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The Carboxyl-terminal Domains of IgA and IgM Direct Isotype-specific Polymerization and Interaction with the Polymeric Immunoglobulin Receptor*

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Mucosal surfaces are protected by polymeric immunoglobulins that are transported across the epithelium by the polymeric immunoglobulin receptor (pIgR). Only polymeric IgA and IgM containing a small polypeptide called the "joining" (J) chain can bind to the pIgR. J chain-positive IgA consists of dimers, and some larger polymers, whereas only IgM pentamers incorporate the J chain. We made domain swap chimeras between human IgA1 and IgM and found that the COOH-terminal domains of the heavy chains (Cα3 and Cμ4, respectively) dictated the size of the polymers formed and also which polymers incorporated the J chain. We also showed that chimeric IgM molecules engineered to contain Cα3 were able to bind the rabbit pIgR. Since the rabbit pIgR normally does not bind IgM, these results suggest that the COOH-terminal domain of the polymeric immunoglobulins is primarily responsible for interaction with the pIgR. Finally, we made a novel chimeric IgA immunoglobulin, containing the terminal domain from IgM. This recombinant molecule formed J chain-containing pentamers that could, like IgA, efficiently form covalent complexes with the human pIgR ectodomain, known as secretory component.

At least 80% of all antibody-secreting plasma cells of the body are located in the gastrointestinal and respiratory mucosae, and most of them are committed to immunoglobulin A (IgA) production (1, 2). All immunoglobulin isotypes consists of two heavy (H) and two light (L) chains, but for IgA, this H_2L_2 monomeric unit can polymerize further. Mucosally produced IgA consists predominantly of dimers and some larger polymers, collectively called polymeric IgA (pIgA).[1] IgA polymerization is regulated by the incorporation of the joining chain (J chain) in that its presence greatly stimulates polymerization (3–9). J chain is also essential in forming a docking site on pIgA for the polymeric immunoglobulin receptor (pIgR) (5, 10–14). This 110-kDa glycoprotein binds pIgA at the basolateral epithelial cell surface. Receptor-IgA complexes are internalized and then transcytosed to the apical surface, where secretory IgA (S-IgA) is released into the lumen by proteolytic cleavage of the receptor ectodomain (2, 15). Cleavage of unoccupied receptor releases the five extracellular immunoglobulin-like domains (D1–D5), known as free secretory component (SC).

IgM also has the ability to polymerize, mainly to pentamers with incorporated J chain and to hexamers without (3, 16, 17). As for IgA, only J chain-containing IgM polymers bind the pIgR (11, 18–20). IgM is believed to be ancestral to all immunoglobulin classes (21). During evolution of IgG, the ability to form polymers, incorporate the J chain, and bind to the pIgR was lost. Thus, the IgM and IgA heavy chains (μ- and α-chain, respectively) have a number of features that are absent in IgG. Both IgM and IgA have a COOH-terminal extension of 18 amino acids called the secretory tailpiece, which includes a cysteine required for polymerization in the penultimate position (Cys-575 in μ tailpiece; Cys-495 in α tailpiece). Besides the secretory tailpiece cysteines, Cys-337 and Cys-414 are available for disulfide bonding in IgM. Whereas Cys-337 most likely forms intramonomeric bonds between two Cμ2 domains, Cys-414 (located in Cμ3) forms intermonomeric bonds (22–26). Conversely, Cys-309 in IgA (equivalent to Cys-414 in IgM) is used for covalent bonding to pIgR during transcytosis (27). Moreover, during evolution of IgA the polymerization process has altered to favor dimerization rather than formation of larger polymers. Monomers are also secreted from IgA-producing plasma cells, whereas they are mainly retained and degraded in IgM-producing plasma cells (24).

J chain incorporation is an early event in IgA polymerization, and this peptide is found in all polymeric forms of this isotype (3, 4). For IgM, however, the J chain is incorporated late in the polymerization process and is found only in pentamers (17). Furthermore, the mode of pIgR binding differs significantly between the two isotypes, and in some species (most notably the rabbit) the ability of the pIgR to bind pentameric IgM has been selectively lost (28, 29). To address the structural basis for the disparate polymerization properties of IgA and IgM and their differential mode of interaction with pIgR, we made a series of domain swap mutants between human IgA1 and human IgM. Our results demonstrate that the COOH-terminal domains of IgA and IgM contain the most important structural elements involved in isotype-specific polymerization. We also found that swapping Cμ4 with Cα3 made IgM able to bind to the rabbit pIgR, indicating that the COOH-terminal domain is also most important for the differential receptor binding.

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[1] The abbreviations used are: pIgA, polymeric IgA; pIgR, polymeric Ig receptor; S-IgA, secretory IgA; J chain, joining chain; SC, secretory component; NIP, 5-iodo-4-hydroxy-3-nitrophenylacetyl; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; MDCK, Madin-Darby canine kidney; mAb, monoclonal antibody; BSA, bovine serum albumin.

TABLE I
Antibodies used in ELISA

Specificity	Coat	Dilution	Primary antibody	Dilution	Secondary antibody	Dilution
NIP-specific IgA	NIP-BSA	2.5 μg/ml	Anti-human IgA mAb[a]	1:50,000	AP-conjugated anti-mouse Ig[b]	1:1000
NIP-specific IgM	NIP-BSA	2.5 μg/ml	Anti-human IgM mAb[a]	1:50,000	AP-conjugated anti-mouse Ig[b]	1:1000
IgA	Rabbit anti-human IgA[b]	1:6000	Anti-human IgA mAb[a]	1:50,000	AP-conjugated anti-mouse Ig[b]	1:1000
IgM	Rabbit anti-human IgM mAb[c]	1:500	Biotinylated anti-human IgM mAb[b]	1:4000	AP-conjugated streptavidin[d]	1:3000
IgG	Goat anti-human IgG[e]	1:1000	AP-conjugated anti-human IgG[e]	1:3000	Not relevant	
SC	Mouse anti-human SC mAb[f]	1:1000	Rabbit anti-human SC[b]	1:1000	AP-conjugated anti-rabbit Ig[b]	1:4000

[a] Dr. T. Lea (Institute of Immunology, Rikshospitalet, Oslo, Norway).
[b] DAKO (Glostrup, Denmark).
[c] Diatec AS (Oslo, Norway).
[d] SouthernBiotech (Birmingham, AL).
[e] Sigma.
[f] Dr. J. Bartek (Research Institute of Clinical and Experimental Oncology, Brno, Slovakia).

