The Role of Constant Region Carbohydrate in the Assembly and Secretion of Human IgD and IgA1*

Received for publication, April 5, 2002, and in revised form, May 9, 2002
Published, JBC Papers in Press, May 22, 2002, DOI 10.1074/jbc.M203258200

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Immunoglobulins are glycoproteins, containing N-linked carbohydrates in the heavy chain constant regions of all isotypes and O-linked carbohydrates in the hinge regions of human IgA1 and IgD. A previous study showed that IgD synthesized in the presence of tunicamycin and lacking the three N-linked glycans on the heavy chain was not secreted (Shin, S. U., Wei, D. F., Amin, A. R., Thorbecke, G. J., and Morrison, S. L. (1992) Hum. Antibodies 3, 65–74). The contribution of each of the carbohydrates in the Fc of IgD to assembly and secretion was now analyzed by eliminating the carbohydrate addition sequence, Asn-X-Ser/Thr, through site-directed mutagenesis. Only the carbohydrate nearest the sole disulfide bond between heavy chains, which remained high mannose and appeared to be buried within the folded molecule, was found to be essential for secretion. When IgD lacked that glycan, assembly reached only the heavy/light chain half-molecule stage, and heavy chains were held inside the endoplasmic reticulum. Using benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADG) to inhibit complete O-linked glycosylation, we found that IgA1 and IgD with incomplete hinge carbohydrates were assembled and secreted from cells. Thus, one N-linked glycan plays a structural role in IgD and is required for proper assembly and secretion, but the O-linked carbohydrates in the hinge of IgD and IgA1 are not required for folding and export.

Like all immunoglobulin isotypes, human IgD and IgA1 are glycoproteins, containing N-linked carbohydrates on their heavy (H)1 chains. They are unique also in that they bear O-linked carbohydrates in the hinge region. The two types of glycans differ in several ways. Asparagine- or N-linked carbohydrates are added as large GlcNAc,Man,Glc precursors to proteins co-translationally in the endoplasmic reticulum (ER) prior to folding. O-linked glycans are assembled sequentially onto folded proteins in the Golgi apparatus, where N-glycans may continue to be enzymatically processed to their mature form (1). The consensus sequence for addition of the precursor carbohydrate to asparagine residues of nascent proteins is Asn-X-Ser/Thr, where X may be any amino acid except proline, and if Ser is in the third position, X may only rarely be Asp, Glu, Trp, or Leu (2–5). Less is known about the regulation of O-glycan addition to serine or threonine residues. There exists no defined addition sequen for the attachment of the first N-acetylgalactosamine (Galfac) residue, although through statistical methods some trends in the surrounding sequences have been identified (6, 7).

N-linked glycans are large and contain a pentasaccharide core derived from the precursor, consisting of two N-acetylgalactosamine (GlcNAc) and three mannose residues. To this may be attached mannose, glucose, galactose (Gal), and/or GlcNAc residues as well as sialic acid and fucose. Mature carbohydrates may be high mannose, hybrid, or complex. High mannose glycans are the least processed and resemble a trimmed version of the precursor. Hybrid glycans are trimmed to a greater degree, with some residues added to terminal mannoses. Complex carbohydrates are the most highly processed and appear in a variety of structures with a greater number of residues added to the pentasaccharide core. O-linked carbohydrates are comprised of a smaller, more diverse core consisting of a GalNAc-Gal pair or a GalNAc-GlcNAc and Gal attached via different possible linkages. More residues also may be present on the mature carbohydrate (8–10).

Glycosylation may play a role in protein stability, prevention or induction of aggregation, secretion, ligand/receptor recognition and binding affinity, activity, protease and heat resistance, and cell-cell interactions, among others (11–14). In particular, the carbohydrate present in the Fc of IgG has been shown to be important for effective activation of the complement cascade and recognition by Fc receptors (15–17).

Earlier reports have shown that N-linked carbohydrates may be necessary for assembly and secretion of IgA and IgD antibodies. Although murine IgA1 was not secreted when the glycosylation sites were removed by site-directed mutagenesis (18), the assembly and secretion of murine-human chimeric IgA1 was unaffected by removal of glycosylation sites (19). Human IgD bears three addition sites, Asn-354, Asn-445, and Asn-496 (20). Murine-human chimeric IgD was not secreted from a transfected myeloma cell line when produced in the presence of tunicamycin (Tm), an inhibitor of N-linked glycosylation. IgD lacking N-linked glycans was assembled into HL half-molecules rather than fully formed H2L2 antibodies (21), and these remained within the cell. Although this study did not identify the role of individual carbohydrate molecules, it was hypothesized that the glycan at Asn-354 may be critical for the proper conformation of the hinge region in which a single...
disulfide bond links the two heavy chains at Cys-290. In the absence of this glycan the conformation may have been altered, blocking assembly and resulting in retention in the secretory pathway.

