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The Chicken Yolk Sac IgY Receptor, a Functional Equivalent of the Mammalian MHC-Related Fc Receptor, Is a Phospholipase A_2 Receptor Homolog

Anthony P. West, Jr.,[1] Andrew B. Herr,[1,3] and Pamela J. Bjorkman[1,2,*]

[1]Division of Biology 114-96 and
[2]Howard Hughes Medical Institute
California Institute of Technology
Pasadena, California 91125

Summary

In mammals, IgG is transferred from mother to young by the MHC-related receptor FcRn, which binds IgG in acidic endosomes and releases it at basic pH into blood. Maternal IgY, the avian counterpart of IgG, is transferred to embryos across yolk sac membranes. We affinity-purified the chicken yolk sac IgY receptor (FcRY) and sequenced its gene. FcRY is unrelated to MHC molecules but is a homolog of the mammalian phospholipase A_2 receptor. Analytical ultracentrifugation and truncation experiments suggest that FcRY forms a compact structure containing an IgY binding site at acidic pH but undergoes a conformational change at basic pH that disrupts the site. FcRY is thus unrelated to mammalian FcRn in both its structure and mechanism for pH-dependent binding, illustrating distinct routes utilized by evolution to transfer antibodies.

Introduction

Acquired immunity in the form of immunoglobulin is passed from mother to young in many vertebrate species (Brambell, 1970). In mammals, this occurs in utero (as for humans) or after birth through ingested milk (as for rodents). In both cases, immunoglobulin G (IgG) is transported across a cellular barrier via transcytosis by the neonatal Fc receptor (FcRn), a receptor specific for the Fc portion of IgG (Ghetie and Ward, 2000). The first FcRn proteins and genes to be characterized were from rodents, where FcRn is expressed in the neonatal small intestine (Simister and Mostov, 1989), the fetal yolk sac (Roberts et al., 1990), mammary gland (Cianga et al., 1999), liver, vascular endothelium (Borvak et al., 1998), and respiratory epithelium (Spiekermann et al., 2002). In humans, because of a different organization of extraembryonic membranes, the placental syncytiotrophoblast rather than the yolk sac is the major site of FcRn-mediated maternal immunoglobulin transport (Leach et al., 1996; Simister et al., 1996).

Mammalian FcRn is a heterodimer with two polypeptide chains: a membrane-bound heavy chain sharing 22%–29% sequence identity in its extracellular domains with class I MHC molecules and a noncovalently attached light chain, β2-microglobulin, which also serves as the class I MHC light chain (Simister and Mostov, 1989). The crystal structures of the ectodomains of rat FcRn (Burmeister et al., 1994a) and human FcRn (West and Bjorkman, 2000) confirm the structural similarity with class I MHC molecules predicted by the sequences, but the FcRn counterpart of the groove used by class I MHC molecules for presentation of antigenic peptides to T cells is narrowed and nonfunctional. The significance of the structural similarity with MHC molecules is unclear as FcRn binds to IgG using residues from the side of its counterpart of the MHC peptide binding domain and from β2-microglobulin (Martin et al., 2001) in an interaction that bears no resemblance to MHC interactions with peptides or macromolecules.

Biochemical studies of membrane-bound and soluble forms of FcRn demonstrate that IgG binding is pH dependent: there is strong binding (nanomolar K_D) under the acidic conditions (pH ~6.0) found in endosomal compartments and in rodent intestines, while at the slightly basic pH of blood (pH 7.4), there is no detectable binding (Ghetie and Ward, 2000). From mutagenesis and crystallographic studies, the mechanism of this pH dependence has been revealed to involve chemical rather than conformational changes (Kim et al., 1994; Martin et al., 2001; Vaughn and Bjorkman, 1998). Specifically, there are attractive interactions at acidic pH between protonated histidines on Fc and negatively charged side chains on FcRn, which are lost upon deprotonation of the Fc histidines at basic pH (Martin et al., 2001). The sequences of FcRn genes isolated from other organisms, including pig (Schnulle and Hurley, 2003), sheep (Mayer et al., 2002), macaque (GenBank accession number AAL92101), brushtail possum (Adamski et al., 2000), and cow (Kacskovics et al., 2000), suggest that the features described above for rodent and human FcRn proteins are shared in the other mammalian orthologs.

Transfer of passive immunity to offspring is not confined to mammals. In birds and some reptiles, immunoglobulin Y (the avian/reptilian counterpart of IgG) is packaged in the yolk compartment of eggs. During late embryonic development, IgY is transported across the yolk sac membrane into the embryonic bloodstream (Kowalczyk et al., 1985). Chicken yolk sac membranes contain an IgY binding activity with characteristics similar to mammalian FcRn (nanomolar binding at pH 6, no binding at pH 8) (Linden and Roth, 1978; Tressler and Roth, 1987), but the protein responsible for pH-dependent binding of IgY had not been isolated or characterized. Here we report the isolation, cloning, and characterization of the chicken yolk sac membrane IgY receptor, which we have named FcRY. The receptor is a 1459 residue protein that is unrelated to mammalian FcRn. Instead, FcRY appears to be the avian homolog of the secretory phospholipase A_2 receptor (PLA_2R) (Figure 1). Like PLA_2R, the FcRY ectodomain contains an N-terminal cysteine-rich (CysR) domain, a fibronectin type II (FNII) repeat, and eight C-type lectin-like domains (CTLDs). Binding studies using isolated FcRY fragments reveal that IgY binding requires regions from the first two domains and the CTLDs, which interact with each

*Correspondence: bjorkman@caltech.edu
[3]Present address: Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267.

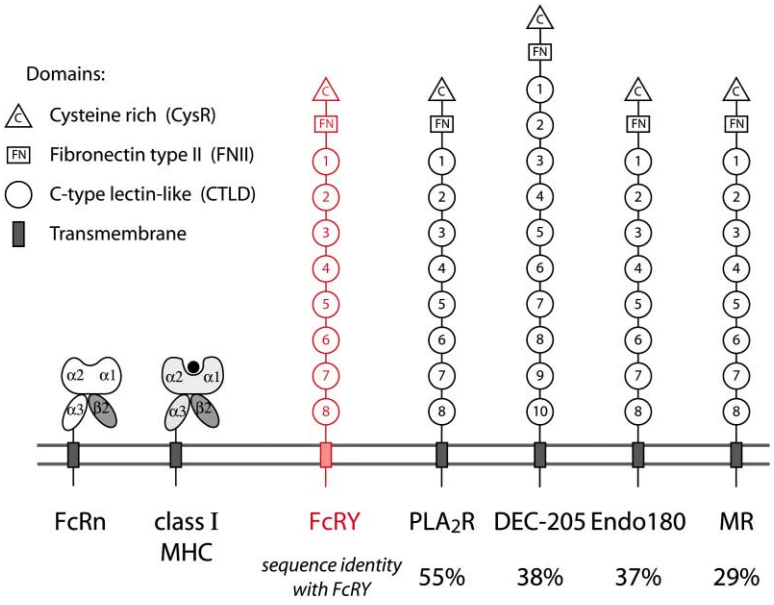

Figure 1. Schematic Representations of the Structures of the MHC-Related Mammalian FcRn, a Class I MHC Molecule, Chicken Yolk Sac IgY Receptor FcRY (red), and Mannose Receptor Family Members

Sequence identities between FcRY and other mannose receptor (MR) family members are based on pairwise alignments to human MR family proteins.

other at acidic pH to form a pH-dependent IgY binding site. These results are used to propose a conformational change model for pH-dependent recognition of IgY and to discuss the evolution of Fc receptor function and the MHC fold.