EXPERIMENTAL PROCEDURES

Construction of Domain Swap Mutants—Oligonucleotide primers were purchased from British Biotechnology Products (Abingdon, UK), and enzymes were purchased from New England Biolabs (Beverly, MA). A 1.9-kb *Hin*dIII/*Bam*HI fragment from pUC19μ or a 2.7-kb *Hin*dIII/*Bam*HI fragment from pUC19α (30) was subcloned into M13mp19 to form M13μ or M13α, respectively. For M13α, a *Spe*I recognition site downstream of the α heavy chain gene was removed by restriction enzyme digestion, T4 DNA polymerase end filling, and blunt end ligation. Single-stranded DNA was prepared for *in vitro* mutagenesis with the Muta-Gene® M13 Mutagenesis Kit, Version 2, (Bio-Rad) as described (31). Restriction sites for *Spe*I were introduced into introns between the exons that encode Cμ1 and Cμ2 and between the exons that encode Cα1 and Cα2, with the primers (mutated nucleotides underlined) 5′-GCCCGCCACTAGTGCCCCTTCGG-3′ and 5′-CACGGGTGCAGGACTAGTGTCAGGGCAC-3′, respectively. Similarly, *Mlu*I sites were created between the exons that encode Cμ3 and Cμ4 and between the exons that encode Cα2 and Cα3 by the primers 5′-GAAGAGGGGCACGCGTGGGGCCTA-3′ and 5′-AGGCCAGGAATACGCGTTGCAAACCAGA-3′, respectively. A 1.3-kb *Dra*III/*Sac*II DNA fragment from mutated M13μ replaced the corresponding fragment in pUCμ, thereby forming wild-type IgM (Fig. 1). The mutated α gene was subcloned into pUC19 as a 2.7-kb *Hin*dIII/*Bam*HI DNA fragment to make IgA (Fig. 1). Mutations were verified by restriction enzyme digestion and DNA sequencing. By means of the introduced *Spe*I and *Mlu*I restriction sites, domain swap mutants were constructed (Fig. 1). The wild-type and domain swap gene constructs were subcloned as *Hin*dIII/*Bam*HI fragments into the expression vector pSV2gptV_{NIP} (courtesy of Dr. M. S. Neuberger, MRC Laboratory of Molecular Biology, Cambridge, UK) downstream of a V_H gene specific for the hapten 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP).

Murine J558L cells, which show constitutive synthesis of the J chain and a λ_1 light chain specific for NIP (courtesy of Dr. S. L. Morrison, Department of Microbiology, Molecular Biology Institute, UCLA), were grown in RPMI medium with penicillin (100 IU/ml) and streptomycin (100 μg/ml; Bio Whittaker, Walkersville, MD) and 10% fetal calf serum (Invitrogen) at 37 °C in an atmosphere with 5% CO_2. Stable transfection of J558L was achieved by electroporation as described previously (30), and clones were selected in medium containing 1 μg/ml mycophenolacid (Invitrogen) and 250 μg/ml xanthin (Sigma). The cells were incubated for 2–3 weeks before the supernatant fluids were harvested and screened for immunoglobulin production by enzyme-linked immunosorbent assay (ELISA). Four or five immunoglobulin-producing colonies from each construct were selected and expanded for further analysis.

Constructs of pIgR/SC—The human SC-his6 expression vector and the chimeric pIgR with rabbit D1 and human D2-to-COOH terminus (rD1-h pIgR) have been described previously (6, 28). The chimeric rD1-h SC with a six-histidine tag was made by amplifying the five immunoglobulin-like domains with the forward primer 5′-ATCTCTAGAAGCTTACCAACTGGCCAGCAG-3′ and the reverse primer 5′-CTCCTCGAGAAGGAGCCGAGG-3′, which introduced *Hin*dIII and *Xho*I restriction sites (underlined) for subcloning into pCDNA(zeo)-his6 (6). COS-1 cells, grown in Dulbecco's modified Eagle's medium (Bio Whittaker) supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, and 1 mM L-glutamine (Invitrogen), were transiently transfected with human SC-his6 or rD1-h SC-his6 expression vectors by means of FuGENE™ (Roche Diagnostics). After 3 days, the supernatants were harvested, and the amount of secreted rD1-h SC with histidine tag was measured by ELISA.

ELISA—Microtiter plates were coated with the indicated coat reagent (Table I) in 0.05 M $NaHCO_3$ (pH 9.6) at room temperature overnight. The plates were washed six times in phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBS/T) after coating and before each new incubation. The plates were blocked with 1% (w/v) BSA in PBS/T for 3 h before the sample (50 μl) was added and incubated overnight at room temperature. Primary and secondary antibody reagent were incubated for 90 min each, and the plates were finally incubated with *p*-nitrophenyl phosphate (Sigma) in diethanolamine buffer at room temperature for 10–60 min. Absorbance was measured at 405 nm with a Tecan Sunrise Microplate Reader (Tecan Austria Gesellschaft, Salzburg, Austria). Standard curves were generated from 2-fold dilutions of purified human IgA, IgM, IgG, or free SC (from colostrum).

SC Binding Analysis—The different immunoglobulin preparations were incubated overnight at room temperature in NIP-BSA-coated microtiter plates. Then 1 μg/ml human SC or rD1-h SC was allowed to react with the bound immunoglobulin. Bound SC was detected as in the SC ELISA described above. The SC and antibody incubations were performed at 4 °C for 90 min to increase the stability of S-IgM. The slope of the line of a 2-fold dilution series of the immunoglobulin preparations was calculated to determine the relative binding of the different chimeric immunoglobulins for either human SC or rD1-h SC as described previously (5). All samples were measured in triplicates.

