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Cysteine Residues Required for the Attachment of the Light Chain in Human IgA2[1]

Koteswara R. Chintalacharuvu,[2†] Li J. Yu,[†] Nishant Bhola,[†] Kunihiko Kobayashi,[‡] Christine Z. Fernandez,[†] and Sherie L. Morrison[†]

In humans, there are two subclasses of IgA, IgA1 and IgA2, with IgA2 existing as three allotypes, IgA2m(1), IgA2m(2) and IgA2(n). In IgA1, Cys^{133} in C_H1 forms the disulfide bond to the L chain. Our previous studies indicated that in IgA2 lacking Cys^{133}, a disulfide bond forms between the α-chain and the L chain when Cys^{220} is followed by Arg^{221}, but not when Cys^{220} is followed by Pro^{221}, suggesting that the Cys in C_H1 might be involved in disulfide bonding to the L chain. However, here we show that covalent assembly of the H and L chains in IgA2(n) requires hinge-proximal Cys^{241} and Cys^{242} in C_H2 and not Cys^{196} or Cys^{220} in C_H1. Using pulse-chase experiments, we have demonstrated that wild-type IgA2(n) with Arg^{221} and Cys^{241} and Cys^{242} assembles through a disulfide-bonded HL intermediate. In contrast, the major intermediate for IgA2 m(1) with Pro^{221} assembly was H_2 even though both Cys^{241} and Cys^{242} were present. Only a small fraction of IgA2 m(1) assembles through disulfide-bonded HL. Overall, our studies indicate that for IgA2 covalent assembly of the H and L chains requires the hinge-proximal cysteines in C_H2 and that the structure of C_H1 influences the efficiency with which this covalent bond forms. ***The Journal of Immunology*, 2002, 169: 5072–5077.**

Protein folding, disulfide bond formation, and glycosylation occur during the assembly and secretion of functional Igs. The Ig H and L chains are synthesized on the membrane-bound polysomes and translocated into the lumen of the endoplasmic reticulum where they fold into globular domains of 110 aa stabilized by at least one intrachain disulfide bond. Covalent assembly of the H and L chains follows an ordered pathway determined by the isotype of the H chains (1–4). IgG assembles into an H_2L_2 molecule; however, both IgM and IgA form higher polymers of this basic subunit often with the associated J chain. Both IgM and IgA can be found in the mucosal secretions associated with secretory component, the cleavage product of the polymeric Ig receptor.

There are two subclasses of human IgA, IgA1 and IgA2, with IgA2 existing as several allotypes, IgA2m(1), IgA2m(2), and IgA2(n) (5). The different forms of human IgA differ in their H and L chain disulfide-bonding pattern. In IgA1, IgA2m(2), and IgA2(n), a disulfide bond links the L chain to the H chain. In contrast, the major form of IgA2m(1) lacks disulfide bonds between the H and L chain, although recent studies have shown that a small amount of HL disulfide-linked IgA2m(1) is made (2).

Structural analysis has shown that for IgA1, Cys^{145} and Cys^{204} in C_H1, Cys^{266} and Cys^{323} in C_H2, and Cys^{369} and Cys^{432} in C_H3 form the intradomain disulfide bonds characteristic of the Ig fold (Fig. 1) (6–8). In IgA1, Cys^{196} and Cys^{200} in C_H1 and Cys^{242} and Cys^{299} in C_H2 form additional intradomain disulfide bonds with the remaining Cys residues forming covalent bonds with other chains: 133 to the L chain, 241 and 301 to the H chain, 311 to the secretory component, and 471 to the J chain (9). A similar structure has been proposed for IgA2m(1) except there is no disulfide bond with the L chain since Cys^{133} is now Asp (Fig. 1) (10). Although IgA2m(2) and IgA2(n) also lack a Cys at 133, they form covalent bonds with the L chain. An unresolved question is which Cys residues within IgA2m(2) and IgA2(n) form the bond with the L chain.

IgA2m(1) differs from IgA2m(2) and IgA2(n) at positions 212 and 221 in C_H1; IgA2m(1) has Pro^{212} and Pro^{221} whereas IgA2m(2) and IgA2(n) have Ser^{212} and Arg^{221}. Studies from our laboratory have shown that it is the presence of Pro^{221} in IgA2m(1) that interferes with the formation of an HL disulfide in the absence of Cys^{133}, suggesting that either Cys^{196} or Cys^{220} that had been reported to form an intrachain disulfide in IgA1 and IgA2m(1) might form the bond with the L chain. However, present studies have now shown that an HL disulfide will form in IgA2(n) in the absence of both Cys^{196} and Cys^{220}. In contrast, removal of either Cys^{241} or Cys^{242} in C_H2 interferes with the formation of the HL disulfide bond. Therefore, it appears that the L chain is disulfide bonded to the hinge-proximal region of C_H2 with assembly taking place through an HL intermediate. In contrast, for IgA2m(1) with Pro^{221}, there is efficient formations of H_2 molecules but little disulfide-bonded HL is present as an assembly intermediate and only small quantities of HL, H_2L_2, and H_4L_4J are present in the secretions. Overall, our studies suggest that for IgA2 covalent assembly of the H chain with the L chain involves the hinge-proximal cysteine residues in C_H2 and the structure of C_H1 influences the efficiency with which this covalent bond forms.

