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Melanie J. Lewis; ... et. al

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Structural Requirements for the Interaction of Human IgA with the Human Polymeric Ig Receptor[1]

Melanie J. Lewis, Richard J. Pleass,[2] Margaret R. Batten, Julie D. Atkin,[3] and Jenny M. Woof[4]

Transport of polymeric IgA onto mucosal surfaces to become secretory IgA is mediated by the polymeric Ig receptor (pIgR). To study the interaction of human dimeric IgA (dIgA) (the predominant form of IgA polymer) with the human pIgR (hpIgR), we generated recombinant wild-type dIgA1 and dIgA2m(1) and various mutant dIgA1 and analyzed their interaction with a recombinant human secretory component and membrane-expressed hpIgR. We found that wild-type dIgA1 and dIgA2m(1) bound to recombinant human secretory component with similar affinity and were transcytosed by the hpIgR to the same extent. Mutation of the IgA Cα2 domain residue Cys^{311} to Ser reduced binding to hpIgR, possibly through disruption of noncovalent interactions between the Cα2 domain and domain 5 of the receptor. Within the Cα3 domain of IgA1, we found that combined mutation of residues Phe^{411}, Val^{413}, and Thr^{414}, which lie close to residues previously implicated in hpIgR binding, abolished interaction with the receptor. Mutation of residue Lys^{377}, located very close to this same region, perturbed receptor interaction. In addition, 4 aa (Pro^{440}-Phe^{443}), which lie on a loop at the domain interface and form part of the binding site for human FcαRI, appear to contribute to hpIgR binding. Lastly, use of a monomeric IgA1 mutant lacking the tailpiece revealed that the tailpiece does not occlude hpIgR-binding residues in IgA1 monomers. This directed mutagenesis approach has thus identified motifs lying principally across the upper surface of the Cα3 domain (i.e., that closest to Cα2) critical for human pIgR binding and transcytosis. ***The Journal of Immunology,*** **2005, 175: 6694–6701.**

Immunoglobulin A is the predominant Ab class at mucosal surfaces and in external secretions, where it exists primarily as secretory IgA (SIgA)[5] (1, 2). Transepithelial transport of IgA onto the mucosal surfaces is mediated by the polymeric Ig receptor (pIgR), a receptor present in both mammals and birds (3–6). The pIgR, which is expressed basolaterally on glandular and mucosal epithelial cells, binds to IgA, which has been produced locally in the mucosal lymphoid tissues. This IgA is polymeric in nature, comprising two or more IgA monomers disulfide bonded to an additional 17-kDa polypeptide, termed J chain. On binding, both receptor and ligand are internalized and transcytosed through a series of vesicular compartments to the apical plasma membrane. At this point, the extracellular portion of the pIgR is proteolytically cleaved to form the secretory component (SC), which is covalently bound to polymeric IgA (pIgA) producing the complete form of SIgA (7, 8). By remaining bound to IgA, SC may provide the Ab with some protection against proteolytic degradation by gut and bacterial proteases (9, 10). Furthermore, the carbohydrate residues on SC mediate anchoring of SIgA to the mucus lining of the epithelium, ensuring effective protection of the mucosal surface (11).

SIgA forms the first line of immunological defense against mucosal infection, a role highlighted by the correlation between specific SIgA Abs and increased resistance to infection by a range of pathogens, including HIV-1, human papilloma virus-16, polio virus, and respiratory syncytial virus (12–17). Mucosal IgA defense is mediated not just on the mucosal surfaces, but also within the epithelial cell, as pIgA has been demonstrated to neutralize pathogens and inhibit pathogen-induced proinflammatory responses during the process of transcytosis (18–21). Moreover, IgA-containing immune complexes formed within the submucosa can be cleared from the tissues via the pIgR, thus limiting systemic exposure to Ag (22, 23). SIgA also contributes to innate immune defense through interaction of the *N*-linked oligosaccharide side chains of IgA and SC with bacterial adhesins and plant-derived toxins, thus preventing attachment of pathogens and toxins to the epithelium (24–26).

Interaction of pIgA with the pIgR is Fc mediated (27) and additionally appears to require direct interaction between the pIgR and the J chain (28). The human pIgR (hpIgR) comprises five Ig-like extracellular domains (29), of which the first three domains (D1, D2, and D3) are critical to interaction with human pIgA, whereas domains 4 and 5 (D4 and D5) appear only to contribute to the affinity of the interaction (30). Studies using pIgR knockout mice have confirmed a central role for transepithelial transport of pIgA by the pIgR in immunological defense at the mucosal surfaces and maintenance of mucosal homeostasis (31, 32). However, to date, the hpIgR binding site on human IgA has been localized to regions rather than individual residues of IgA. To gain a better understanding of the structural requirements for the interaction of human IgA with the hpIgR, we have produced recombinant wild-type dimeric IgA (dIgA) Abs of both IgA subclasses and have adopted a mutational approach to identify individual residues on

Division of Pathology and Neurosciences, University of Dundee Medical School, Ninewells Hospital, Dundee, United Kingdom

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[2] Current address: Institute of Genetics, Queen's Medical Centre, University of Nottingham NG7 2RD, U.K.

[3] Current address: Howard Florey Institute, The University of Melbourne, Parkville, VIC 3010, Australia.

[4] Address correspondence and reprint requests to Dr. Jenny M. Woof, Division of Pathology and Neurosciences, University of Dundee Medical School, Ninewells Hospital, Dundee DD1 9SY, U.K. E-mail address: j.m.woof@dundee.ac.uk

[5] Abbreviations used in this paper: SIgA, secretory IgA; CHO, Chinese hamster ovary; dIgA, dimeric IgA; FPLC, fast protein liquid chromatography; FSC, free SC; hpIgR, human pIgR; MDCK, Madin-Darby canine kidney; mIgA, monomeric IgA; NIP, 3-nitro-4-hydroxy-5-iodophenylacetate; PBST, PBS with 0.02% Tween 20; pIgA, polymeric IgA; pIgR, polymeric Ig receptor; SC, secretory component.