Metabolic Labeling, Immunoprecipitation, and Gel Electrophoresis—Approximately 5×10^6 cells were incubated in 0.5–1 ml of methionine/cysteine-free Dulbecco's modified Eagle's medium (Bio Whittaker) supplemented with 50–100 μCi of [^{35}S]methionine-cysteine (Amersham Biosciences) for 6 h (or overnight with 2% fetal calf serum). Secreted immunoglobulins were immunoprecipitated from the supernatant with rabbit anti-IgM, anti-IgA or anti-IgA/IgM/IgG (1/100; DAKO, Glostrup, Denmark) at 4 °C with gentle agitation for periods between 2 h and overnight. The immune complexes were harvested with ~0.3 mg of Dynabeads® (10 μl) coated with sheep anti-rabbit IgG (Dynal AS, Oslo, Norway) at 4 °C for periods between 1 h and overnight with gentle agitation. The Dynabeads were collected with a Dynal Magnetic Particle Concentrator (Dynal MPC®), washed three times in ice-cold PBS with 1% Nonidet P-40 (Sigma), and eluted in 10 μl of sample buffer (1% SDS, 30% glycerol, 0.02 M phosphate buffer, and bromphenol blue) for 3 min at 95 °C. Aliquots were analyzed without reduction by 4% SDS acrylamide-agarose as described previously (30). Rainbow™ ^{14}C-methylated protein molecular mass markers (46–220 kDa; Amersham Biosciences) were included. The gel was run in 0.1 M phosphate buffer (pH 7.0) with 0.1% SDS in a Bio-Rad mini protean II gel electrophoresis apparatus at 50 V for 3 h, fixed in 30% methanol with 10% acetic acid for 30 min, immersed in Amplify (Amersham Biosciences) for 30 min, dried, exposed to BIOMAX-MR film (Eastman Kodak Co.), and finally analyzed by a phosphor imager (GS-250 Bio-Rad molecular imager) and the Molecular Analyst™/PC software (version 1.4).

Purification of Recombinant Immunoglobulins—Supernatants were harvested from outgrown cultures (700–1000 ml) and loaded onto ~1 ml of packed NIP-coupled Sepharose (Amersham Biosciences) at a flow rate of ~0.6 ml/min. Bound immunoglobulins were washed with 20 column volumes of PBS and eluted with 6 ml of 0.5 mM NIP-CapOH (Genosys Biotechnologies, Pamisford, UK) and 0.05% NaN_3 in PBS. Total immunoglobulin in 0.5-ml fractions was quantified by ELISA. Peak fractions were pooled and dialyzed extensively against PBS.

Western Blot—NIP-purified immunoglobulins were fractionated by nonreducing 4% (w/v) SDS acrylamide/agarose gel electrophoresis and blotted onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA) at 100 V for 1 h. The membranes were blocked overnight with 5% milk powder and probed with a rabbit antibody reagent raised against human J chain (1:1000) (32) or with rabbit anti-human IgA/IgM/IgG (1:3000; DAKO), followed by a donkey anti-rabbit horseradish peroxidase conjugate (1:3000; DAKO). Horseradish peroxidase reactivity was revealed with SuperSignal™ solution (Pierce) for 5 min and exposure to x-ray film.

To examine the heavy chain, light chain, or J chain separately, the NIP-purified immunoglobulins were reduced by treatment with 100 mM dithiothreitol (Sigma) for 5 min at 95 °C and analyzed on a 12% SDS-PAGE with a 5% stacking gel. The gel was transferred to a nitrocellulose membrane (Amersham Biosciences) as described above and probed sequentially with polyclonal rabbit anti-human J chain (1:1000), rabbit anti-mouse λ chain (1:5000; SouthernBiotech, Birmingham, AL), and rabbit anti-IgA/IgM/IgG (1:3000). The membrane was stripped between each probing by incubation in a stripping buffer (53 mM Tris, pH 6.8, 1.6% SDS, and 14.3 mM β-mercaptoethanol) at 60 °C for 15 min and then blocked again with 5% (w/v) milk powder in PBS/T. Bands were visualized with the SuperSignal™ solution and scanned by Chemidoc (Bio-Rad), and densitometry was performed by band analysis with Quantitation One® software.

Transcytosis Assays—Madin-Darby canine kidney (MDCK) cells, untransfected or stably transfected with human pIgR, rabbit pIgR, or the chimeric rD1-h pIgR as described previously (28, 33), were used to study transcytosis of the domain swap mutants. Approximately 5.0×10^5 cells were seeded on 1-cm^2, 3.0-μm collagen-coated PTFE filters (Transwell-COL 3494; Costar). The cells were incubated for 3 days at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 μg/ml gentamicin, and 1 mM L-glutamine. At that time, the transepithelial resistance was about 150–200 ohms. The filters were incubated for 20 h at 37 °C in fresh medium with 50 mM of the chimeric immunoglobulins, and 50 mM of human IgG (to control for leakage) was added to the basolateral chamber. The apical medium was harvested, and the amounts of immunoglobulins transported by the variant pIgR types were measured by ELISA.

In Vitro Covalent Association of SC with Immunoglobulins—NIP-purified recombinant immunoglobulins (500 ng) were incubated with free human SC (10 ng) for 4 h at 37 °C, corresponding to a molar IgA/SC ratio of ~10:1. The reaction was terminated by the addition of SDS sample buffer; resolved on a nonreducing 4% acrylamide, 0.5% agarose gel; and blotted with a rabbit antiserum to SC (1:3000; DAKO). Purified human colostral S-IgA (34) and free human SC were run as molecular markers.

