Both IgM and IgA exist as polymeric immunoglobulins. IgM is assembled into pentamers with J chain and hexamers lacking J chain. In contrast, polymeric IgA exists mostly as dimers with J chain. Both IgM and IgA possess an 18-amino acid extension of the C terminus (the tail-piece (tp)) that participates in polymerization through a penultimate cysteine residue. The IgM (tp) and IgA (tp) tail-pieces differ at seven amino acid positions. However, the tail-pieces by themselves do not determine the extent of polymerization. We now show that the restriction of polymerization to dimers requires both Cβ3 and tp and that more efficient dimer assembly occurs when Cβ2 is also present; the dimers contain J chain. Formation of pentamers containing J chain requires Cβ3, Cμ4, and the tp. IgM-tp is present mainly as hexamers lacking J chain, and μγμ-tp forms tetramers and hexamers lacking J chain, whereas IgA-μtp is present as high order polymers containing J chain. In addition, there is heterogeneous processing of the N-linked carbohydrate on IgA-tp, with some remaining in the high mannos state. These data suggest that in addition to the tail-piece, structural motifs in the constant region domains are critical for polymer assembly and J chain incorporation.

Antibodies consist of a basic four chain structure arranged to form two Fab arms and an Fc region. Although most Ig isotypes are secreted as monomers, IgM exists only as pentamers and hexamers, and IgA is found as both monomers and polymers of predominantly dimeric form. Both polymeric IgA and IgM are transported into external secretions due to their ability to bind the polymeric Ig receptor. Although all antibody-secreting cells produce J chain, it is incorporated only into polymeric Igs. Polymerization not only increases the avidity of Igs for antigen but also enhances effector functions, such as complement activation and binding to Fc receptors (1, 2). Therefore, understanding how polymerization is regulated and the requirements for polymer assembly is of great interest.

Both IgA and IgM possess an 18-amino acid extension in the C terminus called the tail-piece (tp). The conserved penultimate cysteine residue in the IgA and IgM tail-pieces has been demonstrated to be involved in polymerization (3, 4). Although the tail-pieces of IgA (tp) and IgM (tp) are similar in sequence, there are several differences (Fig. 1A). Both tail-pieces contain an N-linked carbohydrate addition site, the presence of which is required for dimer formation in IgA (5) and J chain incorporation and pentamer formation in IgM (4). However, the structure and composition of the N-linked carbohydrates in the tail-pieces differ, suggesting differences in the accessibility of the glycans to processing by glycosyltransferases (6, 7).

The assembly of dimeric IgA is initiated with the formation of H2L2 monomer units through a pathway in which H + L → H2L2 (8). The penultimate cysteine in the tail-piece of one α chain forms a disulfide bond with J chain, which in turn forms a disulfide bond with the α chain of a second monomeric subunit (5, 9–11). It was suggested that J chain was required for polymerization of Igα, and indeed, polymerization by IgA does not take place in cells lacking J chain (12). However, although IgM pentamers contain J chain, IgM hexamers have been shown to lack J chain (9). In addition, all four isotypes of recombinant human IgGs containing the μtp form polymers without associating with J chain (1, 2).

In the present study, we explore the effects of the tail-pieces and associated constant region domains on the polymerization state of Igs and their interactions with J chain by placing the otp and μtp in the context of human IgM, IgA1, and IgG2 constant regions, as well as generating IgA1/IgG2 and IgM/murine IgG2b domain-exchanged mutants. Although both the otp and μtp can effect polymerization in all contexts, the types of polymers formed were determined by the structural context of the tail-pieces. Analysis of domain-exchanged mutants revealed that the formation predominantly of dimers requires not only the otp but also the Cβ3 domain of IgA. Although all proteins containing the μtp formed primarily higher order polymers, there does appear to be some specificity because wild type IgM formed primarily pentamers, whereas IgM-μtp formed mainly hexamers and cross-linked protein μγμ-μtp formed tetramers and hexamers.

J chain was not found in association with all polymers, indicating that the tail-piece alone is insufficient for J chain incorporation. Studies using IgA1/IgG2 and IgM/murine IgG2b domain-exchanged proteins suggest that the covalent association of J chain with polymeric Igs is determined by the structural context of the tail-piece. In the case of IgA1, the Cβ3

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Polimerization of Igs and Association with J Chain

domain, in addition to either the otp or µtp, is required for incorporation of J chain, whereas IgM requires Cµ3, Cµ4, and the µtp for J chain incorporation. Therefore, although both IgM and IgA associate with J chain, the types of protein-protein interactions made between J chain and the Igs may differ, resulting in the formation of pentamers for IgM and dimers for IgA. In addition, mutating the tail-piece of IgA to that of IgM results in altered carbohydrate processing, polymer assembly, and J chain incorporation.