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the IgA Fc involved in interaction with the hpIgR. We demonstrate that residues Phe^{411}, Val^{413}, and Thr^{414} in the Cα3 are critical for hpIgR binding and transcytosis of IgA, and that Lys^{377}, Pro^{440}, Leu^{441}, Ala^{442}, and Phe^{443} are also required for optimal interaction with the human receptor. Finally, the Cα2 domain of human IgA appears to be involved, to some extent, in binding to the hpIgR, as mutation of Cys^{311} partially inhibits receptor interaction.

Materials and Methods

Generation of mutant IgA1 expression vectors

Recombinant IgA1 expression vectors with specific mutations in the Cα3 domain were prepared by PCR overlap extension mutagenesis (33) using a plasmid containing wild-type α1 gene downstream of a mouse Vnp domain (34) as a template and the same 5′ and 3′ flanking primers as described previously (39). Paired mismatch primers annealed within the Cα3 exon. In mutant 411–414AAA, the internal mismatch primer 5′-GGGCACCAC CACCGCCGCTGCGGCCAGCATACTGCG-3′ and its complement were used to incorporate substitutions encoding replacement of Phe^{411}, Val^{413}, and Thr^{414} with Ala residues. Residue 412 is already an alanine in IgA1. In mutant K377A, the internal mismatch primer 5′-GCTTCAGCCCCGCG GACGTGCTGG-3′ and its complement substitutes AAG with GCG to change Lys^{377} to Ala. In mutant D378R, the internal mismatch primer 5′-TCAGCCCCAAGCGCGTGCTGGTTC-3′ and its complement replace GAC with CGC to substitute Asp^{378} with Arg. In mutant E437L, the internal mismatch primer 5′-TGGTGGGCCACCTGGCCCTGCCGC-3′ and its complement substitute GAG with CTG to replace Glu^{437} with Leu. In each case, mutated PCR products were ligated into unique *Xho*I and *Sal*I restriction sites in the expression vector, replacing the wild-type sequence in this region. To verify incorporation of desired mutations and to check for PCR-induced errors, the entire PCR-generated regions were sequenced on an automated sequencer by the Sequencing Service, School of Life Sciences, University of Dundee.

Generation of a human J chain expression vector

RNA was extracted from 10^7 human B lymphocytes using an RNA isolation kit (Stratagene), and cDNA synthesis was performed using an RT-PCR kit (Stratagene) on 5 μl of extracted RNA. PCR amplification of human J chain sequence was performed using 5 μg of cDNA. The 5′ primer (5′-GCGCGCGCAAGCTTGCC**GCCACC**ATGAAGAACCATTTGCTT-3′) incorporated a *Hind*III restriction site (underlined) and a Kozak sequence (in bold), while the 3′ primer (5′-GCGCGCGCGAATTCTTATTAGT CAGGATAGCAGGCATC-3′) incorporated two stop codons and an *Eco*RI restriction site (underlined). The PCR product was cloned into *Hin*dIII and *Eco*RI restriction sites of a mammalian cell expression vector based on pEE6.hCMV (35). DNA sequencing confirmed that the cloned PCR product had the correct human J chain sequence.

Establishment of stable Chinese hamster ovary (CHO)-K1 transfectants expressing anti-3-nitro-4-hydroxy-5-iodophenylacetate (NIP) Abs

CHO-K1 cells were maintained as described previously (34). To produce monomeric IgA1 (mIgA1) Abs, CHO-K1 cells stably expressing the mouse λ L chain specific for the hapten NIP (34) were transfected by calcium phosphate transfection with the plasmid vectors for each of the mutated IgA1 H chains and positive transfectants selected, as described previously (34). To produce dIgA anti-NIP Abs, CHO-K1 cells stably expressing wild-type mIgA1 and mIgA2m(1) (34) and the 411–414AAA, K377A, D378R, and E437L mIgA1 mutants were subjected to a further round of calcium phosphate transfection with the human J chain expression vector. Previously described stable transfectants for mutant mIgA1 N263A (36), C311S (37), P440R, LA441–442MN, A442R, F443R (38), and N459A (39) were also transfected with human J chain to produce mutant dIgA1. Positive transfectants were selected in medium supplemented with 1 mg/ml Geneticin (Sigma-Aldrich). Cell lines producing the highest yields of J chain-containing Ab were identified by ELISA. Microtiter plates (Maxisorp Immunoplate; Nunc) were coated overnight at 4°C, with 100 μl/well of 2.5 μg/ml NIP-BSA in 0.05 M carbonate buffer, pH 9.6. After washing five times with water, 200 μl of blocking buffer (PBS with 0.02% Tween 20 (PBST) containing 5% nonfat milk powder) was added to each well and incubated for 1 h at room temperature. Following five washes as before, plates were incubated for 1 h at room temperature with 100 μl/well transfectant supernatant. After five washes as before, 100 μl/well of 2.5 μg/ml rat IgG2a anti-human J chain mAb, LO-hJ-3 (a gift of J.-P. Vaerman, Universite Catholique de Louvain, Brussels, Belgium), in PBST was applied for 1 h at room temperature, followed by goat anti-rat IgG-alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1/1000 in PBST. After washing, plates were developed for 20–30 min at room temperature with 100 μl of substrate (1 mg/ml pNPP in 0.2 M Tris buffer) per well, and absorbance was measured at 405 nm on a Dynex MRX II plate reader.