RESULTS

The COOH-terminal Immunoglobulin Domain Is Primarily Responsible for the Polymerization Pattern of IgA and IgM—Previous studies have shown that whereas the secretory tailpiece is required for polymerization of IgA and IgM, differences between the α tailpiece and the μ tailpiece cannot account for the different polymerization pattern of the two isotypes (30, 35–38). Furthermore, Cys-309 (in the α chain)/Cys-414 (in the μ chain) and the amino acids immediately flanking these cysteine residues have only a minor effect on the isotype-specific polymerization (37). To pursue the search for the structural motifs in the α and μ heavy chains needed for isotype-specific polymerization and for interaction with pIgR (or SC), one or more positionally equivalent constant domains were interchanged between IgA and IgM (Fig. 1). The nomenclature for the resulting chimeric heavy chains indicates the origin of each immunoglobulin constant domain (A for IgA and M for IgM, see Fig. 1). The Cμ2 hinge domain was swapped as a unit with Cμ3, because no such domain exists in IgA1. Instead, Cμ2 is replaced by a flexible hinge region that is encoded within the Cα2 exon (39). The chimeric heavy chain genes were transfected separately into J558L cells, which constitutively express a compatible mouse λ light chain and the mouse J chain. Supernatants from transfected clones were tested in ELISA for secretion of immunoglobulin products. The concentration of the different recombinant immunoglobulins in outgrown cultures

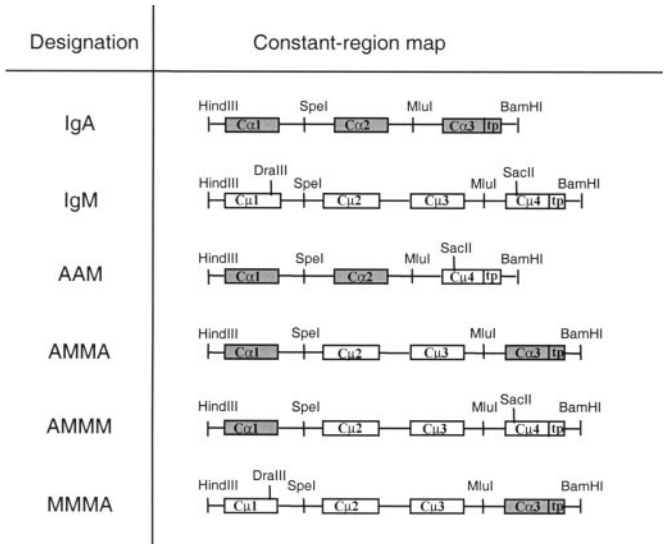

FIG. 1. **Constant region map of IgA, IgM, and recombinant chimeric heavy chain genes.** Recognition sites for the restriction enzymes *Spe*I and *Mlu*I were introduced between the first and second constant domain and in front of the last domain of both IgA and IgM, respectively. Constant domains were exchanged between heavy chain genes of IgA and IgM forming the chimeric AAM, AMMA, AMMM, and MMMA variants. The α- and μ-chain exons are shown as *gray* or *white boxes*, respectively. Secretory tailpieces are indicated by *tp*.

was measured to 2–13 μg/ml supernatant, except for in supernatant of AAM-producing cells, which contained less than 1 μg/ml. Clonal lines of immunoglobulin-producing cells were selected and expanded for further analysis. Metabolically labeled immunoglobulins were immunoprecipitated from supernatants and analyzed by nonreducing SDS-PAGE (Fig. 2*A*). The percentage of the different immunoglobulin polymers was determined by phosphor imager analyses, and the combined results from at least three independent clones of each recombinant immunoglobulin variant (except the AAM chimera) are shown in Table II. AAM polymers were not detected by this method due to low secretion. However, in a similar experiment, all recombinant immunoglobulin variants were affinity-purified before they were separated on a nonreducing SDS-PAGE and Western blotted with polyclonal antibodies specific for IgA and IgM (Fig. 2*C*). The results from this experiment confirmed those obtained by the metabolic labeling and, in addition, allowed for the detection of monomers as well as pentamers and hexamers of the AAM chimera (Fig. 2*C*). Wild-type IgM consisted mostly of pentamers and hexamers, whereas wild-type IgA was secreted as monomers and dimers. Like IgM, the AMMM chimera formed pentamers and hexamers. The AMMA chimera was secreted in different polymeric forms, ranging from monomers to hexamers, but monomers and dimers dominated. This mutant was therefore more similar to IgA than IgM. Polymerization of the MMMA chimera resembled that of the AMMA variant (Table II and Fig. 2, *A* and *C*). In summary, the polymerization pattern was IgM-like when the terminal domain was Cμ4 (the AAM and AMMM variants) and IgA-like when the terminal domain was Cα3 (the AMMA and MMMA variants). The MMMA chimera was omitted in the remaining analyses. Note that IgM as well as the AMMA and AMMM variants migrated more slowly in the gel than equivalent polymeric IgA and the AAM chimera, because the Cμ2 domain is much larger than the IgA1 hinge region (see also Fig. 2*B*, heavy chain blot).

J Chain Incorporation into Recombinant Immunoglobulins—J chain is an integral component of secretory immunoglobulins, but it is known that both IgA and IgM can polymerize with or without concomitant J chain incorporation (13, 16,