Materials and Methods

Reagents and cells

Restriction endonucleases and molecular cloning enzymes were obtained from either New England Biolabs (Beverly, MA) or Stratagene (La Jolla, CA). [^{35}S]Methionine was obtained from ICN Research Products (Costa

*Department of Microbiology, Immunology and Molecular Genetics and Molecular Biology Institute, University of California, Los Angeles, CA 90095; and †Department of Pediatrics, Hokkaido University School of Medicine, Sapporo, Japan

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[2] Address correspondence and reprint requests to Dr. Koteswara R. Chintalacharuvu, Department of Microbiology, Immunology and Molecular Genetics, 611 Charles Young Drive South, Paul Boyer Hall 519, University of California, Los Angeles, CA 90095-1489. E-mail address: kotec@lifesci.ucla.edu

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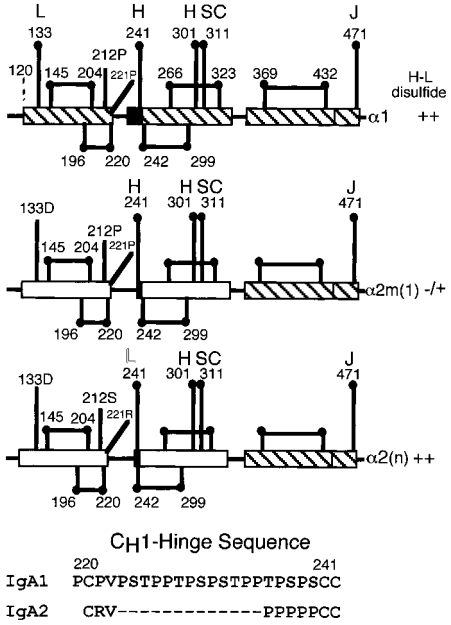

FIGURE 1. Schematic diagram of the human α1-, α2m(1)-, and α2(n)-chains. Filled circles indicate Cys residues. The standard Ig-fold intradomain disulfides and the Cys residues involved in forming disulfide bonds with other chains are shown above the sequence. The residues shown to form additional intradomain links characteristic of IgA are shown below the sequence. The filled box at the 5′ end of C_H2 indicates the hinge region which is 13 aa longer in IgA1; the sequence of the two hinge regions is given at the bottom of this figure. Boxes with diagonal lines have sequences characteristic of α1. The open boxes indicate exons with sequences characteristic of α2. The structure of IgA1 and IgA2m(1) are from Refs. 6–8 and 10. The disulfide pattern for IgA2(n) incorporates information from previous studies as well as the current studies. The light type-faced 241L indicates the Cys residue that is involved in binding to the L chain. Residues are numbered as by Mattu et al. (15), preserving the same numbering system in IgA2 even though it is missing the 13 hinge amino acids.

Mesa, CA). The Sp2/0 myeloma cells were cultured in IMDM containing 5% bovine calf serum (BCS)[3] (HyClone Laboratories, Logan, UT).

Engineering of α2m(1) and mutants of IgA2(n) by site-directed mutagenesis using PCR

The *α2(n)* gene (5) in Bluescript II (Stratagene, La Jolla, CA) was used as a template to generate *α2m(1)* by mutating Ser^{212} and Arg^{221} in α2(n) to Pro^{212} and Pro^{221}, respectively. Two PCR products spanning C_H1 were generated: The first product was generated using primer 1 and primer 2 containing the two mutations shown below. The second mutation generated an *Eco*RI site. The second product was generated using primer 3, the reverse complement of primer 2, and primer 4 that primes in the intron between the C_H1 and C_H2 3′ to a *Sac*II site. The PCR products were sequenced and the *Sal*I-*Sac*II fragment containing the C_H1 of IgA2m(1) was cloned into the *α2(n)* gene to yield the *α2m(1)* gene.

To generate the cysteine mutants in C_H1, primers 1 and 5 containing the *Eco*RI site were used to generate a PCR product including C196S. Primers 6 and 4 were used to generate the PCR product with the C220S mutation. To generate the C199, 220S double mutant, a *Sal*I-*Eco*RI fragment containing the C196S mutation was ligated to the *Eco*RI-*Sac*II fragment containing the C220S mutation. To generate the hinge cysteine mutants, primers 1 and 7 and primers 1 and 8 containing either of the C241S or C242S mutations were used. The PCR products were cloned into the TA vector, sequenced, and cloned into BlueScript$^+$ containing the wild-type *IgA2(n)*. The complete constant regions were cloned into the pSV2gpt vectors containing the dansyl variable regions (11, 12). The constant regions in the expression vectors were sequenced again to confirm the presence of the mutations. Primers used were: 1, 5′-AACAGCTATGACCATG-3′; 2, 5′-CCTCTGACCTGGGCAGGGCACAGTCACATCCTGGCTGGGAATTCGTGTAG-3′; 3, reverse complement of primer 2; 4, 5′-CTGTCCACCACCACCTCCTGGGG-3′; 5, 5′-GCTGGAATTCGTGTAGTGCTTCACGTGGCATGTCACGGACTTGCCGTCTGGGGACTGTGTGGCCGG-3′; 6, 5′-TACACGAATTCCAGCCAGGATGTGACTGTGCCCTCCCGAGGTCAGAGGG-3′; 7, 5′-TCCTCGAGGGCCGGGCGGTGCAGCGACAGTCGGGGGTGGGAGCATGGGGGAGGTGGGGGAAC-3′; and 8, 5′-TCCTCGAGGGCCGGGCGGTGCAGCGACAGTCGGGGGTGGCAGGATGGGGGAGGTGGGGGAAC-3′.