EXPERIMENTAL PROCEDURES

Construction of an IgM Containing the a Tail-Piece—An NcoMI site was introduced just upstream of the otp by PCR overlap mutagenesis, resulting in a silent mutation. In a first PCR, the oligonucleotide with the sequence CACAGCGCAGGTAAACCCACCC (NsmI site underlined) was annealed within the constant region of an IgA gene in plasmid pBS202 was used as the 5’ primer, together with a 3’ anti-sense mutagenic oligo GTGTGATTGGCGCCGAAGGTT, which includes two nucleotide changes (boldface) to introduce an NcoMI site. A parallel reaction was performed with a 5’ oligo complementary to the mutagenic primer and a 3’ primer, AACTAGTGATCCCGCCCTCTCC (with a BamHI site), annealing in the intron downstream of the otp gene. The overlapping PCR was carried out with the external primers (5’-NsmI and 3’-BamHI). After sequencing, the Sma1-BamHI fragment was cloned in a pUC plasmid generating pUC6404. To join the otp on to the µ chain, the Agel-BamHI fragment from µ was replaced with the NcoMI-BamHI otp fragment, producing plasmid pBS6416. The Sall-BamHI cassette, containing the µ constant region with the otp, was then used to produce the anti-DNS H chain expression vector pAG6406. Construction of an IgA Containing the µ Tail-Piece—The µtp was joined to the end of the Cµ3 of IgA by a ScaI to NcoMI Agel to BamHI triple ligation in plasmid pUC6405. The EcoRI-KpnI fragment from this clone, which contains a portion of the IgA Cµ3 with the ºtp, was moved into pBS4213, a subcloning plasmid that lacks a SmaI site resulting in pBS6407. The Sma1-BamHI cassette from this plasmid was replaced by a wild type IgA Cµ3 containing region creating the BamHI-Sall fragment containing the complete IgA constant region fused to the µtp was cloned in the anti-DNS H chain expression vector.

Construction of an IgG Containing the a Tail-Piece—The otp from IgA1 was modified with restriction sites for cloning using PCR. A 5’ primer, CCGCTGCGCAGGTAAACCCACCC, introduced a SmaI site, and the mtp was amplified using plymerase with the sequence CCGCTGCGCAGGTAAACCCACCC (SmaI site underlined) annealed within the constant region of an IgA gene in plasmid pBS202 was used as the 5’ primer, together with a 3’ anti-sense mutagenic oligo GTGTGATTGGCGCCGAAGGTT, which includes two nucleotide changes (boldface) to introduce an SmaI site into the intron downstream of the otp. The Bsp/BamHI PCR fragment was used to replace the µtp in plasmid pBS6446, which contains γ2-µtp. From the resulting plasmid, pBR1724, the γ2-otp was removed and cloned into the anti-DNS H chain expression vector, generating pAG1725.

Construction of Domain-exchanged Mutants—The genomic clones coding for the constant regions of IgA1 and IgG2 were cloned into the multiple cloning site of pBR22 vector, where they can be conveniently modified by domain swapping. A PvuI restriction site was introduced between C1 and C2 to enable us to exchange exons between α1 and α2 (Fig. 1A). The PvuI sites in the intron separating C1 and C2 and the amplicin gene were used to generate γα and γα-otp constant region constructs (Fig. 1B). Similarly, a BglII-Sall fragment, in which the BglII site was blunted, containing C3 was exchanged with a SmaI-Sall fragment containing C3 to generate γα and γα-otp constructs. Genes with exchanged C1-2 exons were generated by using exchanging PvuI fragments from the wild type constructs and Cµ3 domain-exchanged constructs.

The construction of human IgM/murine IgG2b domain-exchanged plasmids has been described previously (13).

Production and Analysis of Recombinant Proteins—The tail-piece and domain-exchanged H chain expression vectors were transfected into the TWS cell line by electroporation. TWS is a derivative of the non-Ig-producing mouse myeloma cell line Sp2/0, which synthesizes J chain and secretes a transfected chimeric anti-DNS Vκ-human Ck light chain (14). Transfectants were selected with mycophenolic acid (3 µg/ ml) and provided with hypoxanthine (125 µg/ml) and xanthine (7.5 µg/ml). Surviving transfected cells were screened by ELISA using danylabeled BSA-coated microtiter plates and anti-human IgG, IgM, or IgA Fc alkaline phosphatase-conjugated polyclonal goat antibody (Sigma). To confirm that the structures of the IgA1/IgG2 domain-exchanged mutants were correct, the cell culture supernatants were screened by ELISA using antigen-coated microtiter plates and detected using monoclonal antibodies kindly provided by Dr. Robert G. Hamilton (15). -specific (HP6053), C1-specific (HP6014), C2-specific (HP6002), and C3-specific monoclonal antibodies (HP6017) were used (Fig. 1B). Positive clones were expanded and maintained in Icsoue’s modified Dubecco’s medium containing 5% calf serum.