Purification and fractionation of NIP-specific Abs

Recombinant Abs were purified from supernatants of CHO-K1 transfectants, as described previously, by affinity chromatography on NIP-Sepharose (34). Samples of affinity-purified Ab were concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore), and subjected to gel filtration on a Superose 6 column connected to an ÅKTA fast protein liquid chromatography (FPLC) system (Amersham Biosciences). Fractions were analyzed by SDS-PAGE and Western blotting, and those corresponding to monomeric and dimeric forms of IgA were pooled separately and quantified by ELISA against a standard curve generated using a 2-fold dilution series (0–1000 ng/ml) of rIgA. ELISAs were conducted as above, except that the detection Ab was goat anti-human IgA-HRP (1 μg/ml; Kirkegaard & Perry Laboratories).

Generation of expression vectors for full-length and truncated forms of hpIgR

A mammalian expression vector for full-length hpIgR was constructed by subcloning hpIgR cDNA (a gift of C. Kaetzel, University of Kentucky, Lexington, KY) as an *Eco*RI fragment into the expression vector pcDNA3 (Invitrogen Life Technologies) featuring a hygromycin resistance gene (a gift of A. Bottger, University of Dundee, Dundee, U.K.). To generate a vector encoding a truncated form (rSC), the hpIgR cDNA *Eco*RI fragment was subcloned into plasmid pSP73 (Promega). The resultant plasmid served as template for PCR mutagenesis. The 5′ primer (5′-CAAACCT CAAGGTAC-3′) annealed at a unique *Kpn*I site within the hpIgR sequence. The 3′ primer (5′-GCGCGCGCCTCGAGTCATCAGTGATGG TGATGGTGATGCTCTGCAAAAAGCCTG-3′) encodes a *Xho*I site, two stop codons, and six histidine residues, resulting in termination of the pIgR sequence after Glu^{589} in domain 6 of the hpIgR and addition of a six-histidine tag to the truncated C terminus. PCR products were digested with *Kpn*I and *Xho*I and ligated into the intermediary plasmid, replacing the wild-type sequence in this region. After sequencing of the entire PCR-generated regions, the truncated hpIgR sequence was subcloned as an *Eco*RI-*Xho*I fragment into the wild-type expression vector, replacing the wild-type pIgR sequence.

Establishment of stable CHO-K1 transfectants expressing rSC

CHO-K1 cells were maintained as above and transfected by calcium phosphate transfection with 10 μg of the plasmid vector encoding the rSC. Selection for the hygromycin-selectable marker was achieved by culture in growth medium supplemented with 200 μg/ml hygromycin B (Roche). Clones producing rSC were identified by ELISA, conducted as above except that microtiter plates were coated with 10 μg/ml rabbit anti-human SC (DakoCytomation) and rSC contained in culture supernatant was detected using 2.5 μg/ml rabbit anti-human SC-HRP (DakoCytomation). Purified human SIgA (2.5 μg/ml) was used as a positive control and culture medium as a negative control. Substrate for the HRP-conjugated Ab was 0.6 mg/ml ABTS and 0.005% H_2O_2 in 0.1 M citrate buffer, pH 4.0. Absorbance was measured at 405 nm after 10–15 min.

Purification of rSC

rSC was purified from culture supernatant by affinity chromatography on polymeric IgA-Sepharose (provided by A. Almogren, University of Dundee). Samples were passed through the column, and bound rSC was washed extensively with PBS, pH 7.0, containing 0.1% NaN_3, and then eluted using 0.1 M citric acid, pH 3.0, directly into neutralizing buffer (0.7 M $Na_2HPO_4 \cdot 2H_2O$, pH 8.8). Purified rSC was dialyzed exhaustively against PBS containing 0.01% NaN_3, pH 7.0, and stored at −20°C.

SDS-PAGE and Western blotting

Purified Abs were analyzed by SDS-PAGE on 5% (nonreducing conditions) or 12.5% (reducing conditions) acrylamide gels. Western blots, using purified SIgA1 and SIgA2 as controls, were probed with either goat anti-human IgA1-alkaline phosphatase or goat anti-human IgA2-alkaline phosphatase (Southern Biotechnology Associates) diluted 1/2000 in PBST for the detection of IgA, or rat anti-human J chain mAb LO-hJ-2 (a gift of J.-P. Vaerman) at 2.5 μg/ml in PBST, followed by goat anti-rat IgG-alkaline phosphatase diluted 1/1000 in PBST, for the detection of J chain. Blots

were allowed to develop for 10–30 min in substrate (0.8 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.16 mg/ml NBT in 100 mM Tris containing 100 mM NaCl and 5 mM $MgCl_2$).

Purified rSC was analyzed on 8.4% acrylamide gels under nonreducing conditions. Western blotting was conducted as above, except that rabbit anti-human SC-HRP (2.5 μg/ml) was used for detection of SC. Blots were allowed to develop for 5–10 min in substrate (10 mg diaminobenzidine, 10 mg NiCl, and 100 μl H_2O_2 in 20 ml of PBS). SC released from reduced human SIgA, purified human free SC (FSC; a gift of A. Almogren), and culture supernatant containing cleaved SC released from the human adenocarcinoma cell line LS174T were used as controls for Western blotting. Purity of rSC preparations was assessed by silver staining of SDS-PAGE gels.

Establishment of a stable Madin-Darby canine kidney (MDCK) transfectant expressing full-length hpIgR

MDCK cells were maintained in MEM with Earle's salts supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 200 U/ml penicillin, 100 ng/ml streptomycin, and 10% FCS. MDCK cells were transfected by calcium phosphate transfection with 10 μg of the plasmid vector encoding the full-length hpIgR. Selection for the hygromycin-selectable marker was achieved by culture in growth medium supplemented with 100 μg/ml hygromycin B. Clones expressing hpIgR were identified using the rSC ELISA described above to detect cleaved SC in the supernatant of transfectants. Membrane expression of hpIgR was confirmed by flow cytometry and immunofluorescence microscopy.