Expression of the wild-type and mutant chimeric H chains in Sp2/0 myeloma cells

The H chain expression vector was transfected into Sp2/0 cells expressing the dansyl-specific chimeric κ L chain gene by electroporation (12). Approximately 6×10^6 cells were washed in cold 0.02 M PBS (pH 6.8) and incubated on ice for 10 min with 15 μg of DNA. Cells were pulsed with an electric field of 200 V and 960 μF in a Gene Pulser apparatus (Bio-Rad, Richmond, CA), washed once, resuspended in 12 ml of IMDM containing 10% FCS, 100 μg/ml gentamicin (Life Technologies, Grand Island, NY), and 100 U/ml nystatin (Life Technologies) and plated in 96-well tissue culture plates at 125 μl/well. After 2 days of growth, an equal volume of medium containing 15 μg/ml hypoxanthine, 250 μg/ml xanthine, and 6 μg/ml mycophenolic acid was added to the wells to select for mycophenolic acid-resistant colonies. After 2 wk, the surviving colonies were screened for Ab production by ELISA using microtiter plates coated with dansyl coupled to BSA with bound Ab detected by alkaline phosphatase-conjugated goat antiserum to human κ L chain (Sigma-Aldrich, St. Louis, MO). Color was developed by adding *p*-nitrophenyl phosphate (Sigma-Aldrich), and the absorbance at 410 nm was determined in a microplate reader (MR 700; Dynatech Laboratories, Chantilly, VA). Clones producing the highest quantities of Ab were expanded in IMDM containing 10% (v/v) BCS.

Large-scale cell culture and purification of IgA Abs

Transfectomas were cultured in roller bottles (BD Labware, Lincoln Park, NJ) in IMDM supplemented with 1% BCS (HyClone Laboratories) and 6 mM L-glutamine (Life Technologies). Supernatants were filtered to remove any cells and cell debris and supplemented with 10 mM phosphate buffer (pH 6.8), 0.45 NaCl, 0.02 M EDTA, and 0.02% NaN_3. Proteins were purified by affinity chromatography using AH-Sepharose beads coupled with the dansyl isomer 2-dimethylaminonapthalene-5-sulfonyl chloride (Molecular Probes, Eugene, OR). Bound Abs were eluted with *N*-(5-carboxypentyl)-2-dimethyl-aminonaphthyl-5-sulfonamide and concentrated and the hapten was removed by extensive dialysis against Tris buffer (pH 7.8) containing 0.45 M NaCl and 0.02% NaN_3. The concentration of proteins was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) and was confirmed by intensity comparison with an Ig standard of known concentration following SDS-PAGE and staining with Coomassie blue.

Biosynthetic labeling and pulse-chase analysis

To biosynthetically label transfectants with [^{35}S]methionine, 2×10^6 cells were washed twice, incubated at 37°C for 30 min in methionine-free medium (Mediatech, Washington, DC), and then incubated for 16 h with 1 ml of methionine-free medium containing 1% (v/v) FCS and 12.5 μCi of [^{35}S]methionine. For pulse-chase experiments, 2×10^7 cells were washed twice, incubated in 0.5 ml of methionine-free medium at 37° for 30 min, and then pulsed by adding 125 μCi of [^{35}S]methionine and incubating at 37°C. After a 5-min incubation, the chase was initiated by adding 10 ml of medium containing 10% BCS and 3 mg/ml (100×) of unlabeled methionine. At various times after the initiation of the chase, 1-ml aliquots of cells were removed and cooled on ice. Cells were pelleted by centrifuging at 13,000 × *g* for 30 s in the cold and lysed by incubation on ice for 30 min in 0.5 ml of 10 mM Tris buffer (pH 7.4) containing 1% (v/v) Nonidet P-40, 0.4% (v/v) deoxycholate, and 66 mM EDTA. The lysates were centrifuged at 13,000 × *g* for 5 min in the cold to remove any unlysed cells and cell debris.

