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### The J Chain Is Essential for Polymeric Ig Receptor-Mediated Epithelial Transport of IgA<sup>1</sup> ✓

Finn-Eirik Johansen; ... et. al

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# The J Chain Is Essential for Polymeric Ig Receptor-Mediated Epithelial Transport of IgA<sup>1</sup>

Finn-Eirik Johansen,<sup>2</sup> Ranveig Braathen, and Per Brandtzaeg

Local production of secretory (S)IgA provides adaptive immunologic protection of mucosal surfaces, but SIgA is also protective when administered passively, such as in breast milk. Therefore, SIgA is a potential candidate for therapeutic administration, but its complex structure with four different polypeptide chains produced by two distinct cell types complicates recombinant production. The J chain is critical in the structure of SIgA because it is required for efficient polymerization of IgA and for the affinity of such polymers to the secretory component (SC)/polymeric (p)IgR. To better understand the role of the J chain in SIgA production, we have generated various mutant forms of the human J chain and analyzed the function of these mutants when coexpressed with IgA. We found that the C terminus of the J chain was not required for the formation of IgA polymers, but was essential for the binding of pIgA to SC. Likewise, we found that two of the intrachain disulfide bridges (Cys<sup>13</sup>:Cys<sup>101</sup> and Cys<sup>109</sup>:Cys<sup>134</sup>) were also required for the binding of pIgA to SC but, interestingly, not for IgA polymerization. Conversely, the last intrachain disulfide bridge (Cys<sup>72</sup>:Cys<sup>92</sup>) was not essential for either of these two J chain functions. Finally, we demonstrated that the presence of only Cys<sup>15</sup> or Cys<sup>69</sup> was sufficient to support polymerization of IgA, but that these polymers were mostly non-covalently stabilized. Nevertheless, these polymers bound free SC with nearly the same affinity as pIgA containing wild-type J chain, but were transcytosed by pIgR-expressing polarized epithelial cells at a reduced efficiency. *The Journal of Immunology*, 2001, 167: 5185–5192.

The mucosal surfaces of the human respiratory and gastrointestinal tracts constitute a large surface area that is vulnerable to pathogens and noxious substances. Adaptive immunity for protection of the mucosae is largely provided by secretory (S)<sup>3</sup>IgA, which cooperates with innate defense mechanisms (1). SIgA differs from IgG in two main ways: it is polymeric in nature and it contains covalently bound secretory component (SC), the cleaved extracellular portion of transmembrane SC which constitutes the polymeric (p)IgR. This receptor mediates the epithelial transport of dimeric and larger polymers of IgA (collectively called pIgA) as well as pentameric IgM to the lumen (2). Its polymeric nature makes SIgA Abs particularly effective at agglutinating viruses and bacteria (3), and the covalent attachment to SC stabilizes SIgA in the secretions by making it more resistant to proteases (4, 5).

SIgA is generated via the active transport of pIgA to the intestinal lumen by SC/pIgR-expressing epithelial cells; this common transport model for pIgA and pentameric IgM was first proposed by Brandtzaeg (6, 7) in 1974. In adult humans, more SIgA is generated per day than the daily production of IgG (8). Furthermore, SIgA production by lactating mammary glands ensures that SIgA is the dominant Ab in human breast milk (9). Such SIgA is nature's

way of passive immunization of infants against Ags in the mother's environment, and numerous studies have documented a protective effect of exclusive breastfeeding against diarrhea (9). The proven efficacy of SIgA in preventing and fighting mucosal infections makes it an attractive candidate for therapeutic purposes (10, 11), and recombinant production in various expression systems has been described (12–15). However, its complex nature with four different polypeptides produced by two distinct cell types imposes restrictions on potential expression systems for efficient production of recombinant SIgA.

Mucosal IgA-producing plasma cells typically also express the J chain (16). This small polypeptide of 15 kDa promotes formation of pIgA and pentameric IgM and is exclusively incorporated into these two types of Ig polymers (17–19). The human J chain gene is encoded by four exons (20). Exon 1 encodes the leader peptide, while exons 2–4 encode the mature protein of 137 amino acids (14, 20). The human J chain contains eight cysteine residues; two (Cys<sup>15</sup> and Cys<sup>69</sup>) are involved in disulfide bridges with the  $\alpha$ - or  $\mu$ -chains, and six are involved in intrachain disulfide bridges (Cys<sup>13</sup>:Cys<sup>101</sup>, Cys<sup>72</sup>:Cys<sup>92</sup>, Cys<sup>109</sup>:Cys<sup>134</sup>) (21, 22). The J chain is well conserved throughout phylogeny, and a homologous peptide has been reported even in the earthworm (19, 23).

The three-dimensional structure of neither the J chain nor pIgA has been reported, but experiments to determine the positioning of J chain in pIgA have suggested that it bridges two  $\alpha$ -chains of opposing IgA monomers which are dimerized tail-to-tail (21, 24, 25). It is often assumed that pIgA and pentameric IgM contain only one J chain molecule (26), but immunochemical studies have suggested that dimeric IgA contains two and pentameric IgM three to four J chains (27, 28). Characterization of J chain released from dimeric, trimeric, and tetrameric IgA purified from the same myeloma serum did indeed suggest that the molar J chain ratio increases with the size of the polymer (29).

The J chain is a key protein in the generation of SIgA because it promotes polymerization of IgA and because its presence in these polymers is believed to be required for their affinity to SC/

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<sup>3</sup> Abbreviations used in this paper: S, secretory; p, polymeric; SC, secretory component; MDCK, Madin-Darby canine kidney; CHO, Chinese hamster ovary; FPLC, fast protein liquid chromatography; NIP, 5-iodo-4-hydroxy-2-nitrophenylacetyl.

pIgR (reviewed in Ref. 19). To better understand these two functions of the J chain, we have used a mutational approach to generate deletion mutants and mutants that abolish J chain intrachain disulfide bonds or disulfide bonds between the J chain and the  $\alpha$ -chain of the IgA. In this report, we demonstrate that the C terminus of the J chain is dispensable for pIgA formation but nevertheless is required for the affinity of such polymers to SC. We also show that the same holds true for two of the three intrachain disulfide bridges. Finally, we document that either one of the two disulfide bonds normally present between the J chain and the  $\alpha$ -chain is sufficient for polymer formation. Although these polymers are mostly noncovalently stabilized, their affinity to SC is nearly the same as that of the recombinant wild-type pIgA, but they appear to be inferior ligands in pIgR-mediated transport compared with covalently stabilized wild-type J chain-containing pIgA.