Antibodies were analyzed by metabolic labeling, Coomassie Blue staining, and Western blotting. In the first method, 3 x 10⁶ cells were washed and resuspended in 1 ml of labeling medium (high glucose Dubecco’s modified Eagle’s medium deficient in methionine: Life Technologies, Inc.) containing 25 µCi of [35S]methionine (Amersham Pharmacia Biotech) and allowed to incorporate label for 3–4 h or overnight with the addition of 1% calf serum at 37 °C under tissue culture conditions. For metabolic labeling, the antibodies were immunoprecipitated with 2.5 µl of a rabbit anti-human IgG, IgM, or IgA Fab and anti-human Fab polyclonal antiserum and a 10% suspension of Staphylococcus aureus with membrane-bound protein A (IgG Sorb, Enzyme Center, Woburn, MA). The samples were resuspended in 50 µl of loading buffer (25 µg Tris, pH 6.7, 0.2% SDS, 10% glycerol, ~3 µg/ml of bromophenol blue) and boiled for 3 min. Alternatively, supernatants were incubated for 1 h at 4 °C with 30 µl of 50% (v/v) danylabeled BSA coupled to Sepharose beads. Antibodies bound to Sepharose beads were pelleted by centrifuging at 13,000 x g for 2 min and washed twice with 1 ml of phosphate buffer, pH 7.8, containing 0.45 M NaCl and then washed twice with 1 ml of PBS. Antibodies were eluted by incubating the beads for 10 min on ice in 30 µl of 3 M Ne-dansyl-l-lysine (Sigma) in phosphate buffer, pH 7.8, containing 0.45 M NaCl. The precipitated labeled antibody was then resuspended in 50 µl of loading buffer and boiled for 3 min. Proteins were analyzed by SDS-PAGE (3.5 or 4% Tris-glycine gels for polymeric Igs or 5% sodium phosphate-buffered (PO₄) gels for monomeric Igs and IgA). To examine H and L chains separately, a portion of the labeled sample was reduced by treatment with 0.15 M β-mercaptoethanol at 37 °C for 30 min and analyzed on 12.5% Tris-glycine gels. To quantitate the relative amounts of monomers and polymers, the gels were analyzed with a phosphorimager (Bio-Rad).

Unlabeled Igs were purified from culture supernatants using danylabeled BSA Sepharose beads and subjected to SDS-PAGE as described above. Igs were detected by staining with Coomassie Blue. Alternatively, Igs were detected by Western blotting. Proteins were transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) according to the method of Towbin et al. (16) using a semidry method. Nonspecific sites were blocked by incubating the blot for 1 h at room temperature in PBS containing 0.2% (v/v) Tween-20 and 5% (v/v) dried milk and then probing with peroxidase-conjugated donkey-anti-κ chain (Sigma) for 1 h at room temperature or overnight at 4 °C. The peroxidase-conjugated antibody was detected using the SuperSignal West Pico Chemiluminescent substrate system (Pierce) as recommended by the manufacturer.

Fast Protein Liquid Chromatography Purification of Polymeric Igs—Igs were purified from culture supernatant by SDS-Sepharose affinity chromatography as described previously (17), and the concentrations of the Igs were determined using the BCA protein assay (Pierce). Polymeric Igs were separated from monomeric Igs by gel filtration on a 30 × 1.5-cm Superose 6 column (Amersham Pharmacia Biotech) in PBS/Tween-20 and washed and resuspended in 1 ml of labeling medium containing 25 µg Tris, pH 7.8, containing 0.45 M NaCl and 5% (v/v) danylabeled BSA conjugated to goat-anti-human Fab (Sigma) for 1 h at room temperature or overnight at 4 °C. Bound antibody was detected using the SuperSignal West Pico Chemiluminescent substrate system (Pierce) as recommended by the manufacturer.