To immunoprecipitate cytoplasmic IgA, lysates were incubated for 1 h on ice with 5 μl of rabbit anti-human α-chain (Sigma) and 2.5 μl of rabbit anti-human Fab followed by incubation for 10 min on ice with 75 μl of 10% fixed *Staphylococcus aureus* cells with surface protein A (IgG Sorb; Enzyme Center, Malden, MA). The bacteria with bound immune complexes were pelleted at 13,000 × *g* for 1 min through a 1-ml layer of 30% sucrose in 10 mM Tris buffer (pH 7.4) containing 0.5% Nonidet P-40, 0.2% deoxycholate, 33 mM EDTA, and 0.15% SDS. The pellet was washed twice with 10 mM Tris buffer (pH 7.4) containing 1% Nonidet P-40, 0.4% deoxycholate, 66 mM EDTA, and 0.3% SDS and once with H_2O. The pellets were resuspended in electrophoresis sample buffer, placed in a boiling water bath for 2 min, and centrifuged at 13,000 × *g* for 2 min and the supernatants were analyzed by SDS-PAGE.

[3] Abbreviation used in this paper: BCS, bovine calf serum.

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Two-dimensional gel analysis

The pattern of disulfide bonding in IgA was determined by two-dimensional "diagonal" electrophoresis (13). The proteins were separated by SDS-PAGE in the first dimension in 5% phosphate gels under nonreducing conditions. To determine the composition of the bands observed in 5% phosphate gels, the lanes were excised and reduced in 0.32 M DTT in sample buffer for 60 min at room temperature. The lanes containing the reduced proteins were placed above separating 12.5% Tris-glycine gels containing SDS and sealed with 1% (w/v) agarose in sample buffer. The reduced proteins were then separated by electrophoresis in the second dimension. The gels were fixed, dried, and exposed to Kodak XAR-5 film.

Western blotting

Five micrograms of wild-type or mutant IgA2(n) were separated by SDS-PAGE in phosphate-buffered 5% gels and transferred to a Millipore Immobilon-P membrane (Millipore, Bedford, MA) according to the method of Towbin et al. (14). Nonspecific sites were blocked by incubating the membrane for 2 h at room temperature in PBS containing 3% (w/v) BSA. The α-chain was detected by incubating for 1 h at room temperature with goat anti-human α-chain (Zymed, San Francisco, CA) diluted 1/5000 (v/v) in PBS containing 1% BSA. The bound goat Abs were detected following incubation for 1 h at room temperature with rabbit anti-goat (Sigma-Aldrich) conjugated to HRP diluted 1/5000 (v/v) in PBS containing 1% BSA, washing, and then exposure to ECL reagent (Amersham, Buckingham, U.K.). Nitrocellulose was exposed to Kodak Hyperfilm MP for 15 s. The L and J chains were detected using goat anti-human κ-chain conjugated to HRP (diluted 1/10, 000) and rabbit anti-human J chain kindly provided by Dr. K. Kobayashi (Hokkaido University School of Medicine, Sapporo, Japan; diluted 1:10, 000), followed by donkey anti-rabbit conjugated to HRP (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. For sequential blots, bound Abs were removed by incubating the blots for 10 min at 60°C in 60.4 mM Tris-HCl (pH 6.7) containing 100 mM 2-ME and 2% SDS. The efficiency of removal of the Abs was confirmed by exposure to film.

Results

Covalent structures in IgA2

IgA2 exists with three alternate H chains: α2m(1), α2m(2), and α2(n) (5). The C_H1 domains of IgA2(n) and IgA2m(2) are identical and differ from α2m(1) at two positions: they have Ser^{212} and Arg^{221} whereas α2m(1) has Pro^{212} and Pro^{221} (Fig. 1). The C_H1 domain of IgA2 has only four cysteine residues and lacks Cys^{133} that forms the disulfide bond with the L chain in IgA1. Nevertheless, only IgA2m(1) fails to efficiently form HL disulfides; in both IgA2m(2) and IgA2(n), disulfides link H and L chains. Our earlier studies had indicated that when Pro^{221} was changed to Arg in C_H1 in IgA2m(1), an HL disulfide formed, suggesting that, like what is seen for IgA1, the HL disulfide for IgA2 was located within C_H1 (2).

To further investigate the cysteine residues important for determining the covalent attachment of the L chain in IgA2, mutants of α2(n) that lacked Cys^{196}, Cys^{220} (data not shown), or both were generated. Surprisingly, the removal of these Cys residues did not impact the formation of HL disulfide-bonded molecules, and in the absence of both Cys^{196} and Cys^{220} covalently assembled IgA2(n) was seen (Fig. 2) with most of the protein present in the secretions as an H_4L_4J dimer with some H_2L_2 and free L chain also observed.