We have now investigated the role of each N-glycosylated site in IgD assembly and secretion using Chinese Hamster Ovary (CHO) cell lines producing IgD proteins mutated to lack a single addition site, two sites, or all three. We found that the presence of carbohydrate at Asn-354, but not at Asn-445 or Asn-496, was required for secretory production. Using immunofluorescent staining and confocal microscopy, the heavy chains from an N354Q IgD mutant were found to remain in the ER and not to traffic to the Golgi apparatus. The carbohydrate at Asn-354 bound effectively by lectin-coated Sepharose resin, suggesting that it was buried between protein domains. Truncation of O-linked glycans in the hinge region of IgD and IgA1 using benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BADG) (22) did not inhibit secretion.

**EXPERIMENTAL PROCEDURES**

**Cloning, Mutagenesis, and Production of Marine-Human Chimeric IgD**—The initial human IgD heavy chain gene expression vector constructed in this laboratory (21) contained the IgD heavy chain gene in its genomic form with six exons. For the current study, a new IgD heavy chain vector was constructed in which the introns within the coding sequence of the constant region were removed using a combination of PCR and cloning. Additionally, the 5′-intron separating V\(_H\) from C\(_{H}1\) was shortened to 223 bases. The heavy chain gene contained the murine 27.44 bp V\(_H\) specific for the hapten dansyl (23) and the 3′-untranslated region from IgG3 (24), and the gene was inserted as an EcorV-BamHI fragment into the multiple cloning site of the expression vector pCDNA3.1 (+) (Invitrogen).

IgD mutants lacking N-linked glycosylation sites were made using nested PCR to produce point mutations in the Asn codons. Primers encoded an Asn → Gln substitution in all three cases plus a XhoI site near the substitutions at Asn-354 and Asn-445. Primer sets were designed as listed below. Asn-354 Accl-HindIII cassette: primer 1, 5′-CT-TGGC/GTCATACGCTGTAACC3′ and primer 2, 5′-GCCGTGCTG-GCCGCT/CTGAGGC3′; primer 3, 5′-GCTGCAAGCGGCAACGAGG- GC3′ and primer 4, 5′-CAGGTCCAGGGAAGCTT/GAAGC3′. Asn-445 HindIII-Fmlll cassette: primer 5, 5′-GCAGCT/CGTGTTG/GACG- GTG-3′ and primer 6, 5′-GAAGTGCTGCAATTACCTGCGT/GCTCAG- GC3′; primer 7, 5′-GCTGCAAGCGGCAACGAGG/GC3′ and primer 8, 5′-GGCA/CAGGT/TAGGTGGTGG-3′. Asn-496 Fmlll-EcoRI cassette: primer 9, 5′-CCA/GACTGTTG/GTCATAGC/AGGAGTCTCCAGGCTTCG/CTGCAG3′ and primer 10, 5′-GCA/GCT/GGAAATC/TCTCGG/GCCAGCAGGGCTTATC/ATGGC-3′. Restriction enzymes are caseins and boldtype, substituted bases are in boldface type, and Asn → Gln is underlined. PCR products were cloned into the TA Cloning Vector (Invitrogen) and analyzed by restriction enzyme digestes. Selected products were then sequenced using reagents and directions by PerkinElmer Life Sciences and the ABI Prism method. Data were collected at the UCLA Core Facility or at Laragen, Inc. (Los Angeles, CA). Mutant cassettes were reconstituted into the complete IgD heavy chain gene, generating sequences lacking one, two, or three carbohydrate addition sites.

Transfection of CHO cells was performed using Lipofectin reagent (Invitrogen). Cells first were transfected with a murine-human chimeric anti-dansyl \(\kappa\) chain expression vector described previously (17), generating a light chain producer. The anti-dansyl heavy chain constructs then were transfected into the light chain producer as described previously (17), with 10 \(\mu\)g of uncut H chain vector per Petri dish containing ~5 \(\times\) 10^5 adherent cells. After transfection, the cells were plated into six 96-well plates in 125 \(\mu\)l/well of Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum and either 0.5 or 0.05 mg/ml histidinol for selection on the light chain vector or 0.5 mg/ml zeocin (Invitrogen) for selection on the heavy chain vector. Surviving clones were screened subsequently by enzyme-linked immunosorben assay using either goat anti-human \(\kappa\) coated or dansyl-BSA coated plates coated with goat anti-human \(\kappa\) antibody conjugated with alkaline phosphatase (Sigma). Selected cell populations were subcloned by limiting dilution, and the clone producing the highest amount of IgD was selected by enzyme-linked immunosorben assay.