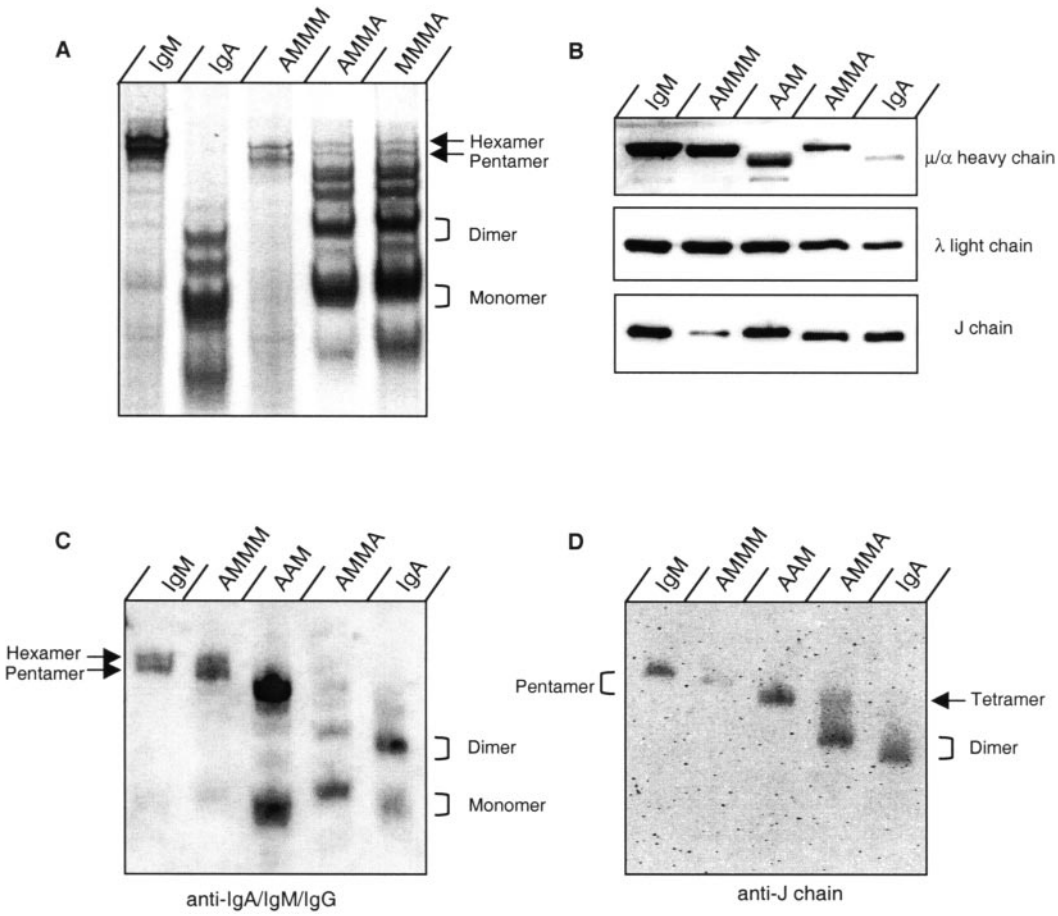

FIG. 2. **Analyses of molecular size and J chain content of IgA-IgM domain swap chimeras and wild-type recombinant immunoglobulin variants.** *A*, metabolically labeled immunoglobulins were immunoprecipitated from the culture medium and resolved by nonreducing SDS-PAGE as described under "Experimental Procedures." The positions of the different polymeric forms are indicated. The relative amounts of the different assembly products were measured by a phosphor imager and are presented in Table II. *B*, NIP-Sepharose-purified recombinant immunoglobulins were reduced by 100 mM dithiothreitol and resolved by 12% SDS-PAGE and blotted onto a nitrocellulose membrane. The membrane was sequentially probed with antisera to J chain, λ light chain, and immunoglobulin heavy chains, with stripping between each probing as described under "Experimental Procedures." *C*, purified immunoglobulins resolved by nonreducing SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and probed with antiserum to IgA/IgM/IgG or *D*, antiserum to J chain. The polymeric forms with incorporated J chain are indicated. Differential migration of some polymeric forms was due to the fact that IgM as well as the AMMA and AMMM variants have four heavy-chain constant domains, whereas IgA and the AAM chimera have three heavy-chain constant domains and a 17-amino acid hinge region.

TABLE II

Composition and size distribution (%) of recombinant immunoglobulin assembly products

Distribution of immunoglobulin assembly products was determined for at least three transfectants of each gene construct as described under "Experimental Procedures" (see also Fig. 2*A*). The quantity of each assembly product is expressed as a percentage of the total amount of secreted immunoglobulin (mean ± S.D.). Only assembly products that formed distinct bands in the gel were included.

Ig variant	Hexamers	Pentamers	Tetramers	Trimers	Dimers	H_3L_3[a]	Monomers	Hemimers
IgM	25.6 ± 11.3	56.4 ± 10.8	3.3 ± 1.2	0.8 ± 0.6	0.9 ± 0.9		6.7 ± 4.5	6.3 ± 9.0
IgA				0.9 ± 0.3	10.9 ± 2.9	9.6 ± 3.9	57.7 ± 8.6	21.0 ± 4.4
AMMM	27.5 ± 7.6	42.0 ± 7.4	4.8 ± 2.1	2.5 ± 1.8	2.7 ± 1.4		10.6 ± 5.1	10.0 ± 6.1
AMMA	1.0 ± 1.0	1.4 ± 1.3	9.5 ± 7.5	7.4 ± 5.6	25.3 ± 6.3		45.5 ± 14.1	9.9 ± 6.9
MMMA	1.3 ± 0.5	1.9 ± 0.6	8.2 ± 2.3	6.0 ± 1.5	18.7 ± 4.7		54.5 ± 7.3	9.4 ± 3.4

[a] Three heavy chains and three light chains.

20, 40–43). To determine the relative J chain content per immunoglobulin monomeric unit, the recombinant immunoglobulins were separated by standard SDS-PAGE, and the amount of J chain was related to the amount of λ light chain by semiquantitative Western blotting (Fig. 2*B*). By arbitrarily defining the ratio of J chain to λ chain in IgA as 1.0, the ratio of J chain to λ chain in IgM, the AAM chimera, and the AMMA chimera was 1.2 ± 0.2, 0.8 ± 0.1, and 0.6 ± 0.0, respectively. Notably, the AMMM chimera had a greatly reduced level of J chain incorporation, with a ratio of J chain to λ chain of only 0.1 ± 0.0.

To determine which polymers had incorporated the J chain, recombinant immunoglobulin variants were resolved by nonreducing SDS-PAGE and immunoblotted with an antiserum specific for J chain (Fig. 2*D*). We found J chain incorporated into the pentamers of IgM and the AMMM and AAM variants but not in the hexameric fraction of any of these three immunoglobulins. Conversely, the J chain was found in IgA dimers as well as in dimers and higher polymers of the AMMA chimera. J chain is normally incorporated into larger IgA polymer (3, 4), but this was not detected, probably because the amount was below the detection level in this Western blot. No J chain was found in the monomeric fraction of any of the recombinant immunoglobulins.