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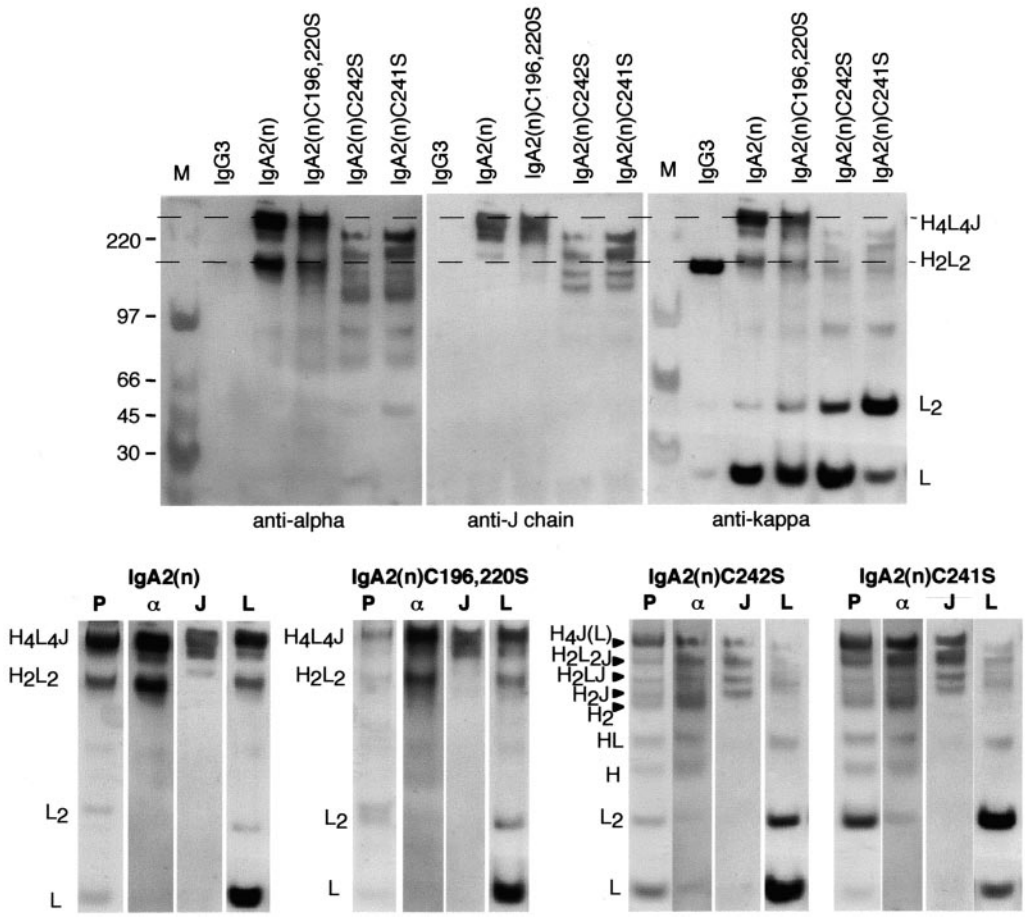

FIGURE 2. SDS-PAGE and Western blotting analysis of purified IgA. IgA2(n) and IgA2(n)C196,220S, IgA2(n)C241S, and IgA2(n)C242S were isolated by affinity chromatography. Five micrograms of protein was analyzed by SDS-PAGE under nonreducing conditions in 5% gels, proteins were transferred to a nitrocellulose membrane, and the H, L, and J chain were detected using anti-α-chain, anti-κ-chain, and anti-J chain antisera followed by secondary Abs conjugated to HRP. IgG3 (3 μg) was included for a control. At the *top* are shown the original membranes. Two lines were added to aid in aligning the different bands. At the *bottom* of this figure, the gels were cut and the different blots for each protein were aligned.

The mutant protein did not differ significantly from wild-type IgA2(n) in its assembly pattern, indicating that neither Cys^{196} nor Cys^{220} are required for disulfide bonding with L chains.

Since neither Cys^{196} nor Cys^{220} appeared to play a role in HL disulfide bond formation, our attention turned to other cysteine residues that might be required. Candidates were Cys^{241}, which had been reported to form a H-H disulfide in IgA1 and IgA2m(1), and Cys^{242}, which has been reported to form an intradomain disulfide bond with Cys^{299} (6, 7, 10). Surprisingly, mutation of either of these residues in IgA2(n) interfered with the formation of the HL disulfide bond (Fig. 2). Multiple molecular forms were isolated from the secretions of the two mutants. Especially noteworthy in IgA2(n)C241S and IgA2(n)C242S was the presence of multiple molecular species with covalently attached J chain. A distinguishing feature of the two proteins was that the free L chains in IgA2(n)C242S were mostly monomers, whereas virtually all of the noncovalently attached L chain present in the IgA2(n)C241S secretions was dimer. Even though most of the L chain is noncovalently associated with the two mutants, there are minor species of several sizes of assembled proteins with covalently attached L chains.

To further examine their assembly state, IgA2(n)C241S and IgA2(n)C242S were analyzed by two-dimensional gel electrophoresis, doing Western blot analysis with anti-J chain and anti-L chain in the second dimension (Fig. 3). At least five different molecular species with covalently attached J chain are seen. Especially striking is the covalent association of the J chain with nonpolymeric forms, including H_2. This is in contrast to what is seen with IgA2(n) and IgA2(n)C196,220S in which the J chain is seen covalently associated only with polymer (Fig. 2). A small portion of the L chain is covalently attached to H chains; some of these species also appear to have covalently associated J chain but it is difficult to precisely define the composition of these polymers since J and L chains are similar in molecular mass.