**Biochemical Synthetic, Immunoprecipitation, and Gel Electrophoresis**—For biosynthetic labeling, cells were washed with phosphate-buffered saline and incubated either 3–4 h or overnight in Dulbecco’s modified Eagle’s medium deficient in methionine, cysteine, and glutamine (Invitrogen) supplemented with 1 \(\mu\)l/ml [35S]methionine/[35S]cysteine and 0.5 mg/ml zeocin (Invitrogen) and assayed for secretion. For shorter incubations 5 \(\mu\)l/ml of 35S-labeled medium was used, and for shorter incubations 5 \(\mu\)l/ml of 35S-labeled medium was used. Following labeling, surviving clones were collected, cytoplasmic lysates were prepared, and these were immunoprecipitated as described previously (25) using goat anti-human IgD (Sigma) and rabbit anti-human Fab followed by rabbit anti-goat IgG (Sigma). Samples were analyzed by SDS-PAGE under non-reducing conditions using 5% phosphate gels or under reducing conditions, as described (25), using 8 or 12.5% Tris-glycine gels.

To inhibit complete O-linked glycosylation in transfected cells, 5 \(\mu\)M BAP (Sigma) was added to culture medium. Proteins containing complete O-linked carbohydrates were precipitated from unlabeled cell supernatants and cytoplasmic lysates using jacinol-coated agarose beads (Vector Laboratories, Burlingame, CA). Following this, the remaining immunoglobulins with truncated O-glycans containing only GalNAc were immunoprecipitated with 40–60 \(\mu\)l of Sepharose 4B (Amersham Biosciences). After IgD mutants were washed and eluted using the immunoprecipitation protocol above, Sepharose beads were washed three times with phosphate-buffered saline, pH 7.8, and eluted by incubating with 40–60 \(\mu\)l of 3 mM dansyl-lucine on ice followed by centrifugation. Samples were analyzed by SDS-PAGE, as described above, with the position of the proteins determined by Western blot (see below).

**Endoglycosidase H Hydrolysis**—Endoglycosidase H (Endo H) treatment of antibody samples was performed as described previously (26). Briefly, immunoprecipitates from the supernatants of ~2 × 10^6 cells were resuspended in 100 \(\mu\)l of Endo H Reaction Buffer (50 \(\mu\)M sodium citrate, pH 5.5, 2 \(\mu\)M phenylmethylsulfonyl fluoride, and 100 \(\mu\)M \(\beta\)-mercaptoethanol (\(\beta\)-Me). 25 \(\mu\)l of each sample was incubated overnight at 37°C with or without 6–9 units of Endo H (Roche Molecular Biochemicals). 5 \(\times\) sample buffer (125 mM Tris, pH 6.7, 1.5% SDS, 50 mM glycerol, and 20 \(\mu\)g/ml bromphenol blue) was added to each tube, and samples were heated in a boiling waterbath for 3 min to elute labeled molecules prior to analysis on a 12.5% Tris-glycine gel.

**Concanavalin A-Sepharose Binding of Glycosylated Antibodies**—5–10 \(\times\) 10^6 transfected cells were biosynthetically labeled overnight, supernatants were collected, and concanavalin A-Sepharose (Sigma) binding was performed as described previously (27). After the resin was removed by centrifugation, the supernatants were immunoprecipitated with anti-IgG or anti-IgD as described above. Antibodies eluted from the resin with 0.5 \(M\) methyl-\(\alpha\)-mannopyranoside also were immunoprecipitated with anti-IgG or anti-IgD. Immunoprecipitated antibodies from the original supernatants and from the ConA-Sepharose were analyzed by SDS-PAGE under reducing conditions as described above.

**Western Blot—**Antibodies in sample buffer containing \(\beta\)-Me were run on 8% Tris-glycine gels and transferred to polyvinylidene difluoride membranes (Immobilon-P®, Millipore Corp., Bedford, MA). Membranes were blocked with 5% nonfat dry milk in buffer containing 100 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20 and then probed with goat anti-human IgD or rabbit anti-human IgA (Sigma) followed by horse-radish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Sigma) or HRP-conjugated donkey anti-rabbit IgG (Promeaga, Madison, WI), respectively. All bands were visualized using Super Signal chemiluminescent substrate (Pierce) and Hyperfilm MP (Amersham Biosciences).