J Chain Detection—An anti-J chain immunoblot was performed using separating purified polymeric antibodies on a 12.5% Tris-glycine gel under reducing conditions and transferring to membrane as described above. The membrane was blocked for 1 h at room temperature in PBS/Tween-20/milk and then probed with peroxidase-conjugated goat-anti-J chain (Nordic Immunology, Capistrano Beach, CA) for 1 h at room temperature or overnight at 4 °C. The peroxidase-conjugated antibody was detected as described above. The blot was then stripped by incubating in stripping buffer (53 mM Tris, pH 6.8, 1.6% SDS, 14.3 mM β-mercaptoethanol) at 60 °C for 15 min and then blocked again using PBS/Tween-20/milk. A donkey-anti-human Fab alkaline phosphatase-conjugated goat antibody (Sigma) was used as a probe and incubated at 1 h at room temperature or overnight at 4 °C. Bound antibody was detected using the SuperSignal West Pico Chemiluminescent substrate system (Pierce). Gels were scanned, and densitometry was performed using the National Institutes of Health Image® program.

Tunicamycin Treatment of Cells—3-5 x 10⁶ cells were washed two
times with PBS and resuspended in 1 ml of Iscove's modified Dulbecco's medium with 5% α-calf serum and pretreated with 8 μg/ml of tunicamycin (Roche Molecular Biochemicals) for 4–5 h. The cells were then metabolically labeled as described above in 1 ml of labeling medium containing 25 μCi of [35S]methionine and 8 μg/ml tunicamycin.

**Endoglycosidase H Treatment—**To analyze the state of processing of N-linked carbohydrates, 8–16 μg of purified proteins was denatured in 25 μl of 0.1 M β-ME + 0.1% SDS by boiling for 5 min. 100 μl of reaction buffer (75 mM sodium citrate + 0.5% (w/v) phenylmethylsulfonyl fluoride) and 2.5–5 microunits of Endoglycosidase H (Roche Molecular Biochemicals) were added. The digestion was allowed to proceed for 18–24 h at 35 °C. After boiling, the antibodies were analyzed by PAGE under reducing conditions on a 12.5% Tris-glycine gel. Western blotting was performed as described above to detect human α H chain using a rabbit-anti-human IgA (Sigma) and peroxidase-conjugated donkey-anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech).

**RESULTS**

The μ and α Tail-pieces Do Not By Themselves Specify the Extent of Polymerization of Igs—Although similar, the tailpieces of IgM (μtp) and IgA (αtp) differ at 7 of 18 amino acid positions (Fig. 1A). Polymeric IgM is present as dimers, whereas polymeric IgM is pentameric and hexameric. To investigate the contribution of the tailpiece to the polymerization state of Igs, we added the tailpiece of IgM or IgA to H chains with different structures (Fig. 1) and expressed them in Sp2/0 myeloma cells expressing a transfected DNS-specific chimeric κ L chain and endogenous J chain. Transfectants secreting DNS-specific antibodies were identified by ELISA. Igs were purified from cell culture supernatants by affinity chromatography and analyzed by SDS-PAGE.

IgM-αtp resembles wild type IgM produced in the same cell system with virtually all of the Ig secreted as pentamers/hexamers (Fig. 2). In contrast to wild type IgA in which most polymers are dimers, IgA-μtp forms mostly pentamers/hexamers, consistent with the recent findings of Sorensen et al. (18) (Fig. 2). We have previously shown that addition of the μtp to all four isotypes of human IgG resulted in the assembly of IgG into dimers, trimers, tetramers, pentamers/hexamers, and monomers (2). We now find that IgG2-αtp is similar to IgG-μtp, forming all species of polymers as well as monomer (Fig. 2). These data indicate that both μtp and αtp are sufficient for Ig polymerization but do not by themselves specify the extent of polymerization.

**Cα2 and Cβ3 of IgA and the αtp Are Important for the Assembly of Dimers—**To further investigate the requirements for the specific assembly of dimers, we generated domain-exchanged mutants. With the hinge and Cα2 considered as a single unit, all possible combinations of α1/γ2 exon-exchanged hybrid genes were generated and expressed (Fig. 1C). Characterization by ELISA using domain-specific monoclonal antibodies confirmed that the structures of the domain-exchanged mutants were correct (Fig. 1C), and SDS-PAGE analysis of biosynthetically labeled proteins under reducing conditions confirmed that the H and L chains were of the expected molecular weight (data not shown). At least five independent clones from at least two transfections for each construct were analyzed to determine the assembly patterns of the domain-exchanged antibodies. The relative amount of each polymeric form was quantitated by densitometry by taking the average of the percentage of the different forms from multiple clones (Table I). Igs from representative clones are shown in Fig. 3. Analysis was done on both 5% PO4 gels and 3.5% Tris-glycine gels to look at all polymerization states.