Covalent assembly pathway of IgA2m(1) and IgA2(n)

Pulse-chase analysis of IgA2(n) confirmed that there was efficient intracellular formation of an HL disulfide with nearly 45% of the total material synthesized present as HL by 15 min (Fig. 4). Thus,

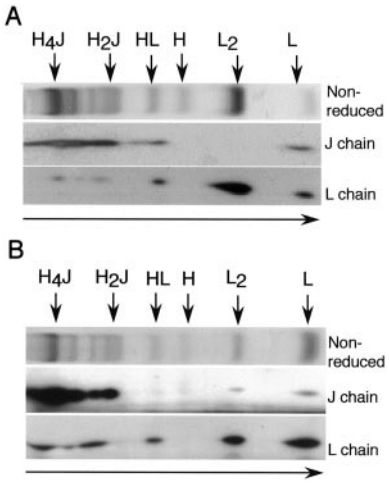

FIGURE 3. Two-dimensional SDS-PAGE and Western blotting analysis of purified IgA(n)C241S (*A*) and IgA(n)C242S (*B*). Purified proteins were separated in a 5% phosphate gel under nonreducing conditions in the first dimension (shown by the arrow at the bottom of each figure) and under reducing conditions in the second dimension and then the position of the L and J chains was determined by Western blot as described. The positions of selected assembly forms are noted. Note that the anti-J chain Ab cross-reacts weakly with reduced L chain.

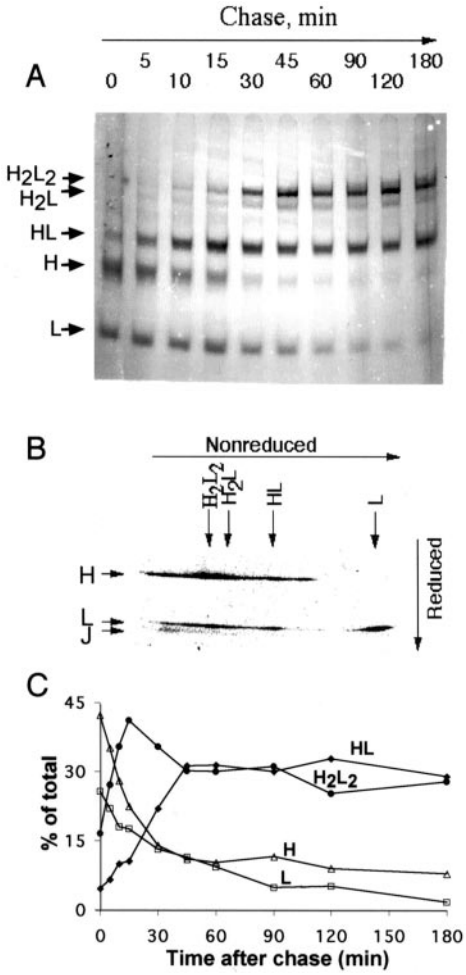

FIGURE 4. Assembly and kinetics of secretion of novel IgA2(n). *A*, Intracellular assembly. Transfectants were pulsed and chased as described in *Materials and Methods.* The immunoprecipitates from the cell lysates were analyzed by SDS-PAGE in a 5% phosphate gel under nonreducing conditions. The gels were dried and analyzed by phosphor imager and quantified using NIH image. The contrast in the photographs was enhanced to aid visualization of the bands using Adobe PhotoShop software. *B*, Two-dimensional SDS-PAGE analysis. The immunoprecipitate from the 120-min time point was separated in a 5% phosphate gel under nonreducing conditions in the first dimension and in a 12.5% Tris-glycine gel under reducing conditions in the second dimension. The position of the labeled protein was detected by phosphor imager analysis. Arrows indicate the direction of electrophoresis in each dimension. *C*, Graphic representation of the kinetics and pathway of intracellular assembly.

the major pathway of assembly was $H + L \rightarrow HL \rightarrow H_2L_2$ with a small amount of H_2L seen. The H_2L_2 molecules formed polymers with associated J chain. The composition of the bands was confirmed by separating the immunoprecipitate from the 120-min time point under nonreducing conditions in the first dimension, reducing it in situ and analyzing by SDS-PAGE in 12.5% gels in the second dimension (Fig. 4*B*). Secretion commenced at 30 min and by 180 min, 7% and 10 of the total counts were secreted as dimeric IgA and mIgA, respectively (data not shown).

Similar analysis of IgA2m(1) showed significant differences and some surprises (Fig. 5). Five minutes after the chase, a band migrating at the position of HL was observed (Fig. 5*A*); this band increased to 18% of the total by 15 min and then rapidly decreased. This suggested that in the presence of Pro^{221} some HL disulfides form but that these intermediates do not efficiently form H_2L_2 molecules. By 30 min, a band of M_r 130,000 constituted the major