**Immunofluorescent Staining and Confocal Microscopy**—Wells of 8-chamber Permanox slides (Nalge Nunc, Naperville, IL) were seeded with CHO transfected cells in Iscove’s modified Dulbecco’s medium + 5% fetal bovine serum and incubated overnight. Cells were gently washed twice with phosphate-buffered saline and fixed in a freshly prepared solution of 0.01 M sodium metaperiodate, 0.075 M lysine, 0.0375 M NaH_2PO_4, pH 7.4, and 2% paraformaldehyde for 2–3 h at room temperature. Cells were rinsed three times with washing buffer, 0.5% IAbbreviations—\(\kappa\) chain: \(\kappa\) chain; Asn: asparagine; Badg: badamyl; ConA: concanavalin A; Fmlll: FokI; H: heavy chain; HAP: hyperactivity; HC: heavy chain; HRP: horseradish peroxidase; IgD: immunoglobulin D; IgG: immunoglobulin G; IgM: immunoglobulin M; IgA: immunoglobulin A; Invitrogen: Invitrogen; M: molecular weight; N: number; p: percent; SDS: sodium dodecyl sulfate; T: transfection; Tris: tris(hydroxymethyl)aminomethane; Tm: transition temperature; V: variable chain; V_{H}: variable region of IgH.
diluted to 1/30; and rabbit anti-Golgi β-coatomer protein (Affinity Bioreagents, Golden, CO), diluted to 1/75. Cells were then washed in Buffer B three times, and secondary antibody was added. Secondary antibodies (Texas Red-conjugated swine anti-goat IgG (EY Laboratories, San Mateo, CA) and FITC-conjugated swine anti-rabbit IgG (Nordic Immunology, Tilburg, The Netherlands)) were diluted 1/25 and 1/100, respectively, in Buffer B. Prior to adding diluted secondary antibodies to wells, the solutions were centrifuged to remove any antibody aggregates, which bind to cells directly and increase background fluorescence. Secondary antibody staining was performed at room temperature for 1 h, and the cells were washed. Chambers were then removed from each slide, and a drop of Prolong (Molecular Probes, Eugene, OR) was laid over each group of cells to reduce quenching of dyes followed by a coverslip, which was sealed to the slide.

RESULTS

Production of Wild Type IgD and Mutant Antibodies—The heavy chain gene used for the expression of murine-human chimeric anti-dansyl IgD is shown in Fig. 1. The gene has a shortened intron separating V H and C H1 and no introns within the constant region. The 3′-untranslated region was derived from IgG3, an antibody produced at high levels in transfectants. Mutant IgD molecules were generated using nested PCR with the heavy chain gene template. Glutamine was substituted for asparagine in each N-glycan addition sequence. Three cassettes, each encompassing a portion of IgD with a mutated addition site, were used to replace wild type sequences, yielding IgD genes with one, two, or all three glycosylation sites mutated.

Glycosylation of Asn-354 Is Required for Complete IgD Assembly and Secretion—To determine the contribution of each of the individual N-linked glycans on IgD to its assembly and secretion, we analyzed wild type IgD and mutants missing glycosylation sites at residues 354, 445, or 496 and combinations thereof. Transfectants expressing wild type or mutant IgD were biosynthetically labeled for 3–4 h, cytoplasmic lysates were prepared, and IgD was immunoprecipitated. Analysis of non-reduced samples by SDS-PAGE (Fig. 2A) showed that all antibodies assembled into HL half-molecules but that only those with heavy chains bearing carbohydrate at site 354 assembled into H L L L antibodies (lanes 2, 4, 5, and 7). Analysis of IgD immunoprecipitated from cell supernatants following overnight labeling showed that only antibodies with asparagine at site 354 of the H chain were secreted as H L L L species (Fig. 2B). Small amounts of HL half-molecules also were present in secretions along with free light chain and light chain dimers. Unexpectedly, we also observed that all heavy chains with carbohydrate at Asn-445 exist as two glycoforms (Fig. 2C), suggesting that the site at 445 is variably glycosylated. The observed band pattern consistent with variable glycosylation of Asn-445 is diagrammed in Fig. 2D. Incompletely Assembled IgD Is Retained in the ER—Immunofluorescent staining was used to determine the intracellular localization of non-secreted IgD HL half-molecules (Fig. 3). Cells fixed to slides were permeabilized with saponin and probed with goat anti-IgD and rabbit antiserum specific for an MR major glycoprotein or the Golgi β-coatomer protein and then stained with either Texas Red-conjugated swine anti-goat or FITC-conjugated swine anti-rabbit secondary antibodies. Comparison of the transfectant stably expressing wild type IgD with that expressing the N354Q heavy chain mutant showed that wild type IgD heavy chain is found in both the ER (panel C) and the Golgi apparatus (panel F), whereas the mutant is found in the ER (panel I), but not in the Golgi apparatus (panel L). Thus, it appears that the heavy chain lacking the hinge-proximal glycan can assemble into HL half-molecules, but these are retained in the ER and do not proceed any further along the secretory pathway.