All Igs containing Cβ3 but lacking a tail-piece were produced only as monomers. In contrast, all of the proteins that contain the αtp were able to form polymers, indicating that the αtp by itself is sufficient to cause polymerization of Igs (Fig. 3A). However, the degree and extent of polymerization varied for the different domain-exchanged mutants, suggesting that other structural determinants in the constant region(s) control the extent of polymer assembly.

As expected, wild type IgA1 showed two predominant bands
of approximately equal intensity, corresponding to the IgA monomer and dimer, respectively. All Igs that contain both the C_3 and atp failed to form significant amounts of higher order polymers (Fig. 3B and Table I). However, dimer assembly was inefficient in the case of _γ_3_α_, α_3_, _μ_2_α_, and _μ_3_α_, in which 80–85% was monomeric. In contrast, dimer formation by _γ_2_α_, which contains both C_2 and C_3, was equivalent to that of wild type IgA. These results suggest that although C_3 can prevent the formation of higher order polymers, both C_2 and C_3 of IgA are required for efficient dimer assembly. C_1 does not appear to influence polymer formation because the assembly state of _γ_2_α_ was indistinguishable from that of wild type IgA1. In addition, the _μ_3 cannot substitute for the atp in IgA for specific dimer assembly because IgA-μ3 atp formed pentamers/hexamers rather than dimers (Fig. 2).

Polymerization of proteins that contain the _μ_3 but lack C_3 was not limited to the formation of dimers. The addition of the atp to IgG2 resulted in the secretion of both dimer and pentamer/hexamer (Table I and Figs. 2 and 3). Among the domain exchange proteins, _α_3_γ_, _α_3_γ_, and γ _γ_ atp all form higher order polymers in addition to dimers (Fig. 3B and Table I), and as noted above, IgM-atp is secreted predominantly as pentamers/hexamers. Thus the atp can affect the polymerization of Igs, but the C_3 is required to specifically limit assembly to the formation of dimers.

Requirements for the Formation of High Molecular Weight Polymers—Earlier studies have shown that IgG-μ3 atp forms not only pentamers/hexamers but also dimers, trimers, and tetramers (2). In contrast, changing the tail-piece on IgA to that of IgM resulted in the formation of pentamers/hexamers but not intermediate polymeric forms (Fig. 2). Taken together, these results suggest that the context of the _μ_3 is important for polymer assembly to be limited to pentamers/hexamers. To further investigate the requirements for specific assembly of pentamers and hexamers, we created domain exchange proteins between IgM and IgG2 (Fig. 1C). Both γ _γ_ _γ_3 and _μ_ _γ_ _γ_ _γ_3 atp proteins (Fig. 3B), as well as IgM-atp (Fig. 2), were secreted as high molecular weight polymers. These studies suggest that there are certain structural motifs in IgA and IgM that are missing from the IgGs that direct the specific formation of pentamers/hexamers.

Incorporation of J Chain into Polymorphic Igs—Previous studies showed that J chain is not required for the formation of polymers (2, 9). To identify the structural requirement for the incorporation of J chain into IgA, IgA1/IgG2 domain-exchanged mutants containing the atp were biosynthetically labeled and analyzed by SDS-PAGE on 12.5% Tris-glycine gels under reducing conditions (Fig. 4A). J chain was found in those proteins that contain both C_3 and atp (wild type IgA1, α _α_ atp, _γ_ _γ_ atp, and _γ_ _γ_ _γ_ atp) but was absent in those containing atp but lacking C_3 (wild type IgG2, _α_ _α_ _γ_ atp, _α_ _γ_ _γ_ atp, and _γ_ _γ_ _γ_ atp). The J chain band in _α_ _α_ _γ_ atp was very faint in multiple experiments. This probably reflects the fact that most of the molecule is secreted as monomers (Table I).

Human IgM/murine IgG2b domain-exchanged proteins were also analyzed for their incorporation of J chain by Western blotting using a polyclonal anti-J chain antiserum. The blot was then stripped and probed using an anti-human κ L chain

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**TABLE I**

**Densitometric analysis of secreted Igs**

Cells expressing recombinant H chains were metabolically labeled and immunoprecipitated as described under “Experimental Procedures.” Igs were analyzed by SDS-PAGE on 3.5% Tris-glycine and/or 5% Po4 gels. To quantitate the relative amounts of monomers and polymers, the gels were analyzed with a phosphorimager. The percentages of monomers and polymers shown are the average for multiple clones. h = hinge, tp = tail-piece.