## Materials and Methods

### Plasmid construction

The plasmid for episomal expression of wild-type J chain, pCEP-wtJ chain, was based on pMEP4 (Invitrogen, De Schelp, The Netherlands) and pCH-J chain (14). A *Bgl*III-*Bam*HI fragment from pCH-J chain that contained the CMV promoter and the human J chain open reading frame was subcloned into *Bgl*III- and *Bam*HI-digested pMEP4. C-terminal deletion mutants were constructed by PCR with J chain-forward (14) and different downstream primers that introduced a stop codon followed by a *Bam*HI recognition site into the J chain sequence with pCH-J chain as template. The resulting PCR products were digested with *Hind*III and *Bam*HI and subcloned into *Hind*III- and *Bam*HI-digested pCEP-wtJ chain. Cysteine-to-serine mutations (except C134S) were made by PCR splice overlap extension with J chain-forward and J chain-reverse (14) as outer primers and different inner primers. PCR products were subcloned as above. C134S was made by PCR with J chain-forward and a reverse primer that mutated the last cysteine codon to serine and introduced a *Bam*HI recognition site immediately after the stop codon, and subcloned as above. For stable expression of C15S and C69S, the inserts were subcloned into pCH. Vectors without inserts were used as negative control (mock) in all transfections. Details of all plasmids will be provided upon request. The complete open reading frame of all expression plasmids was verified by sequencing (Medigenomics, Martinried, Germany).

### Cell culture and transfections

The 5-iodo-4-hydroxy-2-nitrophenylacetyl (NIP)-specific IgA-producing Chinese hamster ovary (CHO) cell line  $\alpha$ 4.2 that has been described previously (14) was cultured in Ham's-F12 supplemented with 10% FCS, 2 mM glutamine, and 50  $\mu$ g/ml gentamicin. For semistable transfections (episomal expression), cells were seeded at 10–15% confluence in six-well trays on day 1. On day 2, cells were transfected with 1.4  $\mu$ g of DNA and 3  $\mu$ l of FUGENE (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. On day 3 the cells received fresh medium, and on day 4 they were trypsinized and seeded into 10-cm plates in medium supplemented with 300  $\mu$ g/ml Hygromycin B (Roche Diagnostics). Cells received fresh medium with Hygromycin B every 3–4 days, and after 10–12 days each 10-cm plate contained >500 Hygromycin B-resistant colonies. The colonies were pooled and seeded into six-well trays for metabolic labeling and IgA production. Stable transfectants were made essentially the same way, except that on day 4 they were seeded at different dilutions into 10-cm plates. On day 12, single colonies were isolated by use of cloning cylinders and expanded for analysis of J chain expression.

### Transcytosis assays

Transcytosis by human pIgR-expressing Madin-Darby canine kidney (MDCK) cells was performed with 8  $\mu$ g/ml IgA and an equal amount of IgG in the basal chamber overnight as described previously (30).

### Metabolic labeling and SDS-PAGE

Approximately 80%-confluent six-well trays of semistable transfectants were starved in DMEM without cysteine, methionine, and FCS for 30 min. Radiolabeled Cys/Met was added and the cells were incubated for 6 h. Supernatants were immunoprecipitated with rabbit antiserum to human IgA (diluted 1/100; DAKO, Glostrup, Denmark) and magnetic beads coated with goat anti-rabbit Ig (DynaL Biotech, Oslo, Norway). The precipitate was washed in PBS with 1% Nonidet P-40 (Sigma-Aldrich, St.

Louis, MO) and eluted in 1 $\times$  sample buffer (1% SDS, 30% glycerol, 0.02 M phosphate buffer, bromophenol blue). Samples were heated to 95°C for 3 min and resolved by SDS-PAGE on a 4% acrylamide agarose gel as previously described (31). The gels were dried under vacuum, and radio-labeled IgA was visualized by exposure to x-ray film.

### ELISA

ELISA specific for human IgG was performed by coating microtiter plates with goat anti-human IgG ( $\gamma$ -chain-specific) (diluted 1/1,000; Sigma-Aldrich). After sample incubation, IgG was detected with alkaline phosphatase-conjugated goat anti-IgG. For all Ag-specific ELISAs, microtiter plates were coated with 3  $\mu$ g/ml NIP-BSA and blocked with 1% (w/v) BSA in PBS for the J chain ELISA and the SC-affinity ELISA. For IgA quantification, the primary Ab was mAb against human IgA (diluted 1/30,000; a gift from T. Lea, Institute of Immunology, Rikshospitalet, Oslo, Norway) and the secondary Ab was alkaline phosphatase-conjugated rabbit anti-mouse Ig Ab (DAKO; diluted 1/1,000). For J chain-specific ELISA, a dilution series of CHO cell supernatants or purified IgA was incubated overnight. The microtiter plates were washed and fixed with 2% glutaraldehyde for 30 min then incubated with 6 M urea (pH 3.0) for an additional 30 min. After washing, the plates were incubated with rabbit antiserum against human J chain (diluted 1/300) (32) and alkaline phosphatase-conjugated goat anti-rabbit Ig (DAKO; 1/2,000). For SC affinity measurement, a dilution series of IgA was incubated overnight. The microtiter plates were washed and incubated with 8  $\mu$ g/ml free SC (from colostrum), then rabbit antiserum against human SC (DAKO; diluted 1/1,000), and finally goat anti-rabbit Ig as above. For the J chain and the SC-affinity ELISA, the values were expressed as the linear regression of OD<sub>405</sub> vs IgA concentration. Pilot experiments demonstrated that this method of calculation yielded the same values for a given IgA sample over at least a four-fold different initial concentration; thus minor errors in determining the IgA concentrations of the different preparations were not carried over to the J chain and SC affinity measurements. However, the relative values of J chain content and SC affinity measured this way could be compared only within a single experiment.

### Purification and fractionation of NIP-specific IgA

NIP-specific IgA was purified from CHO cell supernatants on NIP-Sepharose as described previously (14). For fractionation by fast protein liquid chromatography (FPLC), the eluted IgA was dialyzed against 20 mM Tris-HCl (pH 7.1) and loaded onto a Mono Q column connected to an ÄKTA<sub>FPLC</sub> system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.). The IgA was eluted by a linear salt gradient from 2.5 to 500 mM NaCl in 20 mM Tris-HCl (pH 7.1) and collected in 300- $\mu$ l fractions which were analyzed for IgA concentration by ELISA. Peak fractions were analyzed for molecular size by nonreducing SDS-PAGE followed by immunoblotting, and for SC affinity by ELISA. Several fractions (as indicated in Fig. 4) were pooled before analysis by native PAGE and immunoblotting as well as for transcytosis assays.