Detection of IgA-SC binding by association ELISA

The concentrations of recombinant proteins in culture supernatant were quantified using anti-human J chain ELISA (for dIgA), anti-human IgA ELISA (for mIgA), or anti-human SC ELISA (for rSC), as described above. Microtiter plates were coated overnight at 4°C with 10 μg/ml rabbit anti-human SC in carbonate buffer, pH 9.6. After washing, plates were blocked, as described above. Supernatants containing rSC (25 ng/ml) and 2-fold dilutions (0–700 ng/ml) of the various dIgAs were coincubated at room temperature for 1 h. The corresponding mIgA Abs (0–700 ng/ml) were similarly coincubated with rSC as a negative control. Coincubated proteins were then applied to the ELISA plates and incubated for 1 h at room temperature with shaking. In vitro associated SIgA was detected using 1 μg/ml goat anti-human IgA-HRP (Kirkegaard & Perry Laboratories), and the ELISA was developed, as described above. Purified, quantified recombinant proteins were assessed by association ELISA in the same way. The slope of the linear regression line produced by the 2-fold dilution series of Ab was used to compare the relative SC-binding affinities of the various Abs (28, 40).

Assay of pIgR-mediated transcytosis

hpIgR-transfected and untransfected MDCK cells were seeded at 5×10^4 cells/well in MEM containing 1% nonessential amino acids, 2 mM L-glutamine, 200 U/ml penicillin, 100 ng/ml streptomycin, and 5% FCS (growth medium) into the apical chamber of 6.5-mm, 0.4-μm Transwell-Clear filters (Costar) and incubated for 3 days. Tightness of cell monolayers was confirmed, as described elsewhere (41). Filters were washed twice with fresh growth medium, and the various dIgAs (1 μg/ml in growth medium) were placed in duplicate in the basolateral chambers of the Transwells. The corresponding mIgAs (1 μg/ml) were used as negative controls, and 1 μg/ml purified rIgG1 was included with all Abs as an internal control for monolayer leakage. After incubation for 20 h at 37°C, the amount of Ab transcytosed across the MDCK monolayers into the apical medium was analyzed by ELISA.

Results

Binding of rSC to wild-type dIgA1 and dIgA2m(1) Abs

Recombinant dIgA1 and dIgA2m(1) Abs were generated by transfection of the respective mIgA-producing cell lines with human J chain. Using an ELISA to detect J chain in complex with IgA, CHO-K1 clonal cell lines expressing high levels of J chain were selected for each IgA subclass. The selected clones produced both dIgA and mIgA, as previously described (42). Following Ag-affinity chromatography to purify secreted Igs, the dIgA and mIgA fractions were separated by FPLC. The dimeric and monomeric fractions migrated at the predicted molecular mass (dimer ~340 kDa, monomer ~160 kDa), and only the dIgA fraction contained J chain (Fig. 1).

A truncated version of the hpIgR equivalent to the FSC was produced by PCR mutagenesis. Truncation of the pIgR sequence was confirmed by DNA sequencing and restriction enzyme digestion analysis, and following transfection into CHO cells, a clonal cell line producing rSC was established. The secreted protein was purified in a single step on pIgA-Sepharose and demonstrated by SDS-PAGE and Western blotting to migrate at the predicted molecular mass (~80 kDa) and at a position corresponding to purified human FSC, SC released from reduced human SIgA, and FSC released by the human adenocarcinoma cell line LS174T that endogenously expresses hpIgR (data not shown).

The purification of dIgA Abs is laborious and time consuming. Hence, we chose to establish an assay that could detect the in vitro association of rSC and dIgA Abs using unpurified proteins contained in the supernatant of CHO cell cultures. This approach required prior quantification of dIgA Abs within supernatant that contained IgA in both monomeric and dimeric forms produced by the same cell line. Hence, an anti-J chain ELISA, which would selectively detect IgA Abs that had incorporated J chain, was used, and a standard curve of purified recombinant dIgA1 was included.

Equivalent 2-fold dilutions of dIgA1 and dIgA2 were coincubated with rSC and then captured in an ELISA with a polyclonal anti-SC Ab. Complexes in which rSC had associated with dIgA were detected with an HRP-conjugated polyclonal anti-IgA Ab. This combination of Abs requires that rSC must be bound to IgA to produce a positive signal in the ELISA. Binding specificity was verified by including control incubations of Abs in the absence of rSC, of Abs with supernatant from untransfected CHO cells, and of equivalent mIgA Abs with rSC. Neither mIgA (IgA1 or IgA2) nor

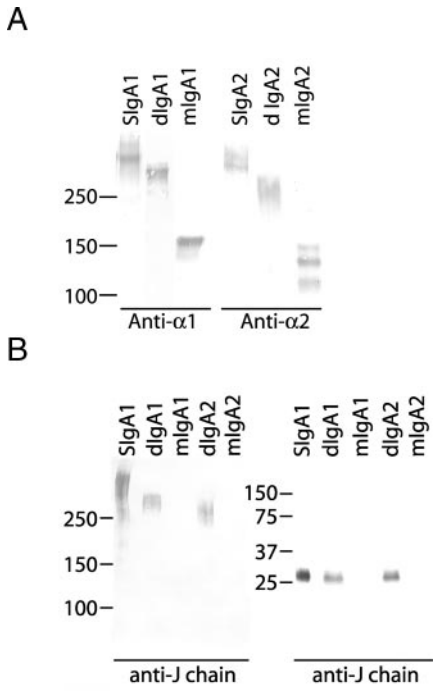

FIGURE 1. Western blot analysis of recombinant dIgA1 and dIgA2m(1). *A*, FPLC-purified dIgA and mIgA fractions resolved under nonreducing conditions and probed with anti-human α1 chain or anti-human α2 chain Abs, as indicated. *B*, FPLC-purified dIgA and mIgA fractions resolved under nonreducing conditions (*left panel*) or reducing conditions (*right panel*) were probed with anti-human J chain Ab. Purified human SIgA1 and SIgA2 were used as controls. The positions of molecular mass markers (in kilodaltons) are indicated on the *left*.