Results

Purification and Cloning of FcRY

To isolate the protein responsible for the pH-dependent IgY binding activity from chicken yolk sacs, we adapted the protocol used to purify mammalian FcRn from rat fetal yolk sacs (Roberts et al., 1990). Yolk sacs from day 18–19 chick embryos were solubilized in detergent and passed over an IgY affinity column at pH 6. After elution of the column at pH 8, a single major band migrating with an apparent molecular weight of 180 kDa was observed by SDS-PAGE (Figure 2A). There were no visible bands in the molecular weight range corresponding to mammalian FcRn (35 to 53 kDa) or β2-microglobulin (11 kDa). The sequence of a proteolytic fragment of FcRY matched two overlapping chicken ESTs (Boardman et al., 2002), allowing a full-length cDNA clone to be obtained by rapid amplification of cDNA Ends (RACE) PCR.

The 5499 bp FcRY cDNA clone encodes a predicted type I transmembrane protein of 1459 amino acids that shares 55% amino acid sequence identity with the human secretory phospholipase A_2 receptor (PLA_2R), a member of the mannose receptor (MR) family (Figure 1 and see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/20/5/601/DC1). Cloning of an avian PLA_2R has not been reported previously, and a BLAST search of chicken ESTs and genomic DNA indicates that FcRY is the closest avian homolog of PLA_2R (data not shown). FcRY and all mammalian

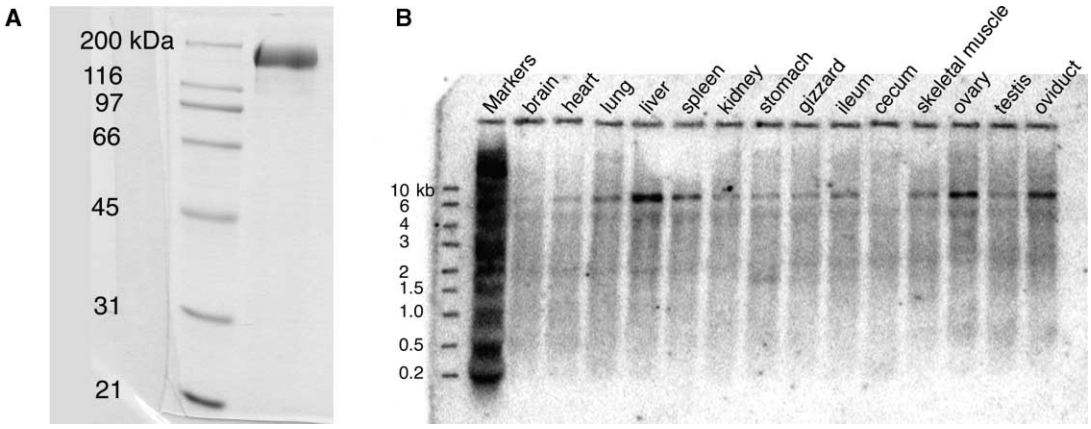

Figure 2. Affinity-Purified FcRY and FcRY Expression Pattern

(A) Coomassie-stained 12% SDS-PAGE of affinity-purified FcRY. The sample was derived from solubilized chicken yolk sac membranes that were passed over an IgY affinity column at pH 6 and eluted at pH 8.

(B) Northern blot analysis of FcRY expression in chicken tissues. The RNA blot was probed with a 695 bp α^{32}P-dATP-labeled PCR-generated FcRY probe.

PLA_2Rs share a common domain architecture with other members of the MR family: an N-terminal signal sequence followed by a cysteine-rich domain, a single fibronectin type II domain, eight to ten C-type lectin-like domains, a transmembrane domain, and a cytoplasmic tail containing ~40 residues (Figure 1). In common with mammalian PLA_2Rs, the FcRY cytoplasmic domain includes two potential endocytosis motifs: a dihydrophobic sequence (EESILI, matching an ExxxØØ motif common to the MR family, where Ø is a bulky hydrophobic amino acid) and an altered tyrosine-based motif (WVRNAYF versus the consensus tyrosine-based motif ØxNxxY) (East and Isacke, 2002).

To determine whether FcRY is expressed in locations other than the yolk sac membrane, an RNA blot of adult chicken tissues was probed with a gene-specific probe (Figure 2B). A strong band corresponding to an ~6.5 kb transcript was observed in liver, ovary, oviduct, and spleen. Lower levels of expression were observed in most tissues except the brain and cecum. FcRY expression in tissues besides the yolk sac membrane may reflect a role protecting IgY from catabolism, similar to FcRn's function extending the serum half-life of IgG in mammals (Ghetie and Ward, 2000).

FcRY Binds IgY with Nanomolar Affinity at Acidic, but Not Basic, pH

To verify that the FcRY gene encodes a functional IgY receptor, full-length FcRY was expressed in Chinese hamster ovary (CHO) cells. FcRY-expressing CHO cells show cell surface staining when incubated with Cy3-labeled FcY (Fc fragment of IgY) at pH 6 but not at pH 8 (Supplemental Figure S2), demonstrating that FcRY is a type I membrane protein expressed at the cell surface, as predicted from its sequence, and that the FcRY cDNA encodes the previously described pH-dependent IgY binding activity of chicken yolk sac membranes (Tressler and Roth, 1987).

To derive quantitative binding data for the FcRY-IgY interaction, we examined the binding of FcY and IgY to a recombinant soluble FcRY ectodomain expressed in baculovirus-infected insect cells using a surface plasmon resonance (SPR)-based binding assay. FcRY was covalently coupled to the surface of a biosensor chip, and IgY or FcY was injected at pH 6, pH 7.4, or pH 8. Binding experiments were also conducted in the reverse orientation by injecting FcRY over immobilized IgY or FcY. In both cases, significant binding of IgY and FcY was observed at pH 6 but not at pH 8 or 7.4 (Figures 3A–3C). The binding was unaffected by the presence or absence of calcium (10 mM calcium acetate) or EDTA (5 mM) (data not shown). To derive equilibrium binding affinities, the pH 6 data were fit to models assuming a 1:1 or a 2:1 FcRY:FcY interaction. A bivalent model that describes the sequential binding of two FcRY molecules to an FcY homodimer fits the data better than a 1:1 model, yielding affinities of 140–220 nM (K_{D1}) and 230–1600 nM (K_{D2}) for the first and second binding events (Figures 3A and 3B). For binding of IgY, the sequential binding affinities are 99 nM (K_{D1}) and 2.8 μM (K_{D2}) (data not shown). As an alternative to this stepwise (microscopic) binding model, the binding can also be described macroscopically as a mixture of high and low affinity binding sites (corresponding to doubly and singly ligated IgY molecules, respectively), yielding a high affinity K_D of 12 nM and a low affinity K_D of ~1 μM for IgY binding to immobilized FcRY. These values can be directly compared to earlier binding studies using yolk sac membranes, in which the macroscopic or apparent K_D values for IgY binding were derived as 15 nM and 340 nM for the high- and low-affinity binding sites (Tressler and Roth, 1987).