### Immunoblotting

IgA (30 ng) was resolved by nonreducing SDS-PAGE on a 5% (w/v) acrylamide gel with the Criterion system (Bio-Rad, Hercules, CA) and transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membrane was blocked by first air drying and then incubation in 10% skimmed milk in PBS plus 0.05% Tween 20 (PBST). Subsequent incubations were all performed in PBST. The blocked membrane was first incubated with a rabbit antiserum to human IgA (diluted 1/3000; DAKO) for 90 min, washed three times for 5 min, and then incubated with HRP-conjugated donkey anti-rabbit Ig (diluted 1/3000; Amersham Pharmacia Biotech) for 90 min. After washing three times for 5 min, the membrane was incubated with SuperSignal (Pierce, Rockford, IL) for 10 min, and the substrate was detected with a light-sensitive camera (Chemidoc; Bio-Rad). For IgA immunoblotting, the membrane was stripped in 0.1% SDS and 100 mM  $\beta$ -mercaptoethanol for 30 min at 37°C, washed in PBST, and incubated with rabbit antiserum to human J chain (previously adsorbed against monomeric IgA) diluted 1/600. Secondary Ab and revealing reaction were as above. For native immunoblots, 60 ng of IgA was resolved on a 5% (w/v) acrylamide gel (acrylamide:bis ratio of 37.5:1), pH 8.5, without stacking gel. SDS was omitted from all buffers and the pH of the loading buffer was 8.5. The gel was transferred to polyvinylidene difluoride membrane and processed as above.

## Results

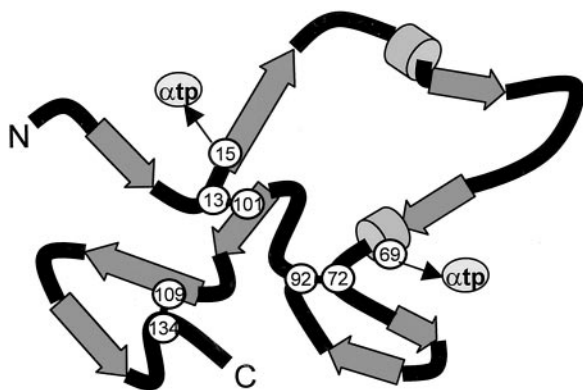
### Structural analysis of the human J chain

There are no three-dimensional structure data available for the J chain of any species. Therefore, we used computer-assisted secondary structure predictions as a first approach to delineate the structure and hence better understand the function of the J chain. A schematic depiction of human J chain that combines the known pattern of disulfide bonding with the predicted secondary structure is shown in Fig. 1. The Jpred program (<http://jura.ebi.ac.uk:8888/submit.html>) predicted that the J chain mostly consists of  $\beta$ -strands and extended coils with two short  $\alpha$ -helices (Fig. 1). Intrachain disulfide bonds between Cys<sup>13</sup> and Cys<sup>101</sup>, Cys<sup>72</sup> and Cys<sup>92</sup>, and Cys<sup>109</sup> and Cys<sup>134</sup> further restrict the possible conformations of the J chain.

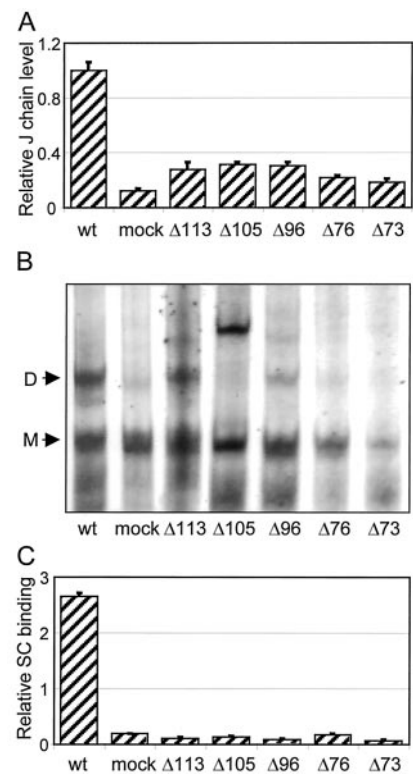
### Role of the J chain C terminus in IgA polymerization and SC binding

The J chain performs two essential functions for SIgA formation: it enhances dimerization (and some formation of larger polymers) of IgA; and it is required for the affinity of pIgA to SC/pIgR. To delineate which regions of the J chain are required for these functions, we first made sequential C-terminal deletions in the J chain sequence. We found that deleting the J chain to amino acid position 113, 105, or 96 appeared to reduce the level of J chain incorporation into IgA (Fig. 2A), but this could not be definitely concluded because the level of incorporation was measured with a polyclonal antiserum to the J chain. Deletion of this C-terminal region might therefore also remove epitopes detected by the antiserum. Deletion from amino acid 76 or more appeared to preclude incorporation of J chain into the coexpressed IgA (Fig. 2A). The properties of IgA produced by CHO cells cotransfected with these J chain mutants were therefore the same as IgA produced by CHO cells transfected with empty vector (Fig. 2, A–C; mock,  $\Delta 76$  and  $\Delta 73$ ).

Deleting the last 25 amino acids (including Cys<sup>134</sup>) did not significantly affect the level of IgA polymerization promoted by the J chain (Fig. 2B;  $\Delta 113$ ). Further deletion of eight or 17 more amino acids still allowed polymer formation, although at slightly reduced efficiency and occasionally giving rise to different polymer sizes (Fig. 2B;  $\Delta 105$  and  $\Delta 96$ , respectively). In agreement with our inability to detect  $\Delta 76$ - and  $\Delta 73$ -mutated J chain associated with



**FIGURE 1.** Schematic depiction of the human J chain structure showing proposed  $\beta$ -strands (broad arrows) and  $\alpha$ -helices (cylinders). The eight J chain cysteines (Cys<sup>13</sup>, Cys<sup>15</sup>, Cys<sup>69</sup>, Cys<sup>72</sup>, Cys<sup>92</sup>, Cys<sup>101</sup>, Cys<sup>109</sup>, and Cys<sup>134</sup>) are indicated. Secondary structure prediction was performed with Jpred (<http://jura.ebi.ac.uk:8888/submit.html>). The three intrachain disulfide bridges (Cys<sup>13</sup>:Cys<sup>101</sup>, Cys<sup>72</sup>:Cys<sup>92</sup>, and Cys<sup>109</sup>:Cys<sup>134</sup>) are indicated. Cys<sup>15</sup> and Cys<sup>69</sup> form disulfide bonds with one  $\alpha$ -H chain tailpiece (atp) of each monomer in the IgA dimer.