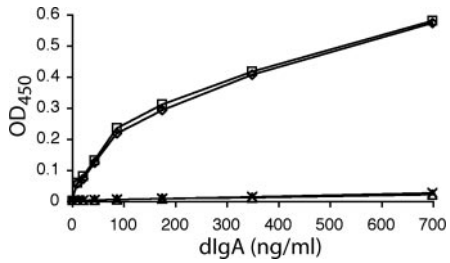

FIGURE 2. Binding of recombinant wild-type IgA1 and IgA2m(1) to rSC measured by association ELISA. Quantified concentrations of monomeric and dimeric forms of IgA1 and IgA2 in purified form were coincubated with rSC. ◇, dIgA1; △, mIgA1; □, dIgA2; ×, mIgA2. The results of a typical experiment, of five performed, are shown. Control incubations of IgA in the absence of rSC, or of IgA with supernatant from untransfected cells gave no absorbance. Only dimeric forms of IgA bound rSC, and binding of dIgA1 and dIgA2 was equivalent.

supernatant from untransfected CHO cells bound to the rSC, whereas both dIgA1 and dIgA2 interacted with rSC to produce a positive signal by ELISA. The two IgA subclasses showed no apparent difference in relative SC binding (data not shown).

To verify that the results obtained above were not influenced by other secretion products or proteins contained in the CHO supernatants, purified recombinant proteins were then tested in the association ELISA. Essentially the same results were obtained, indicating that the ELISA provided reliable results even when unpurified culture supernatant was used. As previously, only coincubated samples containing dIgA and rSC produced a positive signal. Moreover, the relative SC binding of the two IgA subclasses was comparable, confirming that the assay provided a measure of the specific interaction between dIgA and rSC (Fig. 2).

Binding of rSC to mutant dIgA1 Abs

To identify individual IgA H chain residues involved in interaction with the hpIgR/SC, mutant dIgA1 Abs were generated. Mutations were mostly in the Cα3 domain, with the exception of the N263A and C311S mutations, which are within the Cα2 domain (Fig. 7*A*). A previously described mIgA1 tailpiece deletion mutant, PTerm455 (39), in which the H chain terminates at Lys^{454}, was also included to test the hypothesis that the tailpiece in mIgA may mask residues required for binding to the hpIgR and offer an explanation for the nonbinding of mIgA to pIgR (43).

Culture supernatants from transfectants expressing different mutant dIgA1s were tested for binding to rSC using the association ELISA described for wild-type dIgA. For each experiment, using equivalent concentration ranges (0–700 ng/ml), the corresponding mIgA1 versions of the mutants were coincubated with rSC as a control of binding specificity. Two-fold dilutions of wild-type dIgA1 (0–700 ng/ml) coincubated with rSC were included on each ELISA plate for direct comparison. In keeping with a previous study (44), the dIgA1 N263A and N459A mutants exhibited comparable SC binding to that of wild-type dIgA1 (Fig. 3). The dIgA1 mutants D378R and E437L also showed equivalent binding to wild-type dIgA1 (Fig. 3*A*). However, the C311S, K377A, P440R, LA441–442MN, and F443R mutations all resulted in a reduction in SC binding relative to wild-type dIgA1, with the P440R and F443R mutations having the most marked effect (Fig. 3). Interestingly, the A442R mutant exhibited higher levels of SC binding than wild-type dIgA1 at equivalent concentrations. The dIgA1 mutant 411–414AAA and the mIgA mutant PTerm455 demonstrated little, if any, SC binding (Fig. 3).

Those mutant Abs exhibiting a different pattern of SC binding to that of wild-type dIgA1 were purified by NIP-affinity chromatography, and the mIgA1 and dIgA1 fractions were separated by FPLC. The initial peaks eluted had the anticipated elution characteristics of dimers and were analyzed by SDS-PAGE and Western blotting (Fig. 4). The mutants assembled into covalently stabilized dimers, with the exception of the A442R, K377A, and 411–414AAA mutants. Analysis of FPLC fractions corresponding to polymeric IgA for the A442R mutant revealed that it assembled into dimer, trimer, tetramer, and higher polymer, with each of these molecular forms containing J chain (data not shown). FPLC purification did not clearly separate the dimer from the other higher polymeric forms. Hence, for the A442R mutant, a single fraction containing dimer and some trimer was used for additional