The Carbohydrate Attached to Asn-354 Is High Mannose in Wild Type and Mutant Antibodies—It has been reported that the carbohydrates at Asn-354 is high mannos in wild type IgD (28). To determine whether the Asn-354 oligosaccharide processing was also minimal when other heavy chain glycans were absent, cells synthesizing wild type IgD or mutant IgD containing carbohydrate at Asn-354 were biosynthetically labeled. The secreted IgD was immunoprecipitated and digested with Endo H. We observed that all were Endo H-sensitive (Fig. 4). Therefore, the glycan at Asn-354 remains high mannos in antibodies with glycans missing at sites 445, 496, or both as well as in the wild type. The glycan at site 445 appears to remain complex when its neighbor at site 496 is missing, whereas the mutant is found in the ER (panel I), but not in the Golgi apparatus (panel L). Thus, it appears that the heavy chain lacking the hinge-proximal glycan can assemble into HL half-molecules, but these are retained in the ER and do not proceed any further along the secretory pathway.

The Carbohydrate Attached to Asn-354 Is Sequestered within the Folded IgD Molecule—After demonstrating that the carbohydrate attached to Asn-354 is necessary for proper assembly of IgD, we used ConA to investigate whether carbohydrate is sequestered within the folded protein. ConA is a lectin that preferentially binds high mannose sugars but also recognizes complex, bi-antennary oligosaccharides (29). ConA-Sepharose effectively precipitated antibody bearing high mannose carbo-
hydrate in the V region (Fig. 5, lanes 3 and 4) but not IgA1 lacking N-linked carbohydrates (lanes 1 and 2), although the presence of a small amount of protein in lane 1 indicated that ConA-Sepharose beads bound some IgA1 non-specifically. Wild type IgD, which bears Asn-354 high mannose glycan and two complex glycans as well, was bound by the ConA-Sepharose (lanes 5 and 6). However, the IgD mutant with only the Asn-354 high mannose carbohydrate failed to be effectively and specifically bound by ConA-Sepharose at site 354. This suggested that the carbohydrate at site 354 is buried within the folded antibody and is not available on the surface for binding by the lectin.

IgA1 and IgD Lacking O-Linked Glycans Are Secreted—To determine whether the O-linked glycans that are attached to

Fig. 2. SDS-PAGE analysis of IgD containing mutations at N-linked carbohydrate addition sites. Biosynthetic labeling was performed with CHO cell transfectants expressing wild type or mutant proteins or κ light chain only. Supernatants were collected after overnight labeling of 1 × 10⁶ cells with [³⁵S]Met and [³⁵S]Cys. Cell lysates were prepared after labeling 5 × 10⁶ cells with [³⁵S]Met and [³⁵S]Cys for 3 h. Immunoprecipitation was performed using antisera specific for IgD and for IgG Fab (including κ light chain). Immunoprecipitates of cell lysates (A) or supernatants (B) were run on 5% PO₄ gels under non-reducing conditions. Immunoprecipitates from cell lysates (C) were reduced with β-Me and run on a 12.5% Tris-glycine gel. The band pattern observed in C is diagrammed in D.

Fig. 3. Intracellular localization of wild type IgD and an IgD N-linked carbohydrate mutant. Transfected CHO cells were grown on microscope slides, fixed, permeabilized, and stained to determine whether IgD proteins were associated with the endoplasmic reticulum and the Golgi apparatus. Cells were probed with goat anti-human IgD and rabbit anti-merger (panels A–C and G–I) or rabbit anti-hamster Golgi β-coatomer protein (panels D–F and J–L) followed by Texas Red-conjugated swine anti-goat IgG and FITC-conjugated swine anti-rabbit IgG (all panels). The left panels show Texas Red-labeled IgD, the center panels show FITC-labeled organelles, and the right panels show a merged view of these. Panels A–F show cells expressing wild type IgD, and panels G–L show cells expressing N354Q mutant IgD.