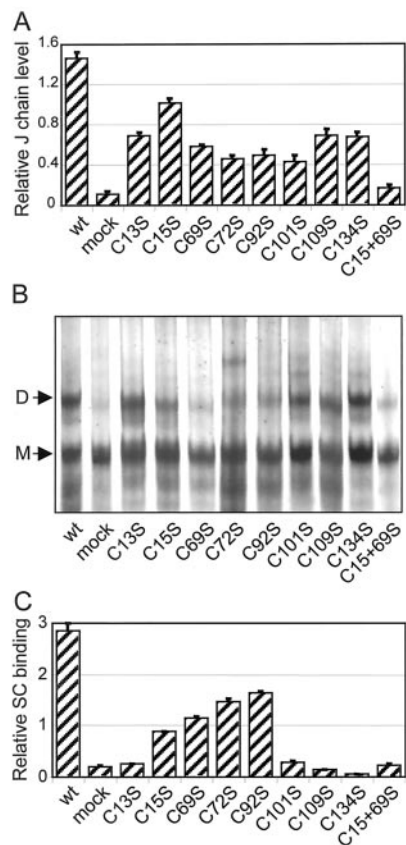


**FIGURE 2.** Analysis of J chain with C-terminal deletions. Mutants were made that deleted the C-terminal sequence including the indicated position ( $\Delta 113$ ,  $\Delta 105$ ,  $\Delta 96$ ,  $\Delta 76$ , or  $\Delta 73$ ), and expression constructs were transfected into IgA-producing CHO cells. A, ELISA was used to measure the extent of incorporation of the wild-type J chain and the C-terminal deletion mutants into coexpressed IgA molecules as described under *Materials and Methods*. B, Molecular size determination of IgA coexpressed with the wild-type or mutants of J chain. Semistable transfectants were metabolically labeled and IgA was immunoprecipitated and resolved by nonreducing 4% SDS-PAGE as described under *Materials and Methods*. The positions of dimers (D) and monomers (M) are indicated. C, The level of binding to free secretory component (SC) of IgA with the wild-type or mutants of J chain was determined by ELISA as described under *Materials and Methods*.

IgA, these mutants did not contribute to enhanced polymerization of IgA. Although the smallest C-terminal deletion in the J chain (25 aa) did not significantly affect polymerization of coexpressed IgA (Fig. 2A;  $\Delta 113$ ), it was clear that this deletion was sufficient to abolish all affinity of the produced pIgA to SC (Fig. 2C;  $\Delta 113$ ). Further deletions of the J chain also resulted in pIgA without affinity to SC (Fig. 2C;  $\Delta 105$  and  $\Delta 96$ ).

### Role of J chain disulfide bridges for IgA polymerization and SC binding

The human J chain forms three intrachain disulfide bridges as well as two bridges to the tailpieces of two opposing IgA  $\alpha$ -chains. To assess the importance of these bonds, we mutated each cysteine individually as well as Cys<sup>15</sup> and Cys<sup>69</sup> in combination (C15+69S). We found that mutating any one of the J chain cysteines reduced the level of J chain incorporation to between 30 and 70% compared with the level of wild-type J chain incorporated into coexpressed IgA (Fig. 3A). However, mutation of Cys<sup>15</sup> and Cys<sup>69</sup> in conjunction appeared to abolish incorporation of the mutated J chain into IgA (Fig. 3A; C15+69S). Mutation of Cys<sup>13</sup>, Cys<sup>101</sup>, Cys<sup>109</sup>, or Cys<sup>134</sup> did not appear to affect polymer formation (Fig. 3B). Surprisingly, all of these mutations abolished the affinity of



**FIGURE 3.** Analysis of J chain with Cys-to-Ser mutations. Each cysteine was mutated to serine alone (C13S, C15S, C69S, C72S, C92S, C101S, C109S, and C134S) or both Cys<sup>15</sup> and Cys<sup>69</sup> were mutated (C15+69S). Expression constructs were transfected into IgA-producing CHO cells and analyzed as described in the legend to Fig. 2. *A*, Analysis of the extent of incorporation of the various cysteine mutants of the J chain into coexpressed IgA molecules. *B*, Molecular size determination of IgA coexpressed with the different J chain mutants. *C*, The extent of binding to free SC of the IgA molecules coexpressed with the different J chain mutants.

the J chain-positive pIgA to SC (Fig. 3C). Mutation of Cys<sup>15</sup>, Cys<sup>72</sup>, and Cys<sup>92</sup> reduced the level of J chain-mediated pIgA formation, while mutation of Cys<sup>69</sup> and the double mutant (C15 + 69S) did not enhance pIgA formation above the level seen without coexpressed J chain (Fig. 3B). Mutation of Cys<sup>15</sup>, Cys<sup>69</sup>, Cys<sup>72</sup>, or Cys<sup>92</sup> still resulted in J chain-positive IgA with significant affinity for SC (Fig. 3C), while the C15+69S mutant did not affect the property of the coexpressed IgA.

The significant affinity of C15S and C69S J chain-positive IgA to free SC prompted us to test whether these Igs could be transcytosed by pIgR-expressing MDCK cells. We found that both mutants of J chain-positive IgA were transported from the basal to apical chamber of polarized pIgR-expressing MDCK cells. However, the level of transport was only 17 (C15S) and 9% (C69S) of the level of transport of pIgA with wild-type J chain (data not shown). Importantly, this low level was significantly above the level of transport or paracellular leakage of monomeric IgA expressed without J chain or of coincubated IgG, which both resulted in ~5% transport compared with pIgA containing wild-type J chain (data not shown).

#### Single CHO cell clones produce different molecular forms of IgA

Molecular size analysis revealed that each pool of IgA and J chain transfectants produced more than one molecular form of IgA (Figs.