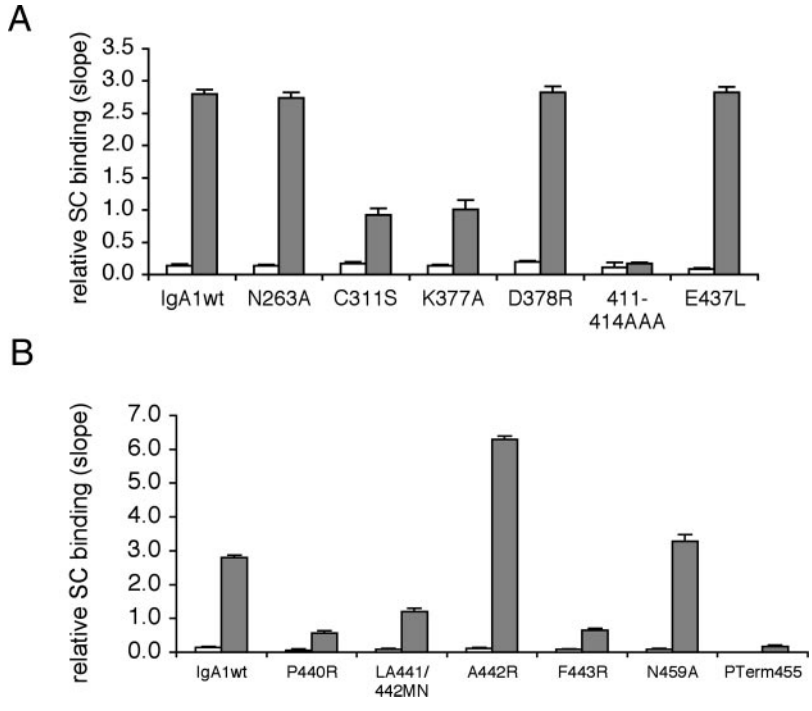

FIGURE 3. *A* and *B*, Relative SC binding of various mutant dIgA1 Abs measured by association ELISA. The specificity of the assay was verified by the inclusion of the mIgA equivalents for each of the mutants coincubated with rSC. □, Indicate mIgA; ▨, indicate dIgA. Note that for the IgA tailpiece deletion mutant the ▨ indicates the mutant mIgA. Figure shows the mean ± SE of three independent experiments.

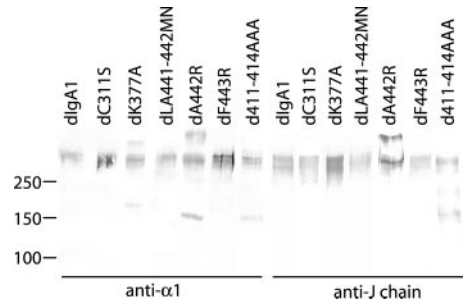

FIGURE 4. Western blot analysis under nonreducing conditions of pooled dimer fractions of FPLC-purified recombinant dIgA1 mutants probed with anti-human α1 chain Ab (*left*) and anti-J chain Ab (*right*). FPLC-purified recombinant wild-type dIgA1 was included as a control. The positions of molecular mass markers (in kilodaltons) are indicated on the *left*.

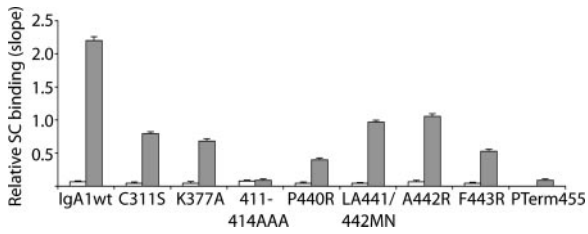

FIGURE 5. Relative SC binding of affinity- and FPLC-purified mutant IgA1 Abs measured by association ELISA. The specificity of the assay was verified by the inclusion of the mIgA equivalents for each of the mutants coincubated with rSC. □, Indicate mIgA; ▨, indicate dIgA. Note that for the IgA tailpiece deletion mutant, the ▨ indicates the mutant mIgA. Figure shows the mean ± SE of three independent experiments.

experiments (Fig. 4). Separation of dIgA from trimeric IgA is known to be extremely difficult (45, 46). Interestingly, SDS-PAGE analysis of the A442R dimer/trimer fraction revealed a J chain-negative band corresponding to mIgA (Fig. 4). This band has also been noted for purified human trimeric myeloma IgA1 (our unpublished observations), suggesting that in some trimeric IgA molecules, one of the mIgA1 units is not covalently bound to the J chain or the other mIgA1 units.

The first peak eluted in FPLC purification of the dK377A mutant comprised principally J chain-positive dimers, but there were also small amounts of J chain-negative monomer and higher molecular mass species present (Fig. 4). Because our assays to estimate dIgA concentration and to measure dIgA-SC interaction both rely on detection of only J chain-positive material, the presence of these minor J chain-negative contaminants was considered unlikely to influence our results.

Although the majority of the first peak eluted in FPLC purification of the d411–414AAA mutant comprised J chain-containing dimers, a small percentage (10.7%, as estimated by densitometric analysis) appears to be present as J chain-positive monomer material (Fig. 4). Thus, for this mutant, a small percentage of some dimers may be made up of one monomer unit covalently attached to J chain associated in a noncovalent way with the second monomer unit.

As anticipated, the tailpiece deletion mutant assembled into predominantly covalently stabilized monomer, with some IgA half molecules (HL) and H chain dimers (H_2), as previously described (39).

The interaction of purified dimeric mutant Abs with rSC was reanalyzed by the association ELISA method used above. Comparable reactivity of the polyclonal anti-IgA detection Ab with each of the purified dimeric mutant Abs was confirmed to ensure that the results of the association ELISA were not affected by reduced detection due to epitope loss from the introduced mutations (data not shown). The results obtained using the purified proteins (Fig. 5) were comparable with those obtained using the proteins contained in crude supernatant (Fig. 3), with the exception of the A442R mutant. Rather than the marked increase in SC binding above that of wild-type dIgA observed for the supernatant preparations, the FPLC-purified dimer/trimer fraction of this mutant revealed a decrease in SC binding to about one-half that of wild-type dIgA1. Hence, the increase in relative SC binding seen for the crude preparation of the A442R mutant is most likely a function of the higher polymeric forms (tetramer and higher polymer) present in this preparation of the mutant. Higher IgA polymer has previously been shown to be a much more potent binder of rSC than dIgA (47).

Transcytosis of IgA across hpIgR-expressing MDCK cells

MDCK cells were transfected with the hpIgR expression vector, and a hpIgR-expressing clonal cell line was selected on the basis of high levels of SC in culture supernatant (through natural cleavage of the receptor) and strong membrane expression (data not shown). To test whether purified recombinant wild-type dIgA (both subclasses) and the various mutant dIgA1s could be transcytosed by the hpIgR-expressing MDCK cells, the MDCK transfectants were grown as polarized monolayers on permeable filters, and the various recombinant Abs were assessed in a transcytosis assay. In agreement with the SC-binding experiments, wild-type dIgA1 and dIgA2m(1) were transcytosed to an equal extent (Fig. 6). The various mutant dIgA Abs showed transcytosis levels that mirrored their relative SC binding, with the C311S and K377A mutants demonstrating transcytosis levels of ~30% that of wild-type, the LA441/442MN and A442R mutants ~40% of wild-type, and the P440R and F443R mutants ~15% of wild type. The 411–414AAA and IgA tailpiece deletion mutants were not transcytosed. As expected, the monomeric equivalents of each of the Abs were not transported across the hpIgR-expressing monolayers (Fig. 6) and neither mIgA nor dIgA were transported across untransfected cell monolayers (data not shown), confirming the specificity of the assay.