<table>
<thead>
<tr>
<th>Hexamers and pentamers</th>
<th>Intermediate polymers</th>
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<th>Monomers</th>
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</tr>
<tr>
<td>wt IgG2</td>
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Fig. 4. J chain incorporation by tail-piece and domain-exchanged mutants. A, transfectants were biosynthetically labeled with [35S]methionine for 16 h, and labeled proteins were precipitated from the culture supernatants with dansylated BSA coupled to Sepharose. The proteins were reduced and then subjected to SDS-PAGE on 12.5% Tris-glycine gels, and the gels were analyzed by autoradiography. B, Igs were purified from culture supernatants, reduced, and subjected to SDS-PAGE as described above. J chain was detected by Western blotting using an anti-J chain antiserum that cross-reacts with H chain and κ L chain. The blot was then stripped and probed with an anti-κ light chain antiserum to control for equal gel loading. The positions of the H chain, κ L chain, and J chain are indicated. Mw, molecular weight marker.
Polymerization of Igs and Association with J Chain

To confirm that J chain was absent from polymers containing the atp but lacking C3, and to extend the studies to include IgM-atp and IgA-atp, we used fast protein liquid chromatography to separate purified IgM-atp, IgG2-atp, IgA1-atp, γγ-atp, and ααγ-atp into polymer and monomer fractions. Analysis of the purified proteins by SDS-PAGE on 6% Tris-glycine gels showed the polymer fractions, with the exception of IgG2-atp, to contain exclusively polymeric Igs (Fig. 5A). The polymers were then analyzed by Western blot for the presence of J chain as described above (Fig. 5B).

Consistent with the biosynthetic labeling experiments, these data indicate that the presence of Cγ3 of IgA is required for the incorporation of J chain into polymers formed by the atp, ααγ-atp, which contains C3, failed to incorporate J chain, whereas γγ-atp, which contains C3, was associated with J chain (Fig. 5B). IgA-atp polymers also contained J chain, suggesting that the atp can substitute for the atp in directing incorporation of J chain into IgA. IgG-atp polymers were shown previously to lack J chain (2), and similarly, J chain was not detected in IgG2-atp polymers. No J chain was present in wild type IgG3, as expected. J chain was found associated with wild type IgM, but not with IgM-atp polymers.

IgM-atp Forms Predominantly Hexamers, Whereas μμγμ-μtp Forms Tetramers and Hexamers. The polymerization of IgM and IgA is directed by the Cγ2 and J chains, respectively. Wild type IgM, IgM-atp, and μμγμ-μtp were subjected to SDS-PAGE under nonreducing conditions on a 4% Tris-glycine gel. The gel was run for 6 h to separate the high molecular weight pentamers and hexamers. Western blotting was performed by probing with an anti-light chain antiserum to control for gel loading (Fig. 4B). The polyclonal anti-J chain antiserum also showed some reactivity with L chain. Similar to what had been observed for IgA, the tail-piece alone was not sufficient for incorporation of J chain into polymeric IgM. J chain was found in wild type IgM and μμγμ-μtp, but not in μμγμ-μtp. These data indicate that Cγ3, Cγ4, and μtp must all be present for J chain to be incorporated into IgM polymers.

To test this possibility, the polymers of wild type IgM, IgM-atp, and μμγμ-μtp were separated by SDS-PAGE. When analyzed by Western blot for k L chain (Fig. 6), IgM-atp showed a slower mobility than wild type IgM, suggesting that IgM-atp is hexameric. Two bands were evident for μμγμ-μtp, but both bands showed a difference in migration from that of wild type IgM (Fig. 6). From these data, we conclude that μμγμ-μtp forms mainly hexamer and tetramer, but not pentamer. Therefore, these data suggest that although atp can substitute for μtp in the formation of IgM polymers, these polymers differ from those seen with wild type IgM in that they are predominantly hexamers lacking J chain. In addition, the replacement of Cγ3 with C2 results in tetramer and hexamer assembly rather than pentamers.