Fig. 4. The carbohydrate at Asn-354 is high mannose in wild type and mutant IgD heavy chains. Transfectant CHO cells expressing wild type IgD or secretion-competent N-linked carbohydrate mutants of IgD were biosynthetically labeled overnight. Secreted proteins were immunoprecipitated with anti-IgD and anti-IgG Fab antibodies and then resuspended in Endo H Reaction Buffer and incubated at 37°C overnight. Samples were analyzed by SDS-PAGE on a 12.5% Tris-glycine gel.

the hinge region of IgD and IgA1 are necessary for antibody secretion, transfectedants producing each isotype were grown in the presence of BADG, an inhibitor of full-length O-glycosylation. Supernatants were collected and cytoplasmic lysates prepared after overnight incubation with or without the drug. Sequential precipitations were performed with each sample,
first with jacalin-coated agarose to bind antibodies containing complete O-glycans, followed by Sepharose 4B coated with dansyl-BSA, for which the antibody variable region is specific. Precipitated proteins were reduced, separated by SDS-PAGE, and transferred to membranes that were probed with anti-IgA (Fig. 6A) or anti-IgD (Fig. 6B). Wild type IgA1 binds to jacalin (Fig. 6A, lane 1), whereas IgA1 with truncated O-glycans does not (lane 2). Although IgA1 lacking complete O-glycans was not bound by jacalin-agarose, it was indeed present in the supernatant and bound to dansyl-BSA Sepharose (lane 4). Under the conditions of the experiment, jacalin-agarose did not remove all antibodies from the supernatant of untreated IgA1 transfectants (lane 3). However, in a subsequent experiment involving three sequential precipitations of supernatant containing wild type IgA1 with jacalin-agarose resin, no IgA1 remained after the second precipitation (data not shown). Therefore, it appeared that all of the IgA1 synthesized by cells not treated with BADG was O-glycosylated.

Similar results were obtained for IgD (Fig. 6B). However, jacalin-agarose bound less than half of the total IgD-bearing O-glycans (lanes 1 and 3). Sequential precipitations with jacalin-agarose, in an experiment like that described above, showed that no IgD remained after the second precipitation, indicating that all wild type IgD was indeed O-glycosylated (data not shown). Interestingly, a comparison of the ratio of the signal seen in lanes 3 and 7 with those in lanes 4 and 8 suggests that less IgD was secreted in the presence of BADG. Therefore, although the O-linked carbohydrates in the hinge of IgD may not be absolutely required, they may facilitate its assembly and secretion. Analysis of cell lysates shows little or no O-glycosylated IgA1 or IgD present prior to secretion (Fig. 6, A and B, lanes 5), consistent with O-glycosylation taking place in the Golgi apparatus. Also, two N-linked glycoforms are visible for IgA1. We occasionally observed variable processing of the N-linked carbohydrates on the heavy chain from this transfectant. Analysis of the intracellular assembly of IgA1 and IgD showed that it was not affected by the presence or absence of complete O-glycans (data not shown).

To further analyze the role of glycosylation in IgA1 secretion, cells were biosynthetically labeled overnight with BADG and/or Tm added to the medium. IgA1 lacking complete O-linked sugars, N-linked sugars, or both is secreted (Fig. 6C). The thin non-specific band migrating below IgA1 (lanes 1 and 2) and above IgA1 (lanes 3 and 4) serves as a marker to discern the small differences in size between each IgA1 glycoform. The apparent molecular weight of the IgA1 heavy chain is largest in lane 1 with a reduction in size observed in successive lanes, representing truncation of O-linked glycans (lane 2), loss of N-linked glycans (lane 3), or both (lane 4). In cell lysates, no change in apparent molecular weight is observed when O-glycosylation is altered, consistent with its being a late event occurring just prior to secretion. Similar analysis of a wild type IgD transfectant showed that antibodies produced in cells treated with BADG and lacking complete O-glycans were secreted, whereas no IgD was secreted in the presence of tunicamycin, as expected (data not shown).