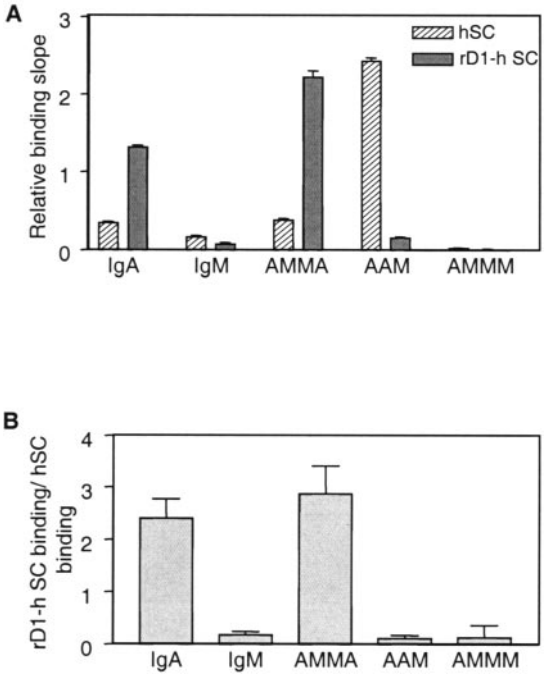

FIG. 3. **Analysis of the binding of different recombinant immunoglobulin products to variants of free SC.** *A*, the level of binding to human SC (*hSC*) and to a chimeric SC composed of rabbit D1 and human D2-to-D5 (*rD1-h SC*) was measured by ELISA as described under "Experimental Procedures." *B*, for each immunoglobulin preparation, the level of binding to rD1-h SC was normalized to the level of binding to hSC. The mean ± S.E.) of seven independent experiments is shown.

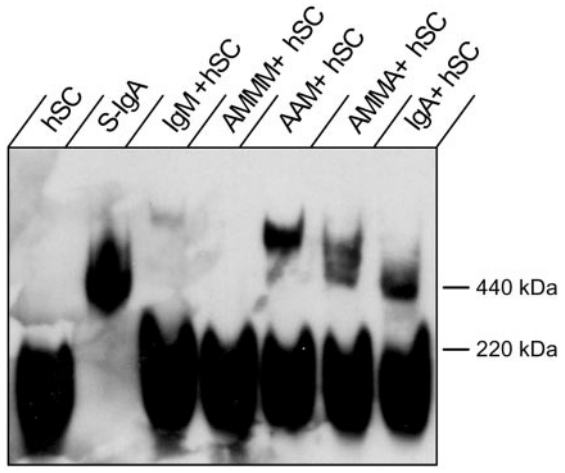

FIG. 4. **Covalent complexes formed in solution between recombinant human SC (*hSC*) and polymeric immunoglobulin variants.** Samples of recombinant immunoglobulins were incubated with human SC at an approximate molar ratio of 10:1 for 4 h at 37 °C. Products formed were resolved by nonreducing SDS-PAGE and detected by immunoblotting with rabbit anti-SC. Native colostral S-IgA and recombinant SC were run as control markers.

Complexing of Free SC with Recombinant Immunoglobulins—J chain-containing pIgA binds to both human and rabbit pIgR, whereas pentameric IgM binds only to human pIgR (28, 29). We have previously shown that this species disparity is caused by differences in D1 of the pIgR, and furthermore, we have made a chimeric pIgR composed of rabbit D1 and human D2-to-COOH terminus (called rD1-h pIgR) that behaves like rabbit pIgR in binding of IgA and IgM (28). An SC variant of this chimeric receptor (rD1-hSC) and recombinant human SC were tested for complexing with the various recombinant immunoglobulins in an ELISA. Importantly, the human region allowed for immunodetection of both human SC and rD1-h SC by the same antibody, such that the binding capacity of either SC type for the different immunoglobulin preparations could be readily compared.

Whereas IgA showed more than a 2-fold increase in binding to rD1-h SC compared with human SC, IgM showed greater than 2-fold decrease in binding to rD1-h SC compared with human SC (Fig. 3, *A* and *B*). These results were in agreement with previous observations of immunoglobulin binding to pIgR-transfected MDCK cells (28). The AMMA chimera demonstrated a similar pattern of binding to the two different SC molecules as IgA. Interestingly, the AAM chimera displayed remarkably high binding to human SC but only little binding to rD1-h SC. The AMMM chimera showed low binding to both human SC and rD1-h SC, consistent with the low J chain content of this polymer. Notably, the Cα1 domain instead of Cμ1 is the only difference between the AMMM chimera and IgM, but at present it is not known how this could influence J chain incorporation. However, like IgM and the AAM chimera, the binding of the AMMM variant to rD1-h SC was reduced compared with that to human SC.

Covalent Association between Human Free SC and Recombinant Immunoglobulins—Although free human SC and pIgR show a much higher initial noncovalent interaction with pentameric IgM than with pIgA (28, 44), covalent bonding occurs only with pIgA. This fact probably explains why we observed better binding of IgA than IgM to human SC (Fig. 3*A*). A disulfide bond is formed between Cys-467 in D5 of human pIgR and Cys-309 of the Cα2 domain (27), which is present both in IgA and the AAM chimera. In IgM, the corresponding cysteine may be involved in intermonomeric bonding (22–26). To investigate the ability of the different domain swap mutants to associate covalently with human SC, the recombinant immunoglobulins were allowed to react with recombinant human SC *in vitro*. Complexes were then separated by nonreducing SDS-PAGE and immunoblotted with rabbit anti-SC. As expected, IgA formed covalently stabilized S-IgA, revealed by a band of ~430 kDa, similar to purified colostral S-IgA (Fig. 4). Also, the AMMA chimera produced several new bands, corresponding to dimers, trimers, and tetramers with covalently attached SC. This finding suggested that Cys-414, present in the Cμ3 domain of this variant, was capable of forming a disulfide bridge with SC. Likewise, the AAM chimera formed a strong band corresponding to SC-containing pentamers. Finally, a weak band was detected for pentameric IgM combined with SC, whereas no SC-containing band was detected for the AMMM chimera. Recombinant human SC formed dimers of ~160 kDa detected as a broad band in the lower part of the gel.