Discussion

This study sought, through a program of site-directed mutagenesis, to investigate the site(s) on human dIgA responsible for binding to hpIgR. The initial association of pIgA with the pIgR is mediated by high affinity noncovalent interactions, which take place within

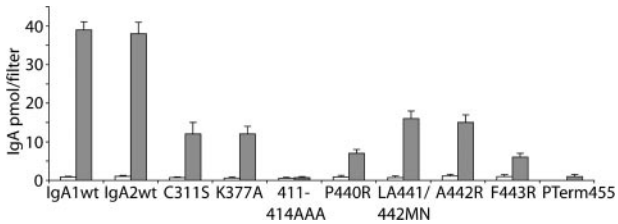

FIGURE 6. Transcytosis of purified IgA across polarized hpIgR-expressing MDCK monolayers. Recombinant Abs were added basolaterally, and the apical medium was harvested after 20 h. □, Indicate mIgA; ▨, indicate dIgA. Note that for the IgA tailpiece deletion mutant, the ▨ indicates the mutant mIgA. Figure shows the results from one of three independent experiments expressed as the mean ± SD of duplicate filters.

5 min of receptor-ligand contact (9). These noncovalent interactions are followed by the formation of a disulfide bond between Cys^{311} in the Cα2 domain of IgA and Cys^{467} in the fifth domain of hpIgR (48), which is thought to increase the stability of the SIgA complex (9, 49).

We found no difference in the relative affinity of rSC binding between the two IgA subclasses, and the amount of IgA that was transcytosed via hpIgR was the same regardless of IgA subclass. This finding is in agreement with earlier studies, which have shown that pIgA2 binds to SC with the same affinity as pIgA1 (9, 27).

Previous work has suggested that both of the Fc domains of human IgA are involved in noncovalent binding to the human receptor, with the Cα3 domain playing the principal role (40). The reduced SC binding and decreased ability to undergo pIgR-mediated transcytosis exhibited by the C311S IgA1 mutant, seen in the present study, are consistent with a role for the Cα2 domain of human IgA1 in interaction with the human receptor. Although disulfide bond formation via Cys^{311} may not be essential for IgA-pIgR interaction (50), it may provide an important stabilizing function in the dIgA-SC complex. Alternatively, the C311S mutation may have a subtle effect on Cα2 domain structure or relative positioning of domains, although such a conservative substitution would not be expected to have a large impact on structure.

It is well established that pIgR interacts only with pIgA (dimers or higher oligomers) and does not bind to mIgA (27, 51, 52). It has been proposed that mIgA might fail to bind the pIgR due to masking of pIgR-binding residues by the IgA tailpiece (43). The IgA1 tailpiece deletion mutant PTerm455 was used to test this possibility. Evidently, the lack of binding of mIgA cannot be explained by hindrance of binding regions by the tailpiece because PTerm455 did not interact with either rSC or cell-bound hpIgR, in line with an earlier study (53). Thus, the requirement for dIgA more likely relates to the presence of J chain and use of pIgR-binding motifs within both IgA monomers. Indeed, within the J chain structure, a C-terminal loop between Cys^{109} and Cys^{134} appears necessary for direct interaction with SC (28).

Previous work investigating the role of the Cα3 domain of IgA1 in hpIgR/SC interaction has implicated three regions, comprising residues 374–391, 402–410, and 430–443 (51, 54). However, only the 402–410 region has been studied by mutational analysis of intact polymeric Abs (51). We sought to identify the extent of the 402–410-binding motif by mutating adjacent IgA residues, and to identify individual amino acids within the 374–391 and 430–443 regions critical for interaction with hpIgR.

Simultaneous mutation of amino acids Phe^{411}, Val^{413}, and Thr^{414} to Ala in the d411–414AAA mutant resulted in complete ablation of hpIgR binding. As the purified preparation of this mutant contained ~10% J chain-containing mIgA, we cannot discount the possibility that this minor monomer contaminant may have contributed to the observed decrease in binding. However, as the effect is so marked, it is reasonable to conclude that the dimeric form of the mutant displays very weak, if any, binding to hpIgR. This triple mutation may affect binding affinity through removal of direct binding interactions, or it may trigger alterations in the conformation of close-lying residues that provide binding contacts. The previously identified 402–410 motif (sequence QEPSQGTTT), with which these residues are continuous, protrudes out from the upper central portion of the Cα3, encompassing part of β strand D, all of the DE loop, and part of β strand E (Fig. 7). Residues 411–414 are located distal to the DE loop within β strand E and appear less accessible (Fig. 7). Thus, residues 411–414 may not be involved in direct interaction with the hpIgR, but may play an indirect role in maintaining the structural conformation of close-lying regions in contact with the receptor. Nevertheless, these residues serve as important markers of the hpIgR binding site.