The ligand binding specificity of FcRY was tested by injecting other potential ligands over an FcRY-coupled chip at pH 6.0. Little or no binding was observed for human IgG (a mixture of IgG1, 2, 3, and 4), human IgE, rat Fc (derived from rat IgG1), or porcine pancreatic PLA_2. In experiments conducted at pH 7.4, no binding was observed for human Fc or mouse IgG (data not shown).

To verify the 2:1 FcRY-IgY stoichiometry suggested by the binding studies, we examined mixtures of FcRY and FcY under binding conditions (pH 6.0) by sedimentation velocity analytical ultracentrifugation (AUC) (Figure 3D). Under conditions in which 1:1 complex formation is favored (an FcRY:FcY molar ratio of 1:25), we observe a species sedimenting at 9.7 S. A 1:1 molar ratio of FcRY:FcY contains species sedimenting at several different rates; these likely correspond to small amounts of free FcY, 1:1, and 2:1 complexes. A 2:1 mixture of FcRY:FcY resulted in species sedimenting at ~13 S, consistent with a 2:1 stoichiometry of FcRY:FcY. Differential sedimentation of species in dynamic equilibrium causes the 1:1 and 2:1 samples to be shifted and broader than theoretical nondissociating complexes. A global fit of data collected at three molar ratios (2:1, 1:1, 1:25), yielded affinity constants for the binding of the first and second FcRY molecules to FcY (K_{D1} = 110 nM, K_{D2} = 280 nM) consistent with those obtained from the SPR analysis.

Multiple Domains of FcRY Are Required for IgY Binding

In order to localize the IgY binding site on FcRY, we expressed truncated forms of FcRY: CysR alone, CysR-FNII, CysR-FnII-CTLD1-4, CTLD1-8, and CysR-FNII-CTLD5-8 (Figure 4A). The FcRY fragments were purified and tested for binding of IgY in an SPR-based binding assay as described above. None of the fragments showed significant binding of IgY (data not shown). However, a mixture of the CysR-FNII fragment and the CTLD1-8 fragment binds IgY at pH 6 (Figure 4B). No binding was observed at pH 8 between IgY and the CysR-FNII and CTLD1-8 mixture (Figure 4B); thus, the pH-dependent binding between FcRY and IgY is preserved when the FcRY ectodomain is fragmented. To further explore the mechanism of IgY binding using FcRY fragments, we tested for binding between the CysR-FNII and CTLD1-8 fragments. In an SPR-based assay in which the CysR-FNII fragment was immobilized on a biosensor chip, CTLD1-8 bound at pH 6 but showed no detectable binding at pH 8 (Figure 4C). These results suggest that FcRY forms an IgY binding structure at pH 6 in which the CysR-FNII region folds back upon the CTLD1-8 region and that disruption of this conformation at pH 8 prevents IgY binding (Figure 4D).

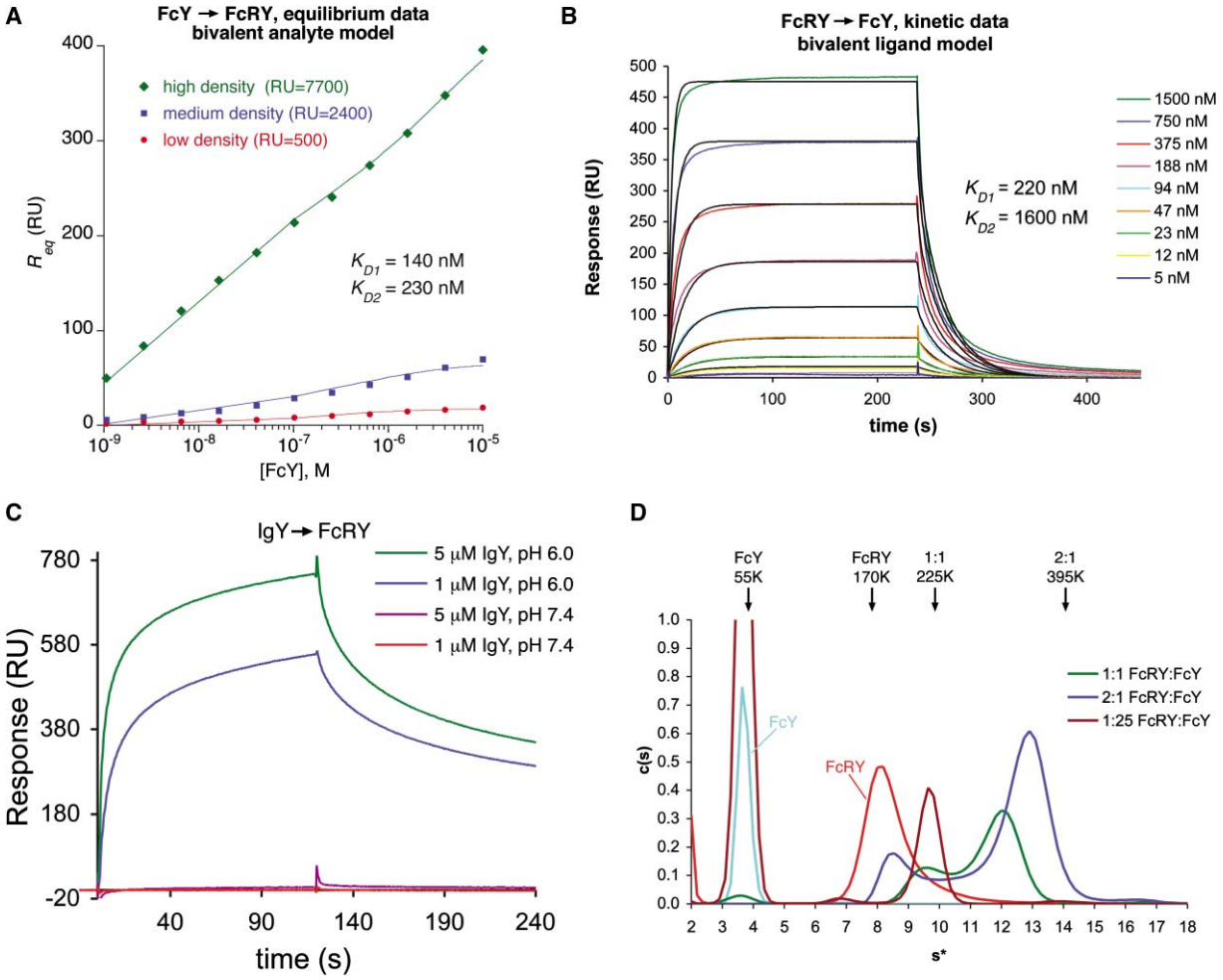

Figure 3. Surface Plasmon Resonance (A–C) and AUC (D) Analyses of the FcRY-FcY Interaction at pH 6

(A) Biosensor data for experiments in which FcY was injected over FcRY immobilized at three different densities: 500, 2400, and 7700 RU. The equilibrium binding response (R_{eq}) is plotted versus the log of the concentration of injected protein. Best-fit binding curves, derived from a bivalent analyte binding model, are superimposed on the binding data. When the same binding data are analyzed assuming a heterogeneous ligand model, in which the coupled population of molecules is assumed to contain two independent classes of binding sites, we derive a high affinity K_D of 4 nM (49% of population) and a low affinity K_D of 1.3 μM (51% of population).