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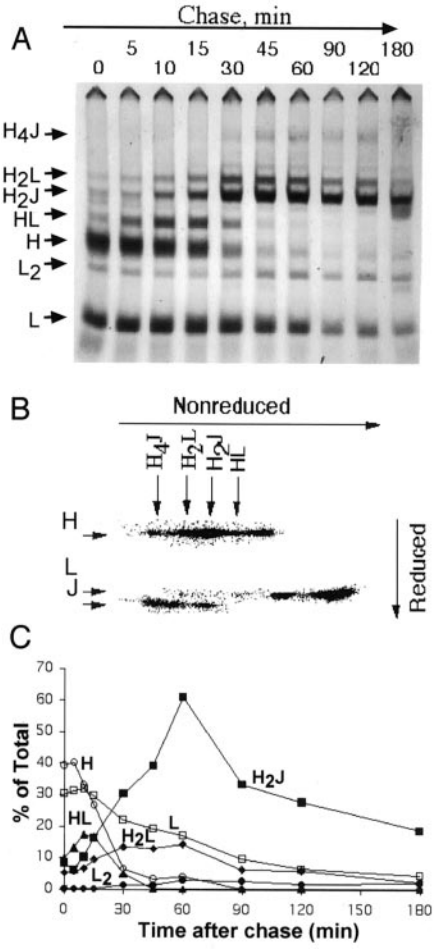

FIGURE 5. Assembly and kinetics of secretion of IgA2m(1). Transfectants secreting IgA2m(1) were pulse labeled and the intracellular IgA was immunoprecipitated as described in Fig. 4 legend. *A,* Intracellular assembly. *B,* Two-dimensional SDS-PAGE analysis. *C,* Graphic representation of the kinetics and pathway of intracellular assembly.

intracellular intermediate and remained as the major intermediate until 180 min. A band of M_r 150,000, comprising 18% of the total counts at 30 min, decreased in quantity when secretion commenced at 180 min. When the immunoprecipitate from 120 min after the chase was analyzed by two-dimensional electrophoresis, the band migrating at M_r 130,000 in the first dimension resolved into H chains, whereas the band at M_r 150,000 resolved into major bands corresponding to H and J chains (Fig. 5*B*). Therefore, the band of M_r 130,000 consists of mostly H_2 and the band of M_r 150,000 consists predominately of H_2J. Thus, IgA2m(1) resembles IgA2(n)C241S and IgA2(n)C242S in that J chains are covalently associated with H_2 molecules. The observation that there is an intracellular accumulation of H_2J in IgA2m(1) implicate it as a major intermediate in the synthesis of dimeric IgA. Together these results suggest that the major assembly pathway of IgA2m(1) is H + H$\rightarrow H_2$ with J chain association and further polymerization. Compared with IgA2(n), IgA2 m(1) with Pro^{211} is less efficient in the formation of disulfide-linked HL molecules and only small amounts of covalently associated L chain are seen in the polymers.

Discussion

Both intrachain and interchain disulfide bonds leading to the covalent assembly of multisubunit proteins are important in determining protein structure. The intrachain disulfide bonds form as a protein assumes its native conformation and function to stabilize its three-dimensional structure. The endoplasmic reticulum, unlike other cell compartments, has an oxidizing environment, making it possible for disulfide bonds to form as proteins proceed through the secretory pathway. Within the endoplasmic reticulum, disulfide isomerase catalyzes the oxidation of free sulfydryl groups to form disulfide bonds and almost all cysteine residues in secreted proteins are disulfide bonded.

The H chain of human IgA has 14 (IgA2) or 15 (IgA1) cysteine residues. These cysteines form intradomain bonds increasing IgA's stability and covalent bonds with other H chains, L chains, J chain, and secretory component. Within human IgG, cysteine residues within the hinge (IgG1) or C_H1 (IgG2, 3, and 4) form the covalent bond with the L chain. Consistent with this bonding pattern, Cys^{133} within the C_H1 domain of IgA1 has been found to be required for the covalent attachment of L chain and its mutation to the aspartic acid found at the same position in IgA2 results in molecules with only noncovalent interactions between the H and L chains (2). Molecular modeling predicts that Cys^{133} will fold into the proximity of the terminal cysteine of the L chain. These same studies showed that HL disulfide bond formation could be restored to IgA2m(1) by mutating the proline next to Cys^{220} to an arginine, suggesting that a cysteine within C_H1 may be responsible for forming the HL disulfide in IgA2. However, we have now shown that neither Cys^{196} nor Cys^{220} in C_H1 are required for the formation of the HL disulfide in IgA2; instead, absence of either Cys^{241} or Cys^{242} in C_H2 disrupts the formation of this disulfide. Thus, either Cys^{241} or Cys^{242} form the HL disulfide bond and alterations in the conformation of C_H1 by the presence of Pro^{220} affect the ability of this bond to form.

No crystal structure exists for IgA; however, molecular models of IgA1 have been published (15, 16). In these models, displacement of the C_H2 domains was required to accommodate the glycosylation and disulfide-bonding pattern leading to steric crowding around the N-terminal region of the hinge compared with IgG1. The disulfide bonds also restrict the movement of the C-terminal region of the hinge and N-terminal region of the C_H2. However, in IgA1 the extended proline-rich, *O*-glycosylated hinge region allows the Fab regions greater conformational freedom relative to the corresponding domains in IgG1 and places the L chain Cys used to disulfide bond with the H chain at a distance from Cys^{241}. In IgA2, the two Fabs are separated from the C_H2 by only nine amino acids. Consequently, the two Fab regions would be closer together in comparison to IgA1 (16) and the penultimate cysteine residues in the two L chains may be positioned closer to each other and/or in close proximity to the hinge cysteines. In IgA2m(1), the presence of Pro^{221} could form a kink in the α-chains, thereby making disulfide bond formation of the L chain with $Cys^{241/242}$ more difficult.