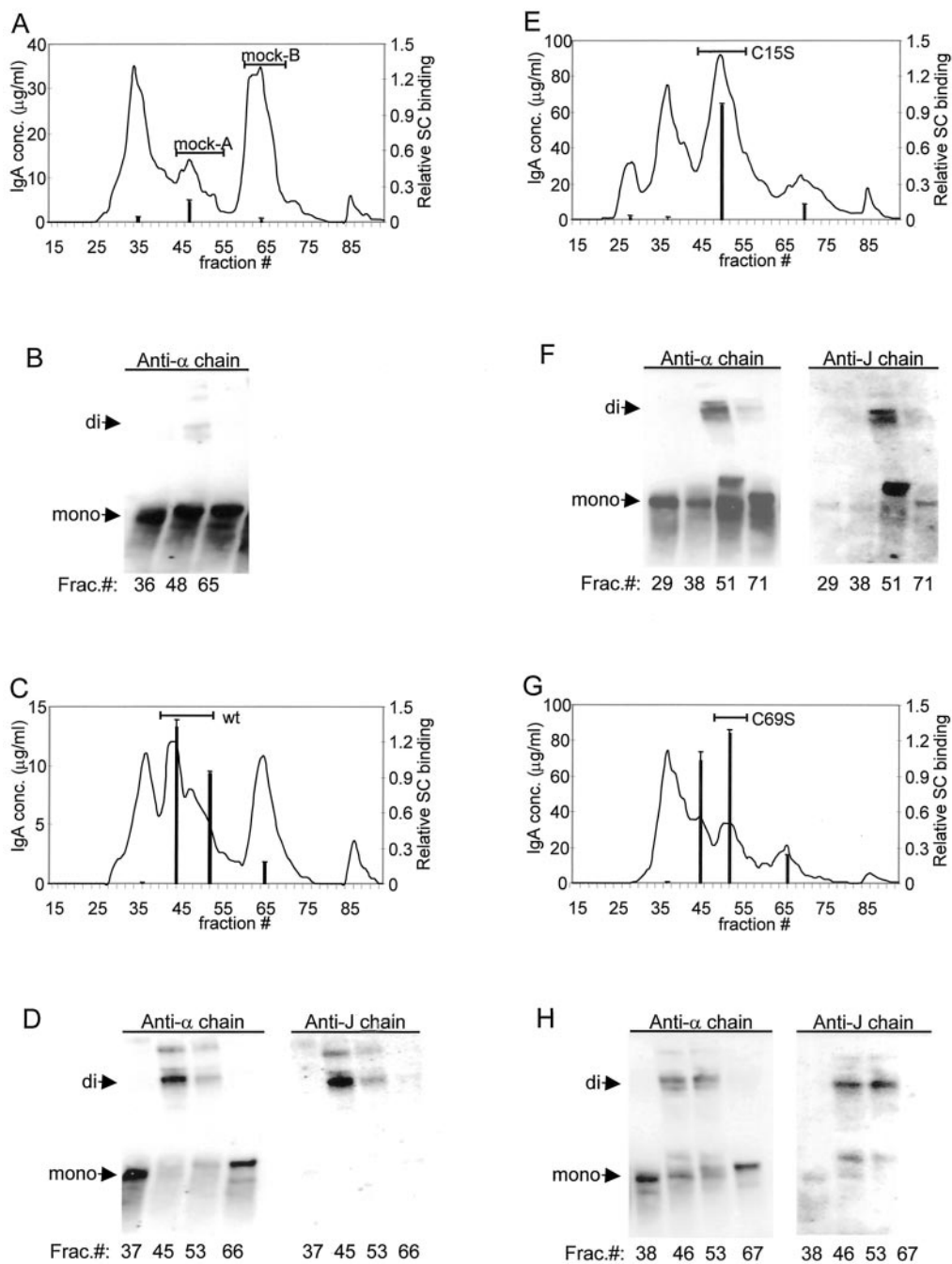
2B and 3B). Furthermore, Western blotting of C15S- and C69S-containing IgA demonstrated that the J chain could be found in both monomers and polymers of IgA (data not shown). To determine whether the discrepancy between the good affinity to SC and the poor ability to be transcytosed by pIgR-expressing polarized epithelial cells of C15S- and C69S-containing IgA might be due to a differential contribution of the various molecular forms in the two assays, we decided to purify the different molecular forms of IgA produced by each transfectant. First, we made stable transfectants of C15S, C69S, and wild-type J chain-expressing, IgA-producing CHO cells and purified IgA from supernatants of each cell line by affinity chromatography. To further fractionate each sample of recombinant IgA we chose Mono Q anion exchange chromatography because gel filtration would presumably be inefficient at separating monomeric IgA with J chain from monomers without. For IgA expressed without J chain, Mono Q fractionation revealed three distinct peaks centered around fractions 36, 48, and 65 (Fig. 4A). Surprisingly, fraction 48 showed low but significant affinity for SC (Fig. 4A), while the other two peaks had no affinity for SC. Nonreducing SDS-PAGE followed by Western blotting revealed that only fraction 48 contained covalently stabilized dimers (Fig. 4B).

IgA with wild-type J chain eluted with a larger peak centered at fraction 45 (with a shoulder extending to fraction 54) from the Mono Q column. Fraction 45 was nearly pure J chain-positive dimeric IgA, while fraction 53 was a mixture of dimeric J chain-positive IgA and monomeric IgA (Fig. 4, C and D). Not surprisingly, fraction 45 demonstrated the highest affinity to free SC (Fig. 4C). IgA with C15S-mutated J chain eluted with an additional peak centered at fraction 51, and IgA with C69S-mutated J chain eluted with two additional peaks centered at fractions 46 and 53 (Fig. 4, E and G). The additional peak from C15S-containing IgA and the two additional peaks from C69S-containing IgA all demonstrated significant affinity to SC, i.e., 72–94% of wild-type fraction 45 (Fig. 4, E and G). Nonreducing SDS-PAGE revealed that these fractions contained a mixture of J chain-positive dimeric IgA, IgA monomers, and J chain-positive monomeric IgA migrating slightly above the ordinary monomers (Fig. 4, F and H).

The Mono Q elution profile of each preparation of IgA prompted us to investigate whether there might be noncovalently stabilized pIgA produced by the C15S and C69S transfectants. Therefore, we pooled the fractions that made up the SC-binding peak (indicated in Fig. 4) and analyzed each pool by native gel electrophoresis (Fig. 5). Indeed, the pooled fractions from the C15S and C69S transfectants revealed that the distribution of IgA dimers and larger polymers in these pools were nearly identical with the distribution of polymers in the wild-type J chain pool.

#### Noncovalently stabilized J chain-positive pIgA shows high affinity for free SC, but is inefficiently transcytosed by pIgR-expressing MDCK cells

IgA from supernatants of CHO clones stably expressing C15S- or C69S-mutated J chain was relatively inefficiently transcytosed by human pIgR-expressing MDCK cells compared with pIgA from wild-type J chain-expressing clones (data not shown). Because these experiments used crude IgA preparations, this result could be due to a less efficient transcytosis of pIgA with mutated Cys<sup>15</sup> or Cys<sup>69</sup> in the J chain. Alternatively, these mutations could result in less efficient assembly of pIgA, but the pIgA formed could be an equally good substrate for epithelial transcytosis as pIgA with wild-type J chain. To test these possibilities, we pooled the fractions from the Mono Q purification of each IgA variant that contained pIgA and tested each pool in our transcytosis assay. We found that polymers containing C15S- or C69S-mutated J chain

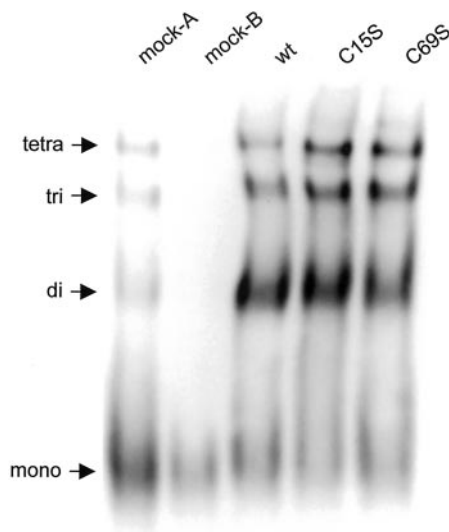


**FIGURE 4.** Purification and analysis of different molecular forms of IgA produced by CHO-cell clones cotransfected with empty vector (mock: *A* and *B*), wild-type J chain (wt: *C* and *D*), Cys<sup>15</sup>Ser (C15S: *E* and *F*), or Cys<sup>69</sup>Ser (C69S: *G* and *H*) J chain variants. *A*, *C*, *E*, and *G*, IgA concentrations of eluted fractions from the Mono Q columns (curve) and their relative binding to free SC of selected fractions (columns). Fractions pooled for native gel electrophoresis and transcytosis assays (Figs. 5 and 6) are indicated by horizontal bars. *B*, *D*, *F*, and *H*, The indicated fractions were resolved by 5% nonreducing SDS-PAGE and immunoblotted with an antiserum to human IgA (*left*). The filter was then stripped and probed with an antiserum to human J chain (*right*). No bands appeared on the blot in *B* when probed with antiserum to human J chain (not shown).

were transcytosed to a level of 19 and 50%, respectively, of wild-type IgA polymers (Fig. 6). The level of transcytosis of each pool correlated well with the level of SC binding of each peak fraction, although reduction in efficiency compared with wild-type IgA caused by the mutations was greater for transcytosis than for SC binding. Finally, we did not find that the fraction of IgA lacking J chain, which contained some pIgA (Fig. 6, mock-A), was transcytosed to a level above that of monomeric IgA (Fig. 6, mock-B) or coincubated IgG (Fig. 6, dotted line), although it demonstrated some binding to SC in the ELISA (Fig. 4A).