(B) Representative sensorgrams (colored lines) derived from injection of different concentrations of FcRY over immobilized FcY. Three hundred and seventy RU of FcY was immobilized. Kinetic data were fit globally using a bivalent ligand model (black lines).

(C) Sensorgrams demonstrating that immobilized FcRY (3620 RU) binds IgY at pH 6 but not at pH 7.4.

(D) Sedimentation velocity AUC analysis of FcRY:FcY mixtures. The sedimentation coefficient distribution was determined for FcRY and FcY alone and for mixtures containing 1:1, 2:1, and 1:25 molar ratios of FcRY:FcY. The arrows indicate the approximate molecular weights and calculated sedimentation coefficients (derived from SedAnal) of free FcY, free FcRY, and 1:1 and 2:1 FcRY:FcY complexes. These s^* values describe how these species would sediment if not in equilibrium with each other.

FcRY Undergoes a pH-Dependent Conformational Change that Correlates with IgY Binding

To explore the possibility of a pH-dependent conformational change in FcRY, we examined its behavior on a gel filtration column. We find that the FcRY ectodomain migrates with the characteristics of an extended protein on a gel filtration column at pH 8, eluting earlier than IgG, which has a similar molecular mass. By contrast, at pH 6, the FcRY ectodomain elutes later from the column, consistent with a more compact conformation (Figure 5A).

To further investigate the possibility of a pH-dependent conformational change in FcRY, we used sedimentation velocity AUC, a technique that provides information on the size and shape of proteins in solution. The sequence of the FcRY ectodomain predicts ten tandem domains, hence a potentially extended structure. Consistent with this prediction, velocity AUC data obtained at pH 8 (Figure 5B) yielded a sedimentation coefficient (s^*) of 7.21 ± 0.01 S and an f/f_0 value (the ratio of the experimental frictional coefficient to that of an ideal nonhydrated sphere) of 1.78. By comparison, typical f/f_0 values for globular proteins range from 1.2 to 1.4 (Tanford, 1961). The values obtained for FcRY at pH 8 are similar to those reported for the MR ectodomain at pH 7.8 ($s^0_{20,w}$ = 7.29 ± 0.02 S; f/f_0 = 1.63) (Napper et al., 2001). In contrast to the monomeric and extended structure predicted for the FcRY ectodomain at basic pH, velocity AUC studies of FcRY at pH 6 suggest a more compact structure with an s^* value of 7.91 ± 0.03 S and f/f_0 =

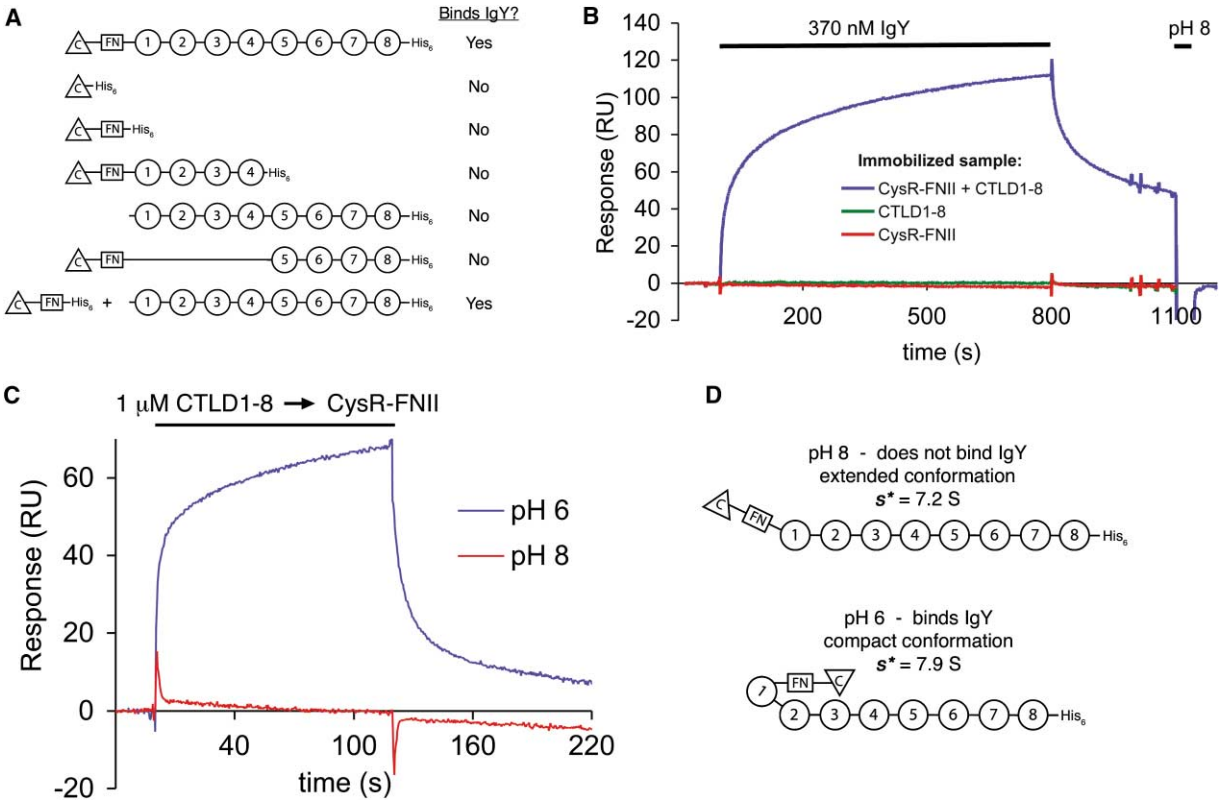

Figure 4. FcRY Deletion Constructs and Their IgY Binding Properties

(A) Schematic representation of soluble FcRY constructs, containing either the entire FcRY ectodomain or fragments thereof, and their IgY binding properties. Fragments listed as not binding to IgY showed no detectable binding to IgY at concentrations up to 5 μM (data not shown).