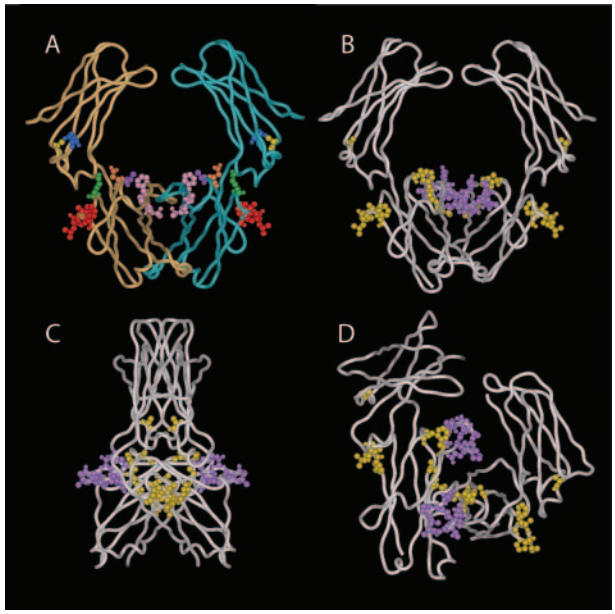

FIGURE 7. Four views of the Fc region of human IgA1 (using coordinates from Protein Data Bank accession code 1OW0). *A–C*, The 2-fold axis of symmetry is vertical, and the Fc is viewed from the front (*A* and *B*) and the side (*C*). *D*, The axis of symmetry is tilted slightly forward from the vertical and to the *right*. The C-terminal tailpieces are omitted. *A*, One H chain is shown in gold and the other in cyan, and the side chains of the residues mutated in this study are highlighted as follows: Cys^{311}, yellow; Asn^{263}, blue; Lys^{377}, purple; Asp^{378}, orange; Phe^{411}, Val^{413}, and Thr^{414}, all pink; Glu^{437}, green; Pro^{440}, Leu^{441}, Ala^{442}, and Phe^{443}, all red. *B–D*, Both H chains are colored silver, and residues critical for interaction with hpIgR are highlighted. The side chains of critical residues implicated in this study are shown as yellow spheres (Cys^{311}, Lys^{377}, Phe^{411}, Val^{413}, Thr^{414}, Pro^{440}, Leu^{441}, Ala^{442}, and Phe^{443}), and those of residues implicated in earlier mutational studies by others (51) as purple spheres (Gln^{402}-Thr^{410}). The highlighted residues appear to form a contiguous surface around the upper surfaces of the CH3 domains.

Two adjacent amino acids (Lys^{377}, Asp^{378}) located within the putative 374–391 hpIgR-binding region (54) were investigated for their relative roles in hpIgR binding. Mutation of Lys^{377} to Ala resulted in reduced hpIgR binding and transcytosis compared with wild-type dIgA1, whereas mutation of Asp^{378} to Arg had no apparent effect. Lys^{377} is located in the center of the BC loop of the Cα3 domain and may be more exposed than its neighbor. Lys^{377} is conserved in most IgA sequences from other species. In contrast, none of the other human Ab isotypes have Lys in this position. Mutation of Lys^{377} to Ala did not completely ablate hpIgR binding, but rather considerably reduced the level of interaction, suggesting that this residue plays a secondary or stabilizing role in the interaction. Spatially, Lys^{377} lies close to the 402–410 hpIgR-binding motif and could combine with it to form a larger interface (Fig. 7).

The remaining putative hpIgR-binding region (54), comprising residues 430–443 in the Cα3 domain, overlaps with the known binding site on human IgA for FcαRI. We investigated Glu^{437} from β strand F and Pro^{440}, Leu^{441}, Ala^{442}, and Phe^{443} from the FG loop for their role in hpIgR binding. Residues Pro^{440}-Phe^{443} compose part of the central core of the FcαRI-binding interface (55), and their mutation results in ablation of FcαRI binding (38, 56). Glu^{437} also contributes to the interaction with FcαRI (55), but

appears to play a more minor role because its mutation to alanine did not have a significant impact on FcαRI-binding affinity (57). We found that mutation of Glu^{437} to Leu had no effect on hpIgR binding, whereas mutation of the FG loop residues resulted in reduced hpIgR binding and transcytosis. Mutation of either Pro^{440} or Phe^{443} to Arg had a more marked effect on hpIgR binding than mutation of Leu^{441} and Ala^{442} jointly to Met and Asn, respectively, or of Ala^{422} individually to Arg. The LA441–442MN mutant changes the sequence of human IgA1 in this region (PLAF) to that of mouse IgA (PMNF). The reduced hpIgR binding and transcytosis seen with this mutant mirror an earlier report that mouse IgA is able to interact with the hpIgR, but is transcytosed somewhat less efficiently than human IgA (49).

SIgA binds FcαRI, but, in contrast to mIgA, is unable to trigger FcαRI-mediated phagocytosis and requires the presence of the integrin coreceptor Mac-1 (CD11b/CD18) to trigger a respiratory burst via FcαRI (58, 59). The overlap of FcαRI and hpIgR binding sites revealed in this study may account for this difference between SIgA and mIgA.

The predicted involvement of all five ectodomains of hpIgR, the Fc region of each monomer of dIgA, and J chain in the interaction between dIgA and hpIgR suggests a relatively large binding interface involving multiple segments from each protein. Despite the identification of critical Ig-binding motifs within D1 of the receptor, and of disulfide bond formation between Cys^{311} in the IgA Cα2 domain and Cys^{467} in D5 of pIgR, specific binding sites within the remaining receptor domains are not localized as yet. However, based on the evidence presented in this work, it is tempting to speculate that in one monomer subunit of dIgA, the Cα3 residues 402–414, together with Lys^{377}, may interact with domain 1 of hpIgR. D1 of hpIgR may form additional contacts with the 440–443 region of IgA of the same monomer, or perhaps these residues in the second IgA monomer interact with an alternative pIgR domain, possibly D5.

In summary, concentrating on the human system, we have identified residues in the Cα3 domain of IgA critical for binding to hpIgR, and confirmed the role of the Cα2 domain Cys^{311} in the interaction. In the absence of data from structural studies, this type of mutational approach lends further understanding to this important and complex interaction.

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Disclosures

The authors have no financial conflict of interest.

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