Translocation of Recombinant Immunoglobulins by pIgR-transfected MDCK Cells—To test whether the recombinant immunoglobulins were functional ligands for pIgR-mediated epithelial transcytosis, we used MDCK cells stably transfected with human pIgR, rabbit pIgR, or the chimeric pIgR containing rabbit D1 and human D2 to COOH terminus (rD1-h pIgR). MDCK transfectants were grown to confluent polarized monolayers on permeable filter supports, and the various immunoglobulins were added to the basal chamber. As expected, IgA was efficiently transcytosed by all pIgR variants (Fig. 5). IgM was also transcytosed by human pIgR, but its transport by rabbit pIgR and rD1-h pIgR was just slightly above background (Fig. 5). All pIgR variants transcytosed the AMMA chimera as efficiently as IgA. The AAM chimera, like wild-type IgM, was only significantly transcytosed by human pIgR; the level of transport mediated by rabbit pIgR, and rD1-h pIgR was again reduced to nearly background levels (Fig. 5). The transcytosis observed for the AMMM chimera by all MDCK cell transfec-

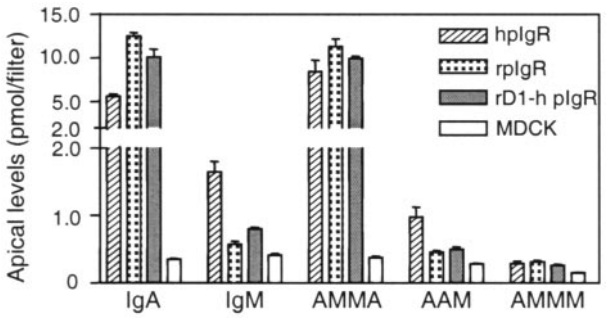

FIG. 5. **Receptor-mediated transport of recombinant immunoglobulins variants through MDCK cells expressing human pIgR (*hpIgR*), rabbit pIgR (*rpIgR*), or a chimeric pIgR containing rabbit D1 and human D2-to-COOH terminus (*rD1-h pIgR*).** Untransfected MDCK cells were included as negative control (MDCK). Samples of recombinant immunoglobulins (50 mM) together with IgG (50 mM), as a control for monolayer leakage, were added basolaterally. Aliquots of apical medium were harvested after 20 h and analyzed by ELISA for the presence of recombinant immunoglobulins and IgG. Less than 0.2 pmol/filter of IgG was found in the apical chamber (not shown). Results from one of three similar experiments are shown, expressed as mean ± S.D. of triplicate filters for each analysis.

tants was only slightly above the background, consistent with the lower level of SC-binding capacity displayed by this polymer. Notably, the transport mediated by rabbit pIgR was similar to that of rD1-h pIgR for all recombinant immunoglobulin variants, justifying the use of the latter receptor variant in the SC binding analysis.

DISCUSSION

IgA and IgM have unique motifs, not found in IgG, which allow them to form polymers and incorporate J chain. Such J chain-containing polymers are selectively transported by pIgR into exocrine fluids to form secretory antibodies (S-IgA and S-IgM). The extensive antigenic exposure of the mucosae underscores the need for a specific first-line defense. However, differences between IgA and IgM exist, both in the size of polymers formed and in their J chain incorporation and mode of interaction with the pIgR. To localize structural motifs that determine these differences, we made domain swap mutants between IgA and IgM heavy chains. The results clearly showed that the COOH-terminal domains (Cα3 and Cμ4) primarily direct the degree of polymer formation. Furthermore, we found that J chain incorporation was restricted to immunoglobulin pentamers containing Cμ4 but occurred in all polymeric forms of immunoglobulins containing Cα3. SC binding and pIgR-mediated transcytosis also demonstrated that the COOH-terminal domains contained the structural elements that determine differential interaction of J chain-containing pIgA and pentameric IgM with rabbit and human pIgR/free SC. However, the COOH-terminal domain was not the only determinant contributing to the functional properties of the chimeric immunoglobulins, because the pentameric AAM chimera showed increased binding to human pIgR/free SC compared with both IgM and IgA and a similar degree of covalent SC complexing as IgA.

Structural Requirements for Immunoglobulin Polymerization—Although the structures of the α- and μ-chains clearly differ, the exact motifs directing the differential polymerization patterns observed for IgA and IgM remain elusive. We (30, 37) and others (35, 36, 38) have previously investigated the secretory tailpiece sequences unique to polymeric immunoglobulins. Recombinant human IgM engineered to contain an α tailpiece (IgMαtp) was found to polymerize like IgM, although with increased hexamer formation (30), whereas the reciprocal mutation with μ tailpiece introduced into IgA led to the formation of some polymers larger than wild-type pIgA (37). Both μ-tailpiece and α-tailpiece sequences induced formation of polymers including pentamers and hexamers when added onto IgG, and such polymerization was most efficient when the secretory tailpiece was introduced in conjunction with a Cys-414/Cys-309 homologue in Cγ2 (30, 35, 36, 38). These results suggested that although the secretory tailpiece is sufficient to drive the polymerization process, it does not by itself direct the number of monomers incorporated into the polymers. The analyses of the domain swap mutants described here were carried out to identify the constant region domain(s) that harbors the elements required for isotype-specific polymerization and pIgR-binding properties of polymeric immunoglobulins.

Together with the light chain and the heavy chain variable domain, the first constant domain forms the so-called Fab portion of the immunoglobulin. The remainder of the heavy chain forms the so-called Fc portion believed to be responsible for most isotype-specific effector functions. To test whether the first constant domain could affect the polymerization pattern of the chimeric immunoglobulins, we exchanged the Cμ1 domain in IgM with the Cα1 domain, producing the AMMM chimera. We also made the same substitution in the MMMA chimera producing the AMMA variant. In both cases, the polymerization pattern of the resulting chimera was nearly identical to that of the parental immunoglobulin. Thus, our findings indicated that the first heavy chain domain does not influence the number of monomers linked during polymerization. This conclusion is supported by the observations that immunoglobulin light chains, which are normally bound to the Cμ1 domain, are not required for efficient polymerization of IgM (45) and that IgM lacking the Cμ1 domain is also secreted as polymers (46).

Other studies have suggested that the Cμ2 region is not essential for IgM pentamer assembly, but the Cμ3 domain may play an important role in the polymerization (3, 47). We have previously reported that mutation of five amino acids flanking Cys-309 in IgA into the corresponding amino acids present in IgM, forming the so-called IgAalm mutant, resulted in only a small increase of trimers and tetramers (37). The AMMA variant, which contained Cμ2 and Cμ3, formed some larger polymers as compared with IgA and the IgAalm mutant. Thus, other structural motifs in Cμ3 may be more important in pentamer formation than the Cys-309 region.