Wild Type IgA and IgA-atp Differ in Glycosylation Status—The atp and μtp do not function equivalently in promoting the polymerization of IgA because a large proportion of IgA-atp was secreted as pentamers/hexamers, whereas wild type IgA is secreted as dimers (Fig. 2). We also observed that the H chain of IgA-atp runs as a doublet in reducing SDS-PAGE on 12.5% Tris-glycine gels. Because treatment of cells with tunicamycin during biosynthetic labeling yielded wild type IgA and IgA-atp H chains of equivalent sizes (Fig. 7), it appears that these two bands reflect heterogeneity in the N-linked carbohydrates. To determine whether the extent of carbohydrate processing differs in IgA and IgA-atp, the Igs were digested with Endoglycosidase H (Endo H), which cleaves high mannose but not complex sugars. Wild type IgA, which is known to contain only complex sugars (6), was resistant to Endo H, as expected. In contrast, at least a portion of IgA-atp was cleaved by Endo H,
indicating that some of the N-linked carbohydrates remain in the unprocessed, high mannose form. Therefore, changing the tail-piece of IgA to that of IgM resulted in differences in the modification of N-linked glycans. Because wild type IgA and IgA-μtp only differ in the tail-piece, the differences in glycosylation most likely represent differences in the tail-piece glycans.

**DISCUSSION**

In contrast to IgG, IgA and IgM exist as polymeric immunoglobulins. However, polymeric IgA is secreted mainly as dimers, whereas IgM is secreted as pentamers and hexamers. An interesting question is what directs IgA to form predominantly dimers, whereas IgM forms pentamers/hexamers. Previous studies and the current investigation have shown that the addition of the otp or μtp to the C terminus of IgM, IgA, or IgG is sufficient for the formation of polymers; however, the selective formation of dimers in those Igs containing the otp and pentamers/hexamers in those containing the μtp was not observed (2, 18, 19), indicating that the tail-pieces by themselves do not contain all of the information necessary for the formation of specific polymers.

We have now used domain-exchanged proteins to define the contribution of the constant region domains to polymer assembly. Because the Ig constant region domains are “modular” in nature, we believe that exchanging the domains between different Ig isotypes would not result in dramatic changes to the overall three-dimensional structure of the Igs. However, the contribution of subtle structural changes in the interdomain regions to polymer assembly and J chain incorporation cannot be ruled out. The results of this investigation are summarized in Table II. Interestingly, we have found that the selective formation of dimers occurred only in the presence of both C, and otp, but efficient dimer assembly was observed only when both C, and C, were present. Mutation of the six amino acids around Cys 414 of IgA to those of IgM did not alter assembly (18), suggesting that other regions in C, contribute to efficient dimer formation. Pentamer assembly seems to require C, C, and the μtp.

J chain has been shown to be necessary for the assembly of IgA dimers (20, 21). We now show that in the presence of otp, the requirements for J chain incorporation closely resemble the requirements for specific dimer formation, namely the presence C, Molecules containing C, otp as well as IgM-otp did not associate with J chain.

Although J chain is associated with IgM pentamers, it is not associated with IgM hexamers (22). The polymerization state of IgM has been shown to be regulated by the amount of J chain synthesized in that increases in J chain production led to an increase in the secretion of pentamers (23). However, we have found that placing the tail-piece of IgA on IgM resulted in the secretion of predominantly hexamers that lack J chain, and replacement of C, with C, resulted in tetramers and hexamers that lack J chain. In these cases, tetramer and hexamer formation does not appear to reflect limiting amounts of J chain because wild type IgM produced by the same cells formed predominantly pentamers associated with J chain. In addition, the preferential formation of IgM-otp hexamers does not appear to be an artifact of this expression system because Sørensen et al. (18) have recently made the same observation in a different cell line, although they did not examine J chain incorporation.

In pentameric IgM, J chain is presumed to bind to two monomers, functioning as a “clasp” to close the circle of monomers (23); in hexameric IgM, it is an additional monomer that closes this circle. It has been proposed that chaperones retain partially assembled IgM polymers in the endoplasmic reticulum (24). Changing the IgM tail-piece to that of IgA may abolish specific structural motifs that are needed to interact with these chaperones for efficient incorporation of J chain into IgM pentamers. Alternatively, the structure of the μtp may be

<table>
<thead>
<tr>
<th>Types of polymers formed</th>
<th>Association with J chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>C, otp</td>
<td>Dimers</td>
</tr>
<tr>
<td>C, μtp</td>
<td>Pentamers/hexamers</td>
</tr>
<tr>
<td>C, otp</td>
<td>All types</td>
</tr>
<tr>
<td>C,C, 4-μtp</td>
<td>Pentamers</td>
</tr>
<tr>
<td>C,C, 4-otp</td>
<td>Hexamers</td>
</tr>
<tr>
<td>C,2,C, 4-μtp</td>
<td>Tetramers and hexamers</td>
</tr>
<tr>
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<td>All types</td>
</tr>
</tbody>
</table>

*More efficient dimer formation occurs when C, is also present.