It is interesting that loss of either Cys^{241} or Cys^{242} disrupts HL disulfide bond formation, making it difficult to directly assign which Cys forms the bond. However, the disulfide-bonding patterns of IgA1 indicates that Cys^{242} and Cys^{299} form an intradomain bond and would therefore be in close proximity in the folded structure. Since Cys^{241} is next to Cys^{242}, it would also be in close proximity to Cys^{299} in the folded Ab. Intramolecular disulfide bond formation between cysteine residues in close proximity would be expected to occur more easily than would the intermolecular disulfide between H and L which requires that two chains that are independently synthesized must noncovalently associate to bring the two Cys residues into close proximity for the formation of the disulfide. It therefore seems likely that if only one Cys is present at either 241 or 242 it would participate in the intradomain disulfide bond. Cys^{301} used to form the H-H disulfide, is oriented

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into the space between the C_H2 domains (15), allowing a disulfide to form even when the Fab is in close proximity to C_H2. Cys^{311} is exposed on the surface consistent with its proposed role in forming a disulfide bond with the secretory component.

L chains contain five cysteine residues, four of which participate in intradomain disulfide bonds while the fifth can form a covalent bond with H chains or another L chain. For L chains to be secreted, the carboxyl-terminal Cys must either be covalently linked to a H or L chain or paired with a free cysteine (17). It is interesting that for IgA2(n)C242S, the majority of the secreted L chain is free, whereas for IgA2(n)C241S, most of the secreted L chain is dimeric. It is possible that the noncovalently associated L chains assume different conformations depending on whether the intradomain bond is between Cys^{241} and Cys^{299} or Cys^{242} and Cys^{299}. Presumably when the intradomain bond is between Cys^{242} and Cys^{299}, the penultimate cysteines in the L chains are brought into close proximity and efficiently form inter-L chain disulfide bonds.

J chain is associated with polymeric IgA and IgM (18). Analysis of human colostral IgA showed that dimers contained 1 mol of J chain for every two monomer units while the tetramers contained one J for every four monomers units (19). J chain contains eight Cys residues, six of which are involved in intrachain disulfide bridges while two are disulfide linked to two penultimate α-chain cysteines in an IgA dimer with the other penultimate Cys of the α-chain forming an intra-H chain bond (8). Substitution of either of the Cys residues of the J chain involved in disulfide bond formation with the α-chain yielded IgA monomers with covalent J chain (20). The disulfide structure is not consistent with previous models of the J chain structure (21, 22) but suggests that the J chain is positioned between two IgA dimers linked tail-to-tail. $C_\alpha3$ with the associated tail piece but not $C_\alpha2$ is required for J chain incorporation into IgA (23). It is therefore puzzling as to why novel forms with covalent J chain are seen in both IgA2(n)C241S and IgA2(n)C242S (Figs. 2 and 3). These forms cannot be solely the consequence of a failure to covalently attach a L chain since they are not present in IgA2m(1) (our unpublished observations).

Traditionally, IgA2m(1) has been described as being a protein lacking covalent bonds between H and L (24–26) while IgA1, IgA2m(2) (27), and novel IgA (5) have covalent HL disulfides. Our studies have shown that this is not strictly true. For IgA2m(1), some HL disulfide bonds do form, albeit only inefficiently (2), suggesting that in IgA2m(1) the cysteine residue involved in forming the HL disulfide is only partially accessible and the majority of the molecules fail to form this bond. For IgA2(n), some noncovalently associated L chains are also secreted (Fig. 2). Since the Abs were purified on Ag columns, this L chain was associated with the H chain during purification. It is interesting that in IgA2m(1) a significant amount of the noncovalent L chains are dimers (2), whereas in IgA2(n) most of the noncovalent L chains are monomers (Fig. 2), again suggesting that the noncovalent L chains assume different orientations in the two molecules.

In IgA2, while the shorter hinge may restrict the movements of the Fab regions to access Ags, it provides a functional advantage by being resistant to bacterial IgA1 proteases. This may explain why IgA2 is more abundant in the intestinal secretions where most of the bacteria reside. In addition, IgA2m(2) and IgA2(n) with covalently linked H and L chains may be more stable than IgA2m(1) in the milieu of the mucosal secretions with varying pH and salt concentrations.

In summary, we have determined the covalent assembly pathways in IgA and identified some of the cysteine residues and the structures in IgA that influence disulfide bond formation in IgA. The covalent assembly of IgA is expected to be essential for it to function efficiently in the hostile environment of the secretions of the respiratory, gastrointestinal, and genito-urinary tracts. Since disulfide bonds among H, L, and J chains are a major force in stabilizing the three-dimensional structure of IgA, the knowledge gained from these studies will enable us to better design and produce efficient immunotherapeutics based on IgA.

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