## Discussion

We have investigated the role of the J chain in the polymerization of human IgA and its contribution to the affinity of such polymers for free SC as well as their ability to be transcytosed by the pIgR. We found that although the J chain C terminus was dispensable for IgA polymerization, it was required to endow the pIgA with affinity to SC. Furthermore, two of three intrachain disulfide bridges (Cys<sup>13</sup>:Cys<sup>101</sup> and Cys<sup>109</sup>:Cys<sup>134</sup>) were shown to be dispensable for pIgA formation but essential for conferring affinity to SC. We

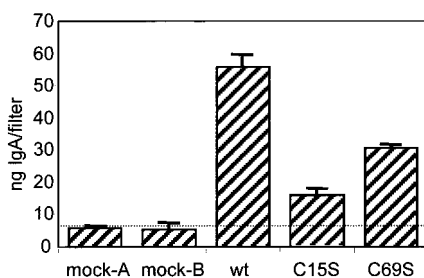


**FIGURE 5.** Molecular size analysis of purified IgA with wild-type or mutant J chain. IgA from the pools indicated in Fig. 4 was resolved by native (to avoid disruption of noncovalent polymers) 5% PAGE and immunoblotted with an antiserum to human IgA. The position of monomers, dimers, trimers, and tetramers are indicated with arrows.

also demonstrated that one disulfide bridge between the J chain and the  $\alpha$ -chain was sufficient to promote the formation of noncovalently stabilized IgA polymers. Such polymers retained high affinity to SC but were poor ligands for pIgR-mediated transcytosis. Finally, we demonstrated that IgA polymers devoid of J chain showed some affinity to free SC but were not actively transcytosed by pIgR-expressing MDCK cells to a level above the paracellular transport of monomeric IgA and IgG.

#### Role of J chain in IgA polymerization

The J chain is not absolutely required for polymerization of IgA, but its coexpression significantly enhances the production ratio of pIgA to monomers (14, 33). The three intrachain disulfide bridges of the human J chain divide the polypeptide into a large loop (Cys<sup>13</sup> to Cys<sup>101</sup>) with a smaller loop within this loop (Cys<sup>72</sup> to Cys<sup>92</sup>) (Fig. 1). Additionally, the C-terminal 29 amino acids form a small loop bridged by Cys<sup>109</sup> to Cys<sup>134</sup>. We found that the C-terminal loop was dispensable for polymerization of IgA. Furthermore, none of the intrachain bridges was absolutely required for pIgA formation. These data suggested that the three-dimensional structure of the J chain is not so important for IgA polymerization



**FIGURE 6.** Epithelial transcytosis of purified IgA with wild-type or mutant J chain. IgA (8  $\mu$ g/ml) from the pools indicated in Fig. 4 and IgG (8  $\mu$ g/ml) were incubated in the basolateral chamber of polarized human pIgR-expressing MDCK cells at 37°C for 16 h. The dotted line shows the average level of IgG transmission, which was nearly identical for all filters, while the columns show the level of IgA transport (+SD) for the different IgA preparations.

as long as there are cysteines in the appropriate places that can be linked to the cysteines of the  $\alpha$ -chain tailpieces. Alternatively, the J chain could fold into two distinct domains, where the N-terminal domain structure is intact in our mutants and sufficient for polymerization of IgA.

The C15S and the C69S mutants of the J chain are precluded from covalently linking two IgA monomers (25) but may still enhance pIgA formation by bringing two monomers together to promote direct covalent bonding between two opposing  $\alpha$ -chain tailpieces. In agreement with this possibility, we found a small increase in covalent pIgA production when IgA was coexpressed with either of these mutants. Surprisingly, we found that J chain-positive IgA containing either the C15S or the C69S mutant had only slightly reduced affinity to free SC compared with IgA containing the wild-type J chain. This result was probably due to noncovalently stabilized dimers present in these IgA preparations. In fact, the third cysteine (equivalent to human Cys<sup>69</sup>) is lacking in the J chain from *Xenopus laevis* (34), suggesting that disulfide bonding between the J chain at this position and IgA or IgM is not required for function in that species. The fact that either Cys<sup>15</sup> or Cys<sup>69</sup> could be mutated without affecting the formation of noncovalently stabilized polymers suggested that covalent bond formation in J chain-mediated polymerization does not occur in a specific sequence. Rather, a complex between two IgA monomers and the J chain is first formed, and then either Cys<sup>15</sup> or Cys<sup>69</sup> can form a disulfide bridge with the adjacent IgA monomer. Presence of this complex also increases the likelihood of disulfide bond formation between the two IgA monomers. However, in the absence of such covalent stabilization, a single bond between the J chain and one of the two IgA monomers appears to be sufficient to form stable polymers. Notably, these polymers disintegrate in SDS because one of the IgA monomers interacts only noncovalently with the covalent IgA monomer-J chain complex. We concluded that covalent bonding between one monomeric IgA molecule and the J chain is required for IgA polymerization, because the double mutant (C15+69S) did not increase polymerization of IgA at all, although its intracellular expression level was similar to that of the wild-type J chain (data not shown). Thus, while the J chain may function as a scaffold for IgA polymerization, it also has a more active role as its covalent interaction with IgA greatly enhances the efficiency of pIgA formation.

#### Role of J chain in SC binding and pIgR-mediated epithelial transcytosis

Knockout mice deficient in J chain or pIgR have demonstrated that both molecules are required for the generation of SIgA in vivo (33, 35–37). Furthermore, rabbit IgG Abs to the J chain and Fab of such Abs could block pIgA-SC interaction in vitro and inhibit pIgR-mediated transcytosis of IgA, suggesting a direct role of the J chain in the binding of pIgA to pIgR (38, 39).