**DISCUSSION**

Previously we observed that in transfectants expressing murine-human chimeric IgD, treatment with Tm halted assembly of antibodies at the HL half-molecule stage, and IgD was not secreted. Tm inhibits all N-linked glycosylation in the cell and prevents carbohydrate addition to all three sites in IgD (30). Therefore, the earlier study could not address the question of whether alterations in the cell machinery were responsible for
the failure in IgD assembly or if the block in assembly was a result of a failure to glycosylate IgD; the study could not define the roles of the three carbohydrates. Studies have demonstrated that in proteins bearing multiple glycans, the lack of some may inhibit assembly, secretion, or function, whereas the lack of others has no effect (31–33). In order to inhibit N-glycosylation of only particular positions within IgD, the N-linked glycosylation sites in the heavy chain were removed singly and in combination by site-directed mutagenesis (Fig. 1). In this way, it was possible to ascertain the relative importance of each carbohydrate in the assembly of IgD and to eliminate any contributions from alterations in the cellular glycosylation machinery.

Human IgD is synthesized at low levels and is not abundant in the serum, representing 0.25% of total immunoglobulins (34). We observed that secretion from Sp2/0 cells transfected with expression vectors was also low (21), and we attempted to increase production by reducing the size of the expression vector, replacing the heavy chain promoter with that of CMV, and exchanging the 3′-untranslated region for that from the IgG3 gene. Although protein production showed a small increase, it remained low for both wild type IgD and the glycosylation mutants compared with what we routinely observe with other isotypes.

We observed the hinge-proximal glycan at Asn-354, but not those at Asn-445 and Asn-496, was required for proper assembly of the molecule (Fig. 2). Only one disulfide bond, located within the hinge at Cys-290, links the IgD heavy chain. In an earlier study, we showed that the order of assembly for IgD is 2 HL → H2L2 (21). It appears that assembly is arrested between these two steps when the Asn-354 carbohydrate is missing. The hinge-proximal glycan at Asn-354 of each heavy chain may be required to obtain the proper conformation for the formation of the interheavy chain bond, and its absence might be sufficient to inhibit assembly and secretion. We did observe the presence (to varying degrees) of HL half-molecules in the secretions of all transfectants (Fig. 2B). This may reflect cell lysis during overnight biosynthetic labeling of cells, resulting in leakage of cytoplasmic material into the supernatants. It also is possible that HL half-molecules may be non-covalently assembled into H2L2 species that are secreted. This has been observed with human IgG4, in which all secreted molecules appear to be H2L2 when examined under non-denaturing conditions; however, SDS-PAGE analysis under non-reducing conditions shows that a portion are only HL covalent species (25, 35, 36).

Other examples of particular glycans on multiply glycosylated proteins being critical for secretion or expression at the cell surface have been described in detail. Human lipoprotein lipase contains glycan addition sites at positions 43, 257 and 359. Substitutions of alanine for asparagine at sites 257 and 359 were shown to minimally affect activity of the enzyme produced. However, loss of glycan at position 43 resulted in production of an enzymatically inactive protein, which accumulated intracellularly and was not secreted (37). The human transferrin receptor (TIR) contains three N-linked oligosaccharides at Asn-251, Asn-317, and Asn-727. When these three addition sites were removed, the aglycosylated TIR expressed in murine NIH-3T3 cells showed a reduced ability to form dimers and reduced expression at the cell surface (38). A subsequent study demonstrated that abolishing the first two glycosylation sites did not affect expression of the TIR at the cell surface; however, the glycan at site 727 appeared to be required (39). Hayes et al. (40) showed that carbohydrates attached to the TIR were high mannose at Asn-727, complex at Asn-251, and hybrid at Asn-317. They postulated that carbohydrates that are less critical for proper glycoprotein folding may be more highly processed because of their location on the surface and therefore accessibility to Golgi complex glycosidases and glycosyltransferases. A carbohydrate with a more structural role (i.e., involved in the folding of a molecule) might be sequenced from these enzymes by the protein backbone or possibly accessory proteins, and therefore may remain high mannose in structure.

Mellis and Baenziger (28) have reported that glycans found on human IgD at Asn-445 and Asn-496 are complex, whereas the glycan at Asn-354 is high mannose (28). We have confirmed that the glycan at Asn-354 remains high mannose even when it is the sole carbohydrate in the Fc. Previously, the high mannose carbohydrate was found to be Endo H-sensitive under non-reducing conditions (21), suggesting that the glycan might be on the surface of the molecule. However, here we found that IgD containing only the oligosaccharide at Asn-354 was not bound by ConA, suggesting that it is not on the surface but is buried within the molecule (Fig. 5A). The ConA-binding experiments were performed at neutral pH, whereas Endo H hydrolysis takes place at acidic pH. It is possible that the non-neutral pH of the earlier experiment altered the conformation of IgD, rendering the glycan at Asn-354 susceptible to cleavage. It is interesting to note that it is a high mannose carbohydrate that plays the critical role in assembly of IgD, as it does in the TIR.

