows that the overall effects are similar to those observed when these mutants dock onto ca Fc $\epsilon$ R $\alpha$ . When assessed for binding to hu Fc $\epsilon$ R $\alpha$ , wild type ca IgE, K426R, F392Y, T337S, and also K75E/T124A show a more rapid association phase than R334K, D362E, and H423F and they also dissociate more slowly than R334K, D362E, and H423F.

The effect of these mutations on  $\beta$ -hexosaminidase release was also investigated using the RBL-2H3.1 cell line transfected with hu  $\alpha$  chain. Compared to wild type hu IgE, which supports maximal release of 58% after a 16 h sensitization period and only 2.5% after 1 h, the ca IgE variants, including wild type ca IgE, F392Y, K426R and K75E/T124A, support a range within the maximal release value of 48–54% during 16 h sensitization assays and 27–34% on 1 h sensitization assays. A comparison of the values for mediator release from ca and hu Fc $\epsilon$ R $\alpha$  chain transfected cells after 1 h sensitization is a clear indication of the relatively higher affinity of ca IgE for hu Fc $\epsilon$ R $\alpha$ .

Ca IgE mutants T337S and D362E are associated with a similar decrease in mediator release compared to wild type hu IgE, although surprisingly, D362E supported much higher release upon sensitization of RBL-2H3.1 cells transfected with hu Fc $\epsilon$ R $\alpha$  compared to cells transfected with ca Fc $\epsilon$ R $\alpha$ , which was also substantiated by kinetic analyses. As expected R334K and H423F effect a significant decrease in mediator release although they support a relatively higher level of release suggestive of a higher affinity for the hu  $\alpha$ -chain than to the ca  $\alpha$ -chain, in accord with the data obtained from release assays after 16 and 1 h cell sensitizations. The 9.6% release for R334K following 16 h sensitization was 10% lower than the 19.6% observed after 1 h, which indicates that this IgE variant dissociates more rapidly from receptor, an observation which has been substantiated by kinetic analysis. H423F supports a reduced level of  $\beta$ -hexosaminidase release between 16% and 19% even when the cells were sensitized for 16 h. The kinetic analysis of H423F indicates a slow association rate followed by a rapid

dissociation, which was mirrored by a low level of release after both 16 and 1 h sensitization periods.

Collectively, our data indicate that homologous residues in dog IgE are involved in the binding to both hu and ca Fc $\epsilon$ R $\alpha$ , indicating a conservation of effector determinant residues between the species. In the present study, the mutation of residues in IgE involved in Fc $\epsilon$ R $\alpha$  binding, originally identified by co-crystal X-ray structure determination between hu IgE/Fc $\epsilon$ R $\alpha$ , confirm their effector determinant function. The outcome of this study should expedite the generation of an IgE variant with a disabled Fc $\epsilon$ R $\alpha$  binding site through mutation of the major effector determinant residues identified in the current studies (R334K, D362E, H423F) where the Fc $\epsilon$ R $\alpha$  effector determinant in the A–B loop is maintained (Sayers et al., 2004). Such a construct, furnished with an antigenic specificity directed to an appropriate cancer antigen may have important applications in the selective activation of target cells expressing Fc $\epsilon$ R $\alpha$ , which give rise to activated oxygen species known to be effective in killing parasites and cancer cells (Helm et al., 1989) without risking a potential adverse anaphylactic response. The cell-lines and reagents described in our current and earlier studies provide the basis for *in vitro* assessments of such constructs without the need for animal experimentation.

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