(B) Sensorgrams obtained in biosensor experiments by injecting IgY at pH 6 over immobilized FcRY fragments. CysR-FNII and CTLD1-8 were immobilized individually (red and green) or together (blue). IgY binding occurs only in the flow cell containing both CysR-FNII and CTLD1-8. The quantities immobilized were 4950 RU of CysR-FNII+CTLD1-8, 1680 RU of CysR-FNII, and 5840 RU of CTLD1-8.

(C) Sensorgrams demonstrating that CTLD1-8 binds to immobilized CysR-FNII (1220 RU) at pH 6 but not at pH 8.

(D) Model of pH-dependent conformational change of FcRY. FcRY has an extended conformation at pH 8 ($s^* = 7.2$ S) with no predicted interaction between the CysR-FNII domains and the CTLDs. At pH 6 the CysR-FNII region folds back and binds to the CTLDs, resulting in a more compact conformation ($s^* = 7.9$ S) that is able to bind IgY. The site on the CTLD1-8 region where the CysR-FNII domains bind is not known; hence, the figure is schematic and shows one possibility.

1.38 (derived from a 30 nM sample of FcRY). A potential complication for interpretation of these results is that the sedimentation velocity AUC data derived from FcRY samples at concentrations greater than 160 nM revealed multiple species (Figure 5B). A global fit of velocity AUC data for all FcRY concentrations at pH 6 suggested that FcRY dimerizes with a K_D of 6.4 μM (Experimental Procedures).

To confirm the velocity AUC results, equilibrium AUC experiments were performed with varying FcRY concentrations at pH 6 and 8 (Supplemental Experimental Procedures and Supplemental Figure S3). At pH 8, the FcRY equilibrium AUC data reveal a single species with a molecular mass of ~170 kDa. In contrast, the pH 6 equilibrium AUC data can best be fit to a dimerization model with a calculated K_D for dimerization of 4.6 μM (Supplemental Experimental Procedures and Supplemental Figure S3), consistent with the dimerization K_D calculated from the pH 6 sedimentation velocity AUC data. At low FcRY concentrations (30–50 nM) at pH 6, FcRY sediments as a single species in both velocity and equilibrium AUC experiments; thus, dimerization at pH 6 is not responsible for the pH-dependent difference in s^* values derived from experiments conducted with low concentrations of FcRY.

Oligomerization of the MR has also been observed by AUC but was calcium dependent and suggested to result from oligosaccharides on one receptor binding to the carbohydrate recognition domain on another receptor (Napper et al., 2001). A similar mechanism is not likely for FcRY because FcRY dimerization is calcium independent and because the FcRY CTLDs lack residues found in functional carbohydrate recognition domains (see Discussion and Supplemental Figure S1). An alternative mechanism for FcRY dimerization, which is consistent with observation of dimers at acidic pH only and with the interaction between the CysR-FNII and CTLD1-8 fragments at pH 6, is formation of a domain-swapped dimer in which the CysR-FNII region of one FcRY molecule binds to the CTLDs of another.

Discussion

Here we report cloning, heterologous expression, and binding studies of an avian Fc receptor present on the chicken yolk sac membrane. Previous studies identified

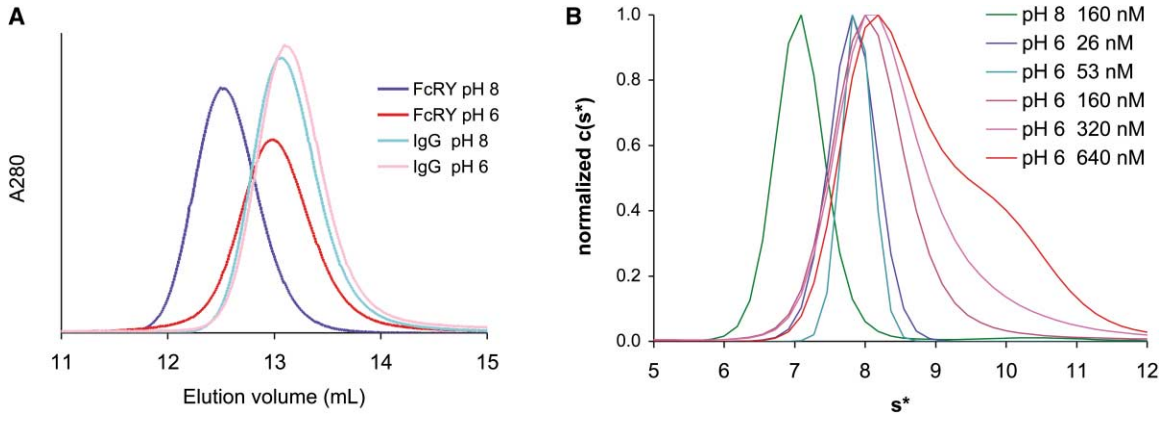

Figure 5. Biophysical Evidence of a pH-Dependent Conformational Change of FcRY

(A) Analytical gel filtration elution profiles of FcRY and IgG at pH 6 and pH 8. FcRY, but not IgG, shows a pH-dependent difference in its elution profile. Both FcRY samples were injected at 800 nM.

(B) Sedimentation velocity AUC of FcRY samples at pH 6 and pH 8. The sedimentation coefficient distribution was determined at several FcRY concentrations. Peak heights were normalized. The pH 8 data collected at 640 nM and 320 nM FcRY yielded sedimentation coefficient distributions essentially identical to the 160 nM curve. At pH 6, the samples of FcRY at higher concentrations have sedimentation distributions indicating partial FcRY dimerization. The lower concentration pH 6 curves converge to a single species with an s^* value of $\sim$7.9 S.

a pH-dependent IgY binding activity in yolk sac membranes (Linden and Roth, 1978; Tressler and Roth, 1987). This binding activity was hypothesized to be responsible for transfer of maternal antibodies to newborn chicks for the following reasons: (1) IgY transport from yolk to chick is specific, thereby requiring a receptor (Brambell, 1970); (2) the site of IgY transport from yolk to embryo is the yolk sac membrane (Brambell, 1970), and (3) the IgY binding protein on the yolk sac membrane has the same pH-dependent binding as FcRn, the mammalian functional equivalent (Tressler and Roth, 1987; Ghetie and Ward, 2000). This pH-dependent binding would permit the IgY binding protein to transfer maternal IgY from egg yolk (pH 6) to the embryonic circulation (pH 7.4), just as rodent intestinal FcRn transports maternal IgG from ingested milk to the neonatal bloodstream (Ghetie and Ward, 2000).