Carbohydrate processing is altered so that some glycans remain in the unprocessed, high mannose form. Therefore, changing the tail-piece of IgA to that of IgM resulted in differences in the modification of N-linked glycans. Because wild type IgA and IgA-μtp only differ in the tail-piece, the differences in glycosylation most likely represent differences in the tail-piece glycans.

**FIG. 8. Sequence alignment of IgM and IgA tail-pieces from various species.** + represents the same amino acid as that of human IgM or IgA at that position. − represents a deletion at that position. *, §, and † indicate residues at positions 8, 12, 13, and 14, respectively, which contain conserved differences between the μtp and otp across species. For the IgA tail-piece, the sequence is that of IgA1 unless otherwise indicated.

**Table II**

Summary of results using tail-piece and domain exchange mutants

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Carbohydrate processing is altered so that some glycans remain in the unprocessed, high mannose form.
such that it leads to preferential formation of hexamers due to changes in the accessibility and/or orientation of the penultimate cysteine involved in polymerization.

The carbohydrate present on both J chain and the α and μ tail-pieces plays a role in polymerization. The carbohydrate in μtp has been shown to be required for J chain incorporation (4), and elimination of the carbohydrate from either J chain or μtp leads to decreased dimer formation (5, 12) and increased formation of higher order polymers (25). The N-glycan on the μtp of IgA1 has been reported to be predominantly a triantennary complex oligosaccharide (6), whereas IgM has been reported to have a high mannose carbohydrate in the tail-piece (7). These differences in carbohydrate structure suggest that the two tail-pieces differ in their accessibility to processing by glycosyltransferases. Indeed, we found that IgA-μtp is heterogeneous in its glycosylation and contains glycans that can be removed by Endo H digestion, whereas all of the glycans on wild type IgA are Endo H-resistant. Polymeric IgM assembly is thought to occur through the stepwise addition of HL half-molecules, presumably limiting the accessibility of the tail-piece carbohydrate to processing by glycosyltransferases (19). The observation that the N-linked carbohydrate in the tail-piece of IgA-μtp is similar to that of the tail-piece glycan of IgM suggests that IgA-μtp is also assembled such that its tail-piece glycan is not available for processing by the glycosyltransferases. These differences in carbohydrate processing suggest that changing the tail-piece alters the overall three-dimensional structure of the Igs, which affects polymer assembly and incorporation of J chain.

Currently, there are no crystal structures available for IgA and IgM, although a theoretical model of monomeric IgA has been published recently (6). Previous models have suggested that two IgA monomers interact through the tail-pieces to form dimeric IgA, which lies tail-to-tail (12). However, our studies indicate that both C3 and μtp are important in the assembly of dimers, suggesting that two IgA monomers may be interacting noncovalently through their C3 domains in addition to forming covalent associations in the tail-piece. In this case, the structure of dimeric IgA would not be a linear molecule of two monomers lying tail-to-tail but rather would be V-shaped. In addition, the formation of pentamers/hexamers also appears to require specific constant region domains of IgA or IgM that are not present in IgG, although the requirements are less clear.

The tail-pieces of IgM and IgA show a high degree of sequence identity and similarity across many different species (Fig. 8B). Human μtp and μtp used in these studies differ at seven positions (amino acids at positions 3, 4, 8, 11, 12, 13, and 14). Residue positions 3, 4, and 11 do not seem uniquely characteristic of either μtp or αtp. In contrast, the leucine at position 8 is present in all μtp except that of the gray opossum, whereas a conserved valine is observed at the corresponding position in the αtp. Also striking is the region containing residues 12–14. Both IgM and IgA contain a negatively charged residue at position 12 in all species analyzed except in ladyfish IgM. Whereas all species except the gray opossum contain a polar residue at position 13 of the μtp, the corresponding position in the αtp is hydrophobic. In addition, all species except for the Norway rat and marsupials, including opposum, contain another negatively charged residue at position 14 in the μtp but not in the αtp. It is tempting to speculate that these differences between the tail-pieces in the context of the various constant region domains contribute to the types of polymers formed and whether they associate with J chain.

In summary, we have shown that although the tail-pieces of both IgA and IgM can effect polymerization of Igs, the incorporation of J chain and the extent of polymerization is determined by additional structural motifs in the constant region domains. In addition, the incorporation of J chain into polymeric Igs is regulated not only by the amount of J chain available but also by structural features in the constant region domains as well as the tail-piece. Based on these findings, we suggest an alternative model for the structure of dimeric IgA in which the two IgA monomers interact noncovalently through their C3 domains as well as through covalent interactions in the tail-piece.

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Structural Requirements for Polymeric Immunoglobulin Assembly and Association with J Chain

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