We used a mutational approach to address the role of J chain structural elements in creating the pIgA docking site for SC/pIgR. Interestingly, the requirements of the J chain structure that allowed for binding of pIgA to SC was much more stringent than those determining IgA polymerization. The C-terminal structure of the J chain was essential to create a binding site for SC on pIgA because even the smallest C-terminal deletion we tested abolished SC interaction. Furthermore, disruption of the Cys<sup>109</sup> to Cys<sup>134</sup> disulfide bond (either C109S or C134S) abolished binding to SC, indicating that this C-terminal loop was required for SC binding, perhaps via direct interaction with the pIgR. Interestingly, the amino acid sequence between Cys<sup>101</sup> and Cys<sup>109</sup> is very highly conserved among different species, suggesting a particular functional importance of this region of the J chain. Alternatively, the C-terminal

loop could be critical for a structural feature of the J chain required for pIgA binding to pIgR. Ablation of the disulfide bond between Cys<sup>13</sup> and Cys<sup>101</sup> also had a dramatic effect on the affinity of pIgA to SC, although in some experiments we could detect significant, although marginal, binding activity even with these incorporated mutants of the J chain. The last intrachain bond, between Cys<sup>72</sup> and Cys<sup>92</sup>, appeared to be less required for binding to SC, perhaps because J chain without this bond is still able to maintain its overall three-dimensional structure. The apparent reduction in IgA dimers formed when this bond was prohibited (Fig. 3B) could be a result of Cys<sup>69</sup> (or Cys<sup>15</sup>) being less accessible for disulfide bond formation with the  $\alpha$ -chain. The relative high affinity of these IgA molecules to SC suggested that the supernatants from C72S- and C92S-transfected CHO cells contained noncovalently stabilized IgA dimers in addition to the covalent dimers seen by SDS-PAGE, although we did not attempt to verify this possibility.

When we fractionated CHO cell-produced IgA without J chain by anion exchange chromatography, we found that the IgA eluted in three distinct peaks, indicating three different species of NIP-specific IgA. The first peak appeared to be slightly smaller than monomers by SDS-PAGE, perhaps reflecting partially assembled IgA lacking a single L chain. The second peak contained some dimers and larger polymers (seen by native acrylamide gel electrophoresis) and, surprisingly, demonstrated marginal affinity to free SC. This result showed that the J chain is not absolutely required for IgA polymerization or for some binding to free SC. Binding of J chain-negative pIgA to SC has also been observed previously, indicating that the  $\alpha$ -chain contains an interaction site for SC (40). The third peak contained only IgA monomers that demonstrated no affinity for free SC. Thus, the  $\alpha$ -chain interaction site for SC apparently requires covalent attachment between two opposing tailpieces to create a novel surface constituting a functional binding site for SC/pIgR. Alternatively, this site is present on monomeric IgA, but polymerization results in higher affinity measurable in our SC binding assays. However, the latter explanation is unlikely, because we failed to detect any affinity between IgA monomers and SC by increasing the concentration of monomers (data not shown). In accord with our findings in this report, J chain-negative myeloma pIgA and human hexameric IgM (without J chain) failed to bind SC properly (7). Also, J chain-deficient myeloma pIgA and murine pIgA isolated from J chain knockout mice were not transcytosed by the pIgR (35, 41).

When we analyzed the chromatography-purified IgA from CHO cells by native gel electrophoresis, we found that mutating Cys<sup>15</sup> or Cys<sup>69</sup> in the J chain yielded a fraction of pIgA that was indistinguishable from the pIgA fraction produced with wild-type J chain (Fig. 5). Furthermore, the peak pIgA fraction from these two mutants showed ~72 (C15S) and 94% (C69S) efficiency of SC binding compared with wild-type pIgA. However, the level of pIgR-mediated transcytosis was reduced to only 19 and 50% of that of wild-type pIgA, respectively. These findings suggested that a binding site for SC/pIgR is not sufficient for efficient transcytosis by the pIgR-expressing epithelium, but that covalent stabilization of pIgA is also required. One possible explanation could be that noncovalently stabilized pIgA is less resistant to the reduced pH in the endosomal compartment of the MDCK cells. Thus, in vivo, there might be an additional level of quality control of pIgA that is to become luminal SIgA.

In conclusion, we have demonstrated that the integrity of the J chain structure is more important for receptor-mediated epithelial transcytosis of pIgA than for polymerization per se. Thus, the striking J chain expression by mucosal plasma cells seems to have production of SIgA and SIgM as its biological goal. Indeed, no other functional role has been identified for this polypeptide (42).

Furthermore, we showed that a single disulfide bond between the J chain and one IgA monomer was sufficient to enhance polymerization of IgA, although the resulting polymers were mostly noncovalently stabilized. Surprisingly, these polymers retained high binding affinity for free SC but were relatively poorly transcytosed.

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## References

- Norderhaug, I. N., F. E. Johansen, H. Schjerven, and P. Brandtzaeg. 1999. Regulation of the formation and external transport of secretory immunoglobulins. *Crit. Rev. Immunol.* 19:481.
- Mostov, K., and C. S. Kaetzel. 1999. Immunoglobulin transport and the polymeric immunoglobulin receptor. In *Mucosal Immunology*. P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R. McGhee, eds. Academic Press, San Diego, p. 133.
- Renegar, K. B., G. D. Jackson, and J. Mestecky. 1998. In vitro comparison of the biologic activities of monoclonal monomeric IgA, polymeric IgA, and secretory IgA. *J. Immunol.* 160:1219.
- Lindh, E. 1975. Increased resistance of IgA dimers to proteolytic degradation after binding of secretory component. *J. Immunol.* 114:284.
- Crottet, P., and B. Corthesy. 1998. Secretory component delays the conversion of secretory IgA into antigen-binding competent F(ab')<sub>2</sub>: a possible implication for mucosal defense. *J. Immunol.* 161:5445.
- Brandtzaeg, P. 1974. Mucosal and glandular distribution of Ig components: differential localization of free and bound secretory component in secretory epithelial cells. *J. Immunol.* 112:1553.
- Brandtzaeg, P., and H. Prydz. 1984. Direct evidence for an integrated function of J chain and secretory component in epithelial transport of immunoglobulins. *Nature* 311:71.
- Conley, M. E., and D. L. Delacroix. 1987. Intravascular and mucosal immunoglobulin A: two separate but related systems of immune defense? *Ann. Intern. Med.* 106:892.
- Hanson, L. A. 1998. Breastfeeding provides passive and likely long-lasting active immunity. *Ann. Allergy Asthma Immunol.* 81:523.
- Renegar, K. B., and P. A. Small. 1991. Passive transfer of local immunity to influenza virus infection by IgA antibody. *J. Immunol.* 146:1972.
- Dickinson, E. C., J. C. Gorga, M. Garrett, R. Tuncer, P. Boyle, S. C. Watkins, S. M. Alber, M. Parizhskaya, M. Trucco, M. I. Rowe, and H. R. Ford. 1998. Immunoglobulin A supplementation abrogates bacterial translocation and preserves the architecture of the intestinal epithelium. *Surgery* 124:284.
- Ma, J. K., A. Hiatt, M. Hein, N. D. Vine, F. Wang, P. Stabila, C. van Dolleweerd, K. Mostov, and T. Lehner. 1995. Generation and assembly of secretory antibodies in plants. *Science* 268:716.
- Chintalacharuvu, K. R., and S. L. Morrison. 1997. Production of secretory immunoglobulin A by a single mammalian cell. *Proc. Natl. Acad. Sci. USA* 94:6364.
- Johansen, F. E., I. Natvig Norderhaug, M. Roe, I. Sandlie, and P. Brandtzaeg. 1999. Recombinant expression of polymeric IgA: incorporation of J chain and secretory component of human origin. *Eur. J. Immunol.* 29:1701.
- Berdoz, J., C. T. Blanc, M. Reinhardt, J. P. Kraehenbuhl, and B. Corthesy. 1999. In vitro comparison of the antigen-binding and stability properties of the various molecular forms of IgA antibodies assembled and produced in CHO cells. *Proc. Natl. Acad. Sci. USA* 96:3029.
- Brandtzaeg, P. 1974. Presence of J chain in human immunocytes containing various immunoglobulin classes. *Nature* 252:418.
- Halpern, M. S., and M. E. Koshland. 1970. Noval subunit in secretory IgA. *Nature* 228:1276.
- Mestecky, J., J. Zikan, and W. T. Butler. 1971. Immunoglobulin M and secretory immunoglobulin A: presence of a common polypeptide chain different from light chains. *Science* 171:1163.
- Johansen, F. E., R. Braathen, and P. Brandtzaeg. 2000. Role of J chain in secretory immunoglobulin formation. *Scand. J. Immunol.* 52:240.
- Max, E. E., and S. J. Korsmeyer. 1985. Human J chain gene: structure and expression in B lymphoid cells. *J. Exp. Med.* 161:832.
- Bastian, A., H. Kratzin, G. E. Fallgren, K. Eckart, and N. Hilschmann. 1995. Intra- and inter-chain disulfide bridges of J chain in human S-IgA. *Adv. Exp. Med. Biol.* 371A:581.
- Frutiger, S., G. J. Hughes, N. Paquet, R. Luthy, and J. C. Jaton. 1992. Disulfide bond assignment in human J chain and its covalent pairing with immunoglobulin M. *Biochemistry* 31:12643.
- Takahashi, T., T. Iwase, N. Takenouchi, M. Saito, K. Kobayashi, Z. Moldoveanu, J. Mestecky, and I. Moro. 1996. The joining (J) chain is present in invertebrates that do not express immunoglobulins. *Proc. Natl. Acad. Sci. USA* 93:1886.
- Garcia-Pardo, A., M. E. Lamm, A. G. Plaut, and B. Frangione. 1981. J chain is covalently bound to both monomer subunits in human secretory IgA. *J. Biol. Chem.* 256:11734.