We used a functional purification procedure involving an IgY affinity column to isolate a pH-dependent IgY binding protein from chicken yolk sac membranes. The resulting protein, which we have named FcRY, binds to the IgY column at pH 6 and is eluted from the column at pH 8. No other yolk sac proteins were observed upon elution of the IgY column at pH 8. After isolating the FcRY cDNA, we expressed recombinant FcRY proteins in transfected cells and as a soluble ectodomain fragment and demonstrated that FcRY binds to IgY with nanomolar affinity at pH 6 and exhibits no significant binding at pH 7.4 or pH 8. This magnitude of a pH-dependent affinity transition near neutral pH is a rare property shared by few other proteins besides FcRn, arguing that the ability to bind ligand at the acidic pH of intracellular vesicles and release ligand at the basic pH of blood is critical for the function of FcRY. Further evidence supporting the identification of FcRY as the receptor responsible for IgY transport is that FcRY exhibits the same immunoglobulin binding specificity as previously described for the IgY transporter (Brambell, 1970; Warr et al., 1995): namely, it binds FcY and IgY, but not mammalian IgGs. Finally, FcRY belongs to the MR family, a group of proteins that characteristically endocytose bound ligands (East and Isacke, 2002), and the FcRY cytoplasmic tail contains a consensus endocytosis motif.

There are two steps in the transfer of immunity from hen to chick. In the first step, antibodies are loaded into the yolk compartment of the developing oocyte. FcRY, which does not bind human IgG, does not appear to be involved in this process, as it has been shown that human IgGs are transported into chicken egg yolk (Mohammed et al., 1998; Morrison et al., 2002). Instead, our data suggest that FcRY is the receptor responsible for the second step in transferring antibodies from yolk to the embryonic circulation, as mammalian antibodies injected into yolk sacs are not transferred to newly hatched chicks (Brambell, 1970).

Despite similarities in function and binding properties, FcRY and mammalian FcRn are not related in sequence or domain organization. Instead FcRY is the chicken counterpart of the mammalian M (muscle)-type PLA_2 receptor, which binds and endocytoses secretory PLA_2 as part of a signal transduction pathway (Hanasaki and Arita, 1999). PLA_2R is a member of the MR family of proteins, which are type I membrane proteins characterized by an ectodomain containing an N-terminal cysteine-rich domain, a fibronectin II repeat, and 8–10 CTLDs (East and Isacke, 2002). Although no transcytosis functions have been ascribed to other MR family members, they contain endocytic sequence motifs in their cytoplasmic domains and perform endocytic roles, recycling between the plasma membrane and endosomal compartments (East and Isacke, 2002). One of the best-characterized members of the family, MR, binds carbohydrates on foreign and host molecules. Two portions of MR bind carbohydrates: two of the CTLDs function as carbohydrate recognition domains to mediate calcium-dependent recognition of terminal mannose, fucose, and N-acetylglucosamine carbohydrates that are com-

monly found on microorganisms, and the cysteine-rich domain of mannose receptor (Cys-MR) binds to glycoproteins containing sulfated sugars (Fiete and Baenziger, 1997; Fiete et al., 1998). In addition to MR and PLA_2R, other members of the MR family include the dendritic cell receptor DEC-205, which is believed to capture extracellular antigens and direct their transport to an antigen-processing compartment (Jiang et al., 1995), and Endo180, which plays a role in remodeling the extracellular matrix (East and Isacke, 2002).

The presence of two potential carbohydrate binding regions within FcRY raises the question of whether FcRY functions as a lectin. The cysteine-rich domain of FcRY is unlikely to recognize sulfated sugars because its sequence, like those of other MR family members except for MR itself, shows alterations in the region known from the crystal structure of a Cys-MR/4-sulfated-*N*-acetylgalactosamine complex (Liu et al., 2000) to interact with sulfated sugars (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/20/5/601/DC1). In addition, the presence of CTLDs within the FcRY sequence does not guarantee carbohydrate binding, as most of the CTLDs in MR family members do not exhibit C-type lectin activity (East and Isacke, 2002). The FcRY and PLA_2R CTLDs lack calcium-coordinating residues that are critical for calcium-dependent carbohydrate recognition in true carbohydrate recognition domains (Weis et al., 1998) (Supplemental Figure S1); thus, FcRY and the PLA_2Rs are not predicted to function as calcium-dependent lectins. Indeed, mammalian PLA_2Rs bind nonglycosylated forms of PLA_2 (Lambeau et al., 1994). Likewise, we propose that FcRY binds IgY by a protein-protein interaction, consistent with the observation that FcRY binding to IgY is unaffected by the presence of EDTA or calcium (data not shown). Carbohydrate recognition in some contexts cannot be completely ruled out, however. For example, PLA_2R shows non-calcium-dependent binding to a mannose conjugate of bovine serum albumin (Lambeau et al., 1994; Nicolas et al., 1995). A high mannose oligosaccharide is attached to the single N-linked glycosylation site of FcY (Suzuki and Lee, 2004); thus, carbohydrate recognition could play a role in FcY binding by FcRY. However, the requirement for regions of the FcRY ectodomain in addition to the CTLDs for binding to IgY (Figure 4A) demonstrates that ligand binding cannot be explained by a lectin activity of any single domain.

To gain insight into which domains of FcRY are involved in FcY recognition, we constructed truncated forms of FcRY and tested their binding to FcY. We find that two regions of FcRY, the CysR-FNII portion and the CTLD1-8, do not individually bind IgY but form a complex that binds IgY at acidic pH. By contrast, a large series of PLA_2R deletion constructs retained significant ligand binding as long as CTLD5 was not removed (Nicolas et al., 1995), suggesting fundamentally different modes of ligand recognition by FcRY and PLA_2R. On the basis of analytical ultracentrifugation and binding studies, we propose that the more compact form of FcRY observed at pH 6, in which the CysR-FNII domain interacts with the CTLDs, is required for IgY binding and that a conformational change at pH 8, which eliminates the interaction between the CysR-FNII region and the CTLDs, results in a more elongated FcRY ectodomain that cannot bind IgY. Interestingly, studies of the MR and Endo180 ectodomains also demonstrate an interaction between the CysR-FNII and CTLD portions of these receptors: protease digestion experiments indicate that MR CysR and FNII interact with CTLD1-2 (Napper et al., 2001), and an electron microscopy structure of Endo180 shows an interaction between CysR and CTLD2 (Rivera-Calzada et al., 2003).

Regarding the FcRY binding site on IgY, our binding studies demonstrate that FcRY binds to FcY and intact IgY with comparable affinities; thus, FcRY specifically recognizes the Fc region of IgY with little or no contribution to binding from the IgY Fab arms. The Fc region of IgY, like mammalian IgE and IgM molecules, contains three constant domains (C_H2, C_H3, C_H4), as compared with mammalian IgG Fc regions, which contain two (C_H2, C_H3). N-terminal sequencing of the FcY used for the binding experiments revealed that it starts at the C_H3 domain (data not shown); thus, the FcRY binding site on FcY can be localized to the C_H3-C_H4 region of IgY. Likewise, FcϵRI binds to the C_H3-C_H4 region of the IgE Fc fragment, with no involvement of the C_H2 domains (Garman et al., 2000). However, unlike the FcϵRI or the FcγR receptors, which bind to their immunoglobulin ligands in an asymmetric interaction with a 1:1 receptor/ligand stoichiometry (Garman et al., 2000; Sondermann et al., 2000), two FcRY molecules bind to each IgY or FcY molecule, implying a symmetric interaction in which one FcRY binds to each chain of the FcY dimer analogous to recognition of IgG by mammalian FcRn (Burmeister et al., 1994b). In the case of the FcRn-Fc interaction, titration of a patch of histidines at the bend between the Fc C_H2 and C_H3 domains (H310, H435, H436 in rat IgG) is responsible for a chemical change that mediates the pH dependence of the FcRn:IgG interaction (Martin et al., 2001). Counterpart histidines are not found in IgY (Parvari et al., 1988); thus, the pH dependence of the FcRY-IgY interaction cannot be ascribed to an analogous chemical change, providing a possible rationalization of the conformational change mechanism proposed for the pH dependence of the FcRY:IgY interaction.