We also have observed variable glycosylation of IgD at Asn-445 with a doublet of bands seen on SDS-PAGE for all proteins containing Asn-445. We know that some N-linked glycosylation sequences are never utilized (2, 41, 42), although the reasons for this are poorly understood. There are also other examples of sites being variably utilized (43–45), and different protein glycoforms may have different activities (13, 45, 46). This variability in activities may reflect carbohydrate-induced conformational differences that affect ligand binding and enzyme activity or may be a consequence of steric hindrance in the proximity of an active site or charge alterations on a protein. Variable N-linked glycosylation also may lead to different clearance rates by the asialoglycoprotein receptor (46). It is unclear at this time what function the variable usage of site Asn-445 on IgD might serve, in part because the role played by IgD in the humoral response has not been well defined.

In another early study we showed that IgD lacking N-linked glycans formed HL half-molecules, which were slowly degraded intracellularly (21). Using immunofluorescent staining and confocal microscopy, here we showed that IgD lacking the glycan at Asn-354 was present in the ER and not in the Golgi apparatus, whereas wild type IgD was present in both locations (Fig. 3). Misfolded proteins have been shown to aggregate in the ER, often cross-linked by aberrant disulfide bonds, and bound non-covalently but stably by the heat shock family protein BiP/GRP78 (38, 47, 48). Aggregated proteins retained in the ER are degraded eventually by the proteasome after retrograde translocation into the cytosol (49, 50).

BADG is a derivative of GalNAc and serves as a competitive inhibitor of β-(1,3)-galactosyltransferase, thereby blocking the elongation of O-linked carbohydrates beyond the addition of the first GalNAc to Ser or Thr residues (22), which results in truncated O-linked carbohydrates. Using BADG, we found that complete O-linked glycans attached to the hinge regions of IgA1 and IgD are not required for assembly and secretion, although IgD appears to be less efficiently secreted in the absence of complete O-glycosylation (Fig. 6). With jacalin-agarose, several sequential precipitations were required to remove all of the molecules with complete O-linked carbohydrates (Fig. 6 and data not shown). The number of O-linked glycans on both IgA1 and IgD is heterogeneous, and it is possible that the
affinity of jacalin for a molecule increases with the number of O-glycans it contains. Antibodies with more O-linked glycans might bind with higher affinity and may be precipitated more efficiently than those with fewer. Additionally, some IgA1 molecules, such as IgA1(Kni) myeloma protein (51), have been reported to lack any O-linked carbohydrate, although in the present study all of the IgA1 could be precipitated by jacalinagarose. IgD bears four or five O-linked trisaccharides, and IgA1 generally bears three to five (52). Although they may not be required for assembly and secretion, they may be necessary for maintaining the proper conformation of the membrane-bound and secreted IgD and IgA1, which have long hinge regions compared with other human isotypes. O-linked carbohydrates serve to confer rigidity to long straight-chain mucins (53, 54), and they may serve the same purpose here, preventing the Fab and Fc regions from folding over each other and interfering with effector interactions in which the Fc participates.

Although N-linked glycans are not necessary for production of human IgA1 (19), they are necessary for secretion of murine IgA (18). Because murine and human IgA1 sequences differ, the contributions made by N-linked glycans to their assembly and secretion might also be different. The two proteins differ both in amino acid sequence and in the positioning of carbohydrates, with human IgA1 containing glycans in CH2 and CH3 and murine IgA in CH1 and CH2. It is unclear at this time exactly why carbohydrates on murine IgA but not on human IgA1 play a structural role critical for the proper assembly of the antibody. Although human IgD requires carbohydrate at Asn-354 for assembly and secretion, murine IgD is secreted in the absence of N-linked glycans (55). The difference in a requirement for glycosylation is perhaps not surprising, given the considerable differences in IgD structure between the species, with murine IgD being shorter than its human counterpart by an entire domain.

Glycosylation is an important post-translational modification of antibodies. Both O- and N-linked glycans can contribute to various antibody functions. In addition, some carbohydrates are critical for antibody assembly and secretion. With increased knowledge regarding the exact nature of the contributions made by glycosylation to both synthesis and function, it may be possible to design better antibodies with improved characteristics for therapeutic and other applications.

Acknowledgments—We thank Ryan Trinh for help in cloning the wild type IgD heavy chain gene and Dr. David Meyer at UCLA for kindly providing the rabbit anti-MERG antibodies.

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