25. Krugmann, S., R. J. Pleass, J. D. Atkin, and J. M. Woof. 1997. Structural requirements for assembly of dimeric IgA probed by site-directed mutagenesis of J chain and a cysteine residue of the  $\alpha$ -chain CH2 domain. *J. Immunol.* 159:244.
26. Zikan, J., J. Mestecky, R. Kulhavy, and J. C. Bennett. 1986. The stoichiometry of J chain in human secretory dimeric IgA. *Mol. Immunol.* 23:541.
27. Brandtzaeg, P. 1975. Immunochemical studies on free and bound J chain of human IgA and IgM. *Scand. J. Immunol.* 4:439.
28. Grubb, A. O. 1978. Quantitation of J chain in human biological fluids by a simple immunochemical procedure. *Acta Med. Scand.* 204:453.
29. Vaerman, J. P., A. Langendries, and M. C. Vander. 1995. Homogenous IgA monomers, dimers, trimers and tetramers from the same IgA myeloma serum. *Immunol. Invest.* 24:631.
30. Roe, M., I. N. Norderhaug, P. Brandtzaeg, and F. E. Johansen. 1999. Fine specificity of ligand-binding domain 1 in the polymeric Ig receptor: importance of the CDR2-containing region for IgM interaction. *J. Immunol.* 162:6046.
31. Sorensen, V., I. B. Rasmussen, L. Norderhaug, I. Natvig, T. E. Michaelsen, and I. Sandlie. 1996. Effect of the IgM and IgA secretory tailpieces on polymerization and secretion of IgM and IgG. *J. Immunol.* 156:2858.
32. Brandtzaeg, P. 1975. Purification of J chain after mild reduction of human immunoglobulins. *Scand. J. Immunol.* 4:309.
33. Hendrickson, B. A., D. A. Conner, D. J. Ladd, D. Kendall, J. E. Casanova, B. Corthesy, E. E. Max, M. R. Neutra, C. E. Seidman, and J. G. Seidman. 1995. Altered hepatic transport of immunoglobulin A in mice lacking the J chain. *J. Exp. Med.* 182:1905.
34. Hohman, V. S., S. E. Stewart, C. E. Willett, and L. A. Steiner. 1997. Sequence and expression pattern of J chain in the amphibian, *Xenopus laevis*. *Mol. Immunol.* 34:995.
35. Hendrickson, B. A., L. Rindisbacher, B. Corthesy, D. Kendall, D. A. Waltz, M. R. Neutra, and J. G. Seidman. 1996. Lack of association of secretory component with IgA in J chain-deficient mice. *J. Immunol.* 157:750.
36. Johansen, F. E., M. Pekna, I. N. Norderhaug, B. Haneberg, M. A. Hietala, P. Krajci, C. Betsholtz, and P. Brandtzaeg. 1999. Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. *J. Exp. Med.* 190:915.
37. Shimada, S., M. Kawaguchi-Miyashita, A. Kushiro, T. Sato, M. Nanno, T. Sako, Y. Matsuoka, K. Sudo, Y. Tagawa, Y. Iwakura, and M. Ohwaki. 1999. Generation of polymeric Ig receptor-deficient mouse with marked reduction of secretory IgA. *J. Immunol.* 163:5367.
38. Brandtzaeg, P. 1975. Blocking effect of J chain and J-chain antibody on the binding of secretory component to human IgA and IgM. *Scand. J. Immunol.* 4:837.
39. Vaerman, J. P., A. E. Langendries, D. A. Giffroy, C. S. Kaetzel, C. M. Fiani, I. Moro, P. Brandtzaeg, and K. Kobayashi. 1998. Antibody against the human J chain inhibits polymeric Ig receptor-mediated biliary and epithelial transport of human polymeric IgA. *Eur. J. Immunol.* 28:171.
40. Crottet, P., S. Cottet, and B. Corthesy. 1999. Expression, purification and biochemical characterization of recombinant murine secretory component: a novel tool in mucosal immunology. *Biochem. J.* 341:299.
41. Vaerman, J. P., A. Langendries, D. Giffroy, P. Brandtzaeg, and K. Kobayashi. 1998. Lack of SC/pIgR-mediated epithelial transport of a human polymeric IgA devoid of J chain: in vitro and in vivo studies. *Immunology* 95:90.
42. Koshland, M. E. 1985. The coming of age of the immunoglobulin J chain. *Annu. Rev. Immunol.* 3:425.