To our knowledge FcRY is the first chicken Fc receptor to be purified and molecularly characterized. Available sequence information for the chicken genome (Boardman et al., 2002) does not reveal obvious homologs of FcRn (besides class I MHC proteins), but the recent identification of the chicken polymeric Ig receptor (pIgR) (Wieland et al., 2004) and evidence of secretory component in avian secretory IgA (Karaca and Naqi, 1997; Peppard et al., 1986) implies that birds express a receptor that transports IgA into secretions. Hence, it appears that the molecules responsible for the transcytosis of IgA are shared by birds and mammals, whereas this is not the case for IgG/IgY. Instead, transcytosis of IgG in mammals and IgY in birds during passive acquisition of maternal immunity by offspring is accomplished by fundamentally different types of proteins: a class I MHC-related Fc receptor for IgG in mammals and a PLA_2R-related Fc receptor for IgY in birds. The functional significance of the relationship between the structures of FcRn and MHC molecules in mammals and FcRY and PLA_2R in birds is unknown. However, the finding that birds do not express an MHC-related Fc receptor that transfers maternal antibodies to newborns but do express class

I MHC molecules (Guillemot et al., 1989) suggests that the peptide binding and T cell receptor recognition properties of the MHC fold evolved prior to the immunoglobulin binding and transport function.

Experimental Procedures

Materials

Chicken IgY and porcine pancreatic PLA_2 were purchased from Sigma (St. Louis, MO). FcY and human IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Human IgE was obtained from Scripps Laboratories (San Diego, CA). Recombinant rat Fc was prepared as described in reference Martin and Bjorkman (1999).

Affinity Purification of FcRY from Yolk Sac Membranes

Yolk sacs were dissected from day 18–19 embryos, drained of yolk, and washed extensively in phosphate-buffered saline (PBS). Yolk sacs from eight to ten eggs were Dounce homogenized at 4°C in PBS containing 3 mM EDTA and protease inhibitors (Complete Mini, Roche, Indianapolis, IN). Homogenates were centrifuged at 100,000 × g for 60 min. The pellet was solubilized by resuspension in a binding buffer consisting of 0.15 M phosphate (pH 6.0), 60 mM n-octyl β-D-glucopyranoside, 10 mM D-gluconic acid lactone, 1 mM EDTA, and 0.02% NaN_3. After 2 hr of stirring, the resuspended material was centrifuged again at 100,000 × g for 30 min. The solubilized membrane fraction was applied to an IgY affinity column (equilibrated in binding buffer) prepared from CNBr-activated agarose (Pharmacia, Uppsala, Sweden). After overnight incubation, the agarose beads were microfuged, and then washed three times in binding buffer and in diluted binding buffer (0.015 M phosphate [pH 6.0], 30 mM n-octyl β-D-glucopyranoside, 10 mM D-gluconic acid lactone, 1 mM EDTA, and 0.02% NaN_3). Bound protein was eluted from pelleted beads by adding 150 μl of the same buffer at a higher pH (0.15 M phosphate [pH 8.0]) and run on a 12% SDS-PAGE gel under reducing conditions.

Peptide Sequencing

N-terminal sequencing yielded the sequence ALSRDELPSL, which does not match any known proteins. To provide additional sequence data, a proteolytic digestion was performed with Endo-Lys, and individual peptides were isolated by HPLC. Three additional peptide sequences were assigned based on a combination of chemical sequencing and mass spectroscopy. One of the internal peptide sequences (YVEDDSRNCGVFK) exactly matched two overlapping chicken ESTs (Boardman et al., 2002) (clone IDs ChEST872p24 and ChEST724b16).

Cloning of FcRY by RACE-PCR

mRNA was prepared from freshly dissected yolk sac membranes using the RNeasy kit (Qiagen, Chatsworth, CA). Primers were initially designed to amplify a 415 bp fragment contained in EST ChEST872p24. RACE-PCR was used to obtain the full-length message (SMART RACE-PCR kit, Clontech, Palo Alto, CA; Gene-Racer Kit, Invitrogen, Carlsbad, CA). Partial clones missing the 5′ end were obtained initially, and these were used to design new primers to obtain the entire coding region.

RNA Blot Analysis

A Northern blot (RNway Laboratories, Seoul, Korea) was hybridized with a α^{32}P-dATP-labeled probe consisting of a 695 bp PCR product (Strip-EZ PCR kit, Ambion, Austin, TX) corresponding to the 5′ region of the FcRY cDNA.

Expression of FcRY

Full-length FcRY (in vector pcDNA3.1) was transfected into CHO cells using Lipofectamine 2000 (Invitrogen). Two days after transfection, cells were washed with a pH 6.0 buffer, incubated on ice with 500 nM Cy3-labeled FcY (Jackson Immunoresearch) for 30 min, and then washed.

Expression of Soluble FcRY and Deletion Constructs

A construct encoding a soluble version of FcRY (including the native signal sequence and residues 1–1370 of the mature protein) with a C-terminal 6×-His tag was subcloned into the baculovirus transfer vector pVL1393 (Pharmingen, San Diego, CA). FcRY was purified from supernatants of baculovirus-infected High 5 cells, exchanged into 50 mM phosphate at pH 8.0, then subjected to Ni-NTA chromatography (Ni-NTA Superflow, Qiagen). Protein from an imidazole elution was further purified by gel filtration chromatography on a Superdex 200 column. C-terminally His-tagged FcRY deletion constructs were prepared by PCR, and expression and purification were as for the full-length soluble fragment. The oligonucleotides (5′ to 3′) used were: TCGAATTCTCAGTGATGATGATGATGATGGGTGT ATGTTTCTTGGAAAGGATGTTCAC for CysR alone (residues 1 to 143 of mature protein), TCGAATTCTCAGTGATGATGATGATGATG CTCAACACCAGGACAAAAGCCCCATTTTTC for CysR-FNII (residues 1 to 197), TTTGCATATCCATTCACGCTTTTCATTGCAG and GAT GCAGATCTTGATGCAGCAGAACC for CysR-FnII-CTLD1-4 (residues 1 to 771), AAGGGAGGGCAGCTCGTCCCGGCTGA and GAA AAATGGGGCTTTTGTCCTGGTGTT for CTLD1-8 (residues 187 to 1370), and AGGACAAAAGCCCCATTTTTCATC and CCAAAAGGTG TTAAACCAAAAAATCC for CysR-FNII-CTLD5-8 (residues 1 to 193 and 773 to 1370).