We identified the Cα3 and Cμ4 domains as most important for isotype-specific polymerization. Whereas an IgA-like polymerization pattern was observed for the AMMA and MMMA variants, both the AAM and AMMM variants formed mainly pentamers and hexamers. This observation accorded with that of Yoo *et al.* (38) on human IgA1-human IgG2 domain swap mutants, which suggested that the Cα3 domain is required for IgA-like polymerization. In the same study, murine IgG2b-human IgM chimeras provided evidence that both Cμ3 and Cμ4 are needed for IgM-like polymerization (38). The fact that our AAM chimera formed mostly pentamers and hexamers, similar to IgM, demonstrated the validity of using IgA-IgM chimeras to identify domains responsible for the differential polymerization pattern of IgA and IgM. Thus, Cα2 or Cμ3, but not Cγ2 (38), could support IgM-like polymerization. Taken together, our results pointed to the COOH-terminal domain as the main focus for further studies of isotype-specific polymerization motifs.

J Chain Incorporation and Interactions with Free SC or pIgR of Polymeric Immunoglobulins—Only J chain-containing polymeric immunoglobulins can bind to the pIgR (5, 11–13, 18, 20), and J chain-specific IgG antibodies or Fab fragments have been shown to inhibit binding of pIgA and pentameric IgM to free SC or the pIgR (10, 14). In the present study, we found that the

stoichiometry of J chain and λ light chain appeared to be similar for all recombinant immunoglobulin molecules except the AMMM chimera, which had a significantly reduced J chain content. Thus, J chain was abundantly present in pentamers from IgM as well as the AMMM and AAM variants but only at a low level in the AMMM variant. In IgA and the AMMA chimera, J chain was found in dimers and, especially with the AMMA chimera, also in larger polymers. Thus, isotype-specific J chain incorporation is determined by the COOH-terminal domain. Both Yoo *et al.* (38) and we (3) have previously found that chimeras of IgM and IgG need motifs from Cμ3 as well as Cμ4 for efficient J chain incorporation. Our present results suggested that Cα2 and Cμ3 are almost interchangeable in this respect. The role of the Cμ1 domain in J chain incorporation remains to be elucidated.

In a recent study, one extra Cα3 domain from IgA2 was added onto IgG1, and the resulting polymers resembled pIgA in that they incorporated J chain and bound to the human pIgR (48). It has been proposed that a predicted exposed loop of the Cα3 domain containing amino acids 402–410 (QEPSQGTTT), constitutes the pIgR binding site of pIgA (49). However, a naturally occurring mutant (protein 511) that lacks 36 amino acids in Cα3 (including amino acids 402–410) was found to complex with iodinated rat free SC and to be transported from blood into bile in a manner indistinguishable from pIgA (50). To identify polymeric immunoglobulin domains involved in the pIgR binding sites, we exploited the fact that the rabbit receptor shows virtually no binding to human pentameric IgM but efficient binding to human pIgA, whereas human pIgR binds both ligands very efficiently. The recombinant rabbit-human chimeric free SC employed in our study (rD1-h SC) behaved like the rabbit receptor and was compared with the human counterpart. We found that all immunoglobulin variants bound quite well to human SC, except for the AMMM chimera, which had incorporated very little J chain. Both IgA and the AMMA chimera showed stronger binding to rD1-h SC than to human SC, in agreement with the higher level of binding shown by human pIgA for rabbit pIgR (28). Our data suggested that elements in Cα3 are responsible for this efficient binding, which was supported by the fact that not only IgM but also the AAM chimera showed weaker binding to rabbit than to human SC. Our transcytosis experiments confirmed the functionality of the recombinant polymeric immunoglobulins by showing efficient transport of IgA and the AMMA chimera in MDCK cells transfected with either human pIgR, rabbit pIgR, or recombinant rD1-h pIgR. Also, IgM and the AAM chimera were transported by human pIgR but virtually not at all by rabbit pIgR or rD1-h pIgR. As expected, no receptor variant was able to transport the AMMM chimera, which had a low J chain content and low SC/pIgR binding capacity. Surprisingly, despite showing a very high level of binding to human free SC, the AAM chimera was not transported more efficiently than IgM by the human pIgR.

The Cys-414 residues of Cμ3 may form disulfide bonds between the monomers in IgM (23), whereas the homologous Cys-309 of Cα2 is not involved in similar bonding between IgA subunits but instead forms a disulfide bridge to human SC (9, 27). Interestingly, the AMMA chimera, which contained Cys-414 in Cμ3, also formed covalent complexes with human free SC. Thus, the Cys-414 residues were presumably not involved in intermolecular bonds in the AMMA dimer but were available for SC binding. In the AAM chimera, the Cα2 domain Cys-309 could apparently not make intermonomer bonds, because, similar to IgA, pentameric AAM formed covalent complexes with free SC (Fig. 4). The failure of such intramolecular disulfide stabilization may explain why the AAM variant was secreted in only very small amounts. Our result clearly showed that pentamer formation in itself does not restrict covalent SC binding. For IgM, we noted only a weak covalent association of SC with the pentamers, probably because most of the Cys-414 residues were engaged in intermonomer bonds in IgM and therefore unavailable for bridging to Cys-467 of human SC (23, 25). It remains to be determined whether the covalent linking of SC to the AAM chimera made this pentamer more resistant to proteases, as has been shown for S-IgA (51). It is also possible that the presence of Cα2 enhanced the noncovalent interactions between the AAM chimera and human SC because of progressive interactions between D2 and or D3 of human SC and Cα2 (52).

We have shown in this study, that the Cμ4 domain was sufficient for pentamer formation. Similarly, the Cα3 domain was sufficient for directing dimer formation. Cα3 also contained the unique motif for dimeric IgA to bind rabbit pIgR. Furthermore recombinant immunoglobulins containing Cμ4 failed to bind rabbit pIgR. We also produced a pentameric IgA-IgM chimeric variant that associated covalently with SC. Although such bonding did not increase ligand transport by human pIgR-transfected MDCK cells, it is known to stabilize S-IgA in secretions.

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The Carboxyl-terminal Domains of IgA and IgM Direct Isotype-specific Polymerization and Interaction with the Polymeric Immunoglobulin Receptor

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