Biosensor Assays

A BIACORE 2000 biosensor system (Pharmacia, LKB Biotechnology) was used to assay the interaction of FcRY with chicken IgY and Fc fragments. This system includes a biosensor chip with a dextran-coated gold surface to which one protein (referred to as the "ligand") is covalently immobilized. Binding of an injected protein (the "analyte") to the immobilized protein results in changes of the surface plasmon resonance that are directly proportional to the amount of bound protein and read out in real time as resonance units (RU) (Malmqvist, 1993).

FcRY was covalently immobilized to three of the four flow cells on a CM5 biosensor chip (Pharmacia) using standard primary amine coupling chemistry (BIACORE manual) at three different densities (500, 2400, and 7700 RU). The fourth flow cell was mock coupled using buffer to serve as a blank. IgY, FcY, and test proteins were injected at room temperature in 50 mM sodium phosphate (pH 6.0, pH 7.4, or pH 8.0), 150 mM NaCl, and 0.005% P20 surfactant. No binding was observed at pH 6.0 for human IgG, human IgE, rat Fc, or porcine pancreatic PLA_2 at concentrations up to 5, 1, 10, and 10 μM, respectively (data not shown). In other experiments, FcY or FcRY fragments were covalently immobilized to a flow cell using primary amine chemistry, and potential binding partners were injected over the chip surface. The binding data were analyzed using the programs BIAevaluation v3.1 (Biacore AB) and Clamp99 (Morton and Myszka, 1998). In the case of FcY injections over coupled FcRY, binding reactions were allowed to reach or closely approach equilibrium, and K_Ds were derived by nonlinear regression analysis of plots of R_{eq} (the equilibrium binding response) versus the analyte concentration (Figure 3A). For FcRY injections over coupled FcY, kinetic constants were derived from sensorgram data using simultaneous fitting to the association and dissociation phases of the interaction and global fitting to all curves in the working set (Figure 3B).

Since each polypeptide chain of an FcY homodimer can be bound by an FcRY molecule, the binding data were analyzed using models assuming sequential binding of FcRY to the first and second binding sites on an FcY homodimer. Thus, reactions involving FcY as the injected protein (analyte) (Figure 3A) were analyzed using the bivalent analyte model described previously (West and Bjorkman, 2000), and reactions involving FcY as the immobilized protein (ligand) (Figure 3B) were analyzed using the bivalent ligand model using Clamp99. Simple 1:1 binding models did not account for the observed data as judged from large residuals in the fits (data not shown). We also fit the binding data to a heterogeneous ligand model involving two classes of noninteracting binding sites as described for the analysis of rat FcRn with IgG (Vaughn and Bjorkman, 1997). This model derives macroscopic equilibrium dissociation constants and the percentage of the total response due to each class of binding site on the biosensor chip.

Analytical Gel Filtration

Analytical gel filtration chromatography was performed on a Superdex 200 10/30 column (Pharmacia) with a flow rate of 0.5 ml/min. The column was eluted with 10 mM Tris, 10 mM bis-Tris, 150 mM NaCl at pH 6.0, or 20 mM Tris, 150 mM NaCl at pH 8.0. FcRY samples (150 μl) were injected at concentrations of 80 and 800 nM. Elution profiles did not change over this concentration range.

Analytical Ultracentrifugation

Sedimentation experiments were performed at 20°C in a Beckman XL-I Ultima analytical ultracentrifuge using absorbance optics. Soluble FcRY for these experiments was further purified by IgY affinity chromatography. The buffer was 50 mM sodium phosphate (pH 6.0 or 8.0) and 150 mM NaCl. Velocity sedimentation experiments with samples (420 μl) of FcRY, FcY, or mixtures of FcRY and FcY were spun at 32,000 rpm. For analysis of the stoichiometry, FcRY-FcY mixtures were spun that contained 2.6 μM FcRY and 1.3 μM FcY (2:1 ratio), 1.3 μM of each FcRY and FcY (1:1), and 1.3 μM FcRY and 32.5 μM FcY (1:25). Two-sector, charcoal-filled epon centerpieces with quartz windows were used with a four-hole Ti-60a titanium rotor. Scans were collected continuously at 228, 230, or 280 nm, with a step size of 0.005 cm. Sedimentation coefficients were determined using the program Svedberg (Philo, 1994), which fits velocity data directly to an approximate form of the Lamm equation. AUC velocity data were also processed using the c(s) analysis routine of SEDFIT (Schuck, 2000). The c(s) analysis produces a sedimentation coefficient distribution in which sample diffusion has been accounted for explicitly. The apparent sedimentation coefficients (s^*) reported in these studies were not corrected to standard conditions; however, all s^* values were determined under identical conditions except for pH. The f/f_0 values reported were calculated using SEDFIT.

AUC data from the stoichiometry experiments were analyzed using the program SedAnal (Stafford and Sherwood, 2004), which fits raw sedimentation velocity data from interacting systems using numerical solutions to the Lamm equation and chemical kinetics. A global fit of data from the three FcRY-FcY mixtures was performed using a five component model (FcY, FcRY, 1:1 complex, 2:1 complex, and FcRY dimer). The affinity constants for the binding of the first and second FcRY molecules to FcY produced from this fitting procedure were $K_{D1} = 110$ nM and $K_{D2} = 280$ nM. The fitted s^* values of the individual components and the 1:1 complex are similar to values obtained from the c(s) analysis of SEDFIT (Figure 3D).

Alternative methods of analyzing the sedimentation velocity data of FcRY at pH 6 yielded similar s^* values. The program SedAnal (Stafford and Sherwood, 2004) was used to model the dimerization of FcRY, and a global fit of data at five concentrations ranging from 50 nM to 3.2 μM yielded a sedimentation coefficient of 7.98 S for monomeric FcRY. The weak dimerization of FcRY at pH 6 is expected to only slightly affect the Svedberg calculated s^* value at low FcRY concentrations. Using a K_D for dimerization of 6.4 μM derived by SedAnal, we calculate that there would be 1.0% (by mass) FcRY dimers at a concentration of 30 nM. Assuming the dimer has a sedimentation coefficient of ~14 S, this would cause the weight averaged s^* value to be 0.1 S units higher than in the absence of any dimerization. Hence, the pH 6.0 s^* values obtained either by correcting the s^* value obtained from the program Svedberg for the effect of dimerization (7.9–0.1 = 7.8 S) or from the SedAnal global fit (which explicitly models dimerization) (7.98 S) are significantly higher than the pH 8.0 value of 7.2 S. Equilibrium AUC data confirm monomeric FcRY at pH 8 and FcRY dimerization at pH 6 with $K_D = 4.6$ μM (see Supplemental Experimental Procedures and Supplemental Figure S3 at http://www.immunity.com/cgi/content/full/20/5/601/DC1).

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Accession Numbers

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