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V Region Carbohydrate and Antibody Expression

François A. Gala and Sherie L. Morrison

N-Linked carbohydrates are frequently found in the V region of Ig H chains and can have a positive or negative effect on Ag binding affinity. We have studied a murine anti-α(1→6) dextran V_H that contains a carbohydrate in complementarity-determining region 2 (CDR2). This carbohydrate remains high mannose rather than being processed to a complex form, as would be expected for glycans on exposed protein loops. We have shown that the glycan remained high mannose when murine-human chimeric Abs were produced in a variety of cell types. Also, when another carbohydrate was present in CDR1, CDR2, or CDR3 of the L chain, the V_H CDR2 glycan remained high mannose. Importantly, we found that when the anti-dextran V_H CDR2 replaced CDR2 of an another carbohydrate was present in CDR1, CDR2, or CDR3 of the L chain, the V_H CDR2 glycan remained high mannose. We have shown that the glycosylation site was used, but H chains were withheld in the endoplasmic reticulum and did not traffic to the Golgi apparatus. These results suggest that inappropriate V region glycosylation could contribute to ineffective Ab production from expressed Ig genes. In some cases, a carbohydrate addition sequence generated by either V region rearrangement or somatic hypermutation may result in an Ab that cannot be properly folded and secreted. The Journal of Immunology, 2004, 172: 5489–5494.

All Iggs are glycoproteins, with N-linked oligosaccharides attached in the Fc region. Human IgG contains one carbohydrate at Asn^297 in the C_H2 domain; however, the average number of carbohydrates on serum IgG is 2.8 (1), reflecting the fact that carbohydrate is frequently attached to the V region of the Ab. The consensus sequence for attachment of carbohydrate is: Asn-X-Ser/Thr, where X may be any amino acid except proline (and if Ser is in the third position, may only rarely be Asp, Glu, Trp, or Leu) (2–5). Although only a few germline V region genes contain the sequon in complementarity-determining region 2 (CDR2)^4 or framework region 3 of the H chain (6), it may be generated during the process of affinity maturation, when somatic hypermutation of V region genes occurs (7). Studies have shown that carbohydrate in the Ag-binding site can influence the affinity of the Ab-Ag interaction (8, 9). Carbohydrate found at the Ag-binding interface may increase affinity by increasing hydrophilic interactions between Ab and carbohydrate epitopes; alternatively, it may decrease affinity through stearic hindrance of the interaction of the Ab with Ag.

Asparagine- or N-linked carbohydrates are added to nascent proteins as they enter the lumen of the endoplasmic reticulum (ER). A dolichol (lipid)-linked precursor carbohydrate consisting of a branched GlcNAc2Mann3Glc3 structure is transferred from the ER membrane-bound dolichyl pyrophosphate to a target asparagine residue. It then undergoes enzymatic modification by glycosidases and glycosyltransferases in the ER and Golgi apparatus. Mature carbohydrates may be high mannose, hybrid, or complex. High mannose glycans undergo the least processing, yielding a trimmed version of the precursor, with only terminal mannose units. Hybrid carbohydrates are trimmed to a greater extent and contain some terminal mannose residues, as well as other terminal sugars. Complex carbohydrates are processed to the greatest degree as they migrate through the ER and Golgi complex. They often contain terminal fucose and/or sialic acid molecules and may take one of several different structural forms: biantennary, branched, or bisected.

The factors governing the extent of carbohydrate processing at each site on a glycoprotein have not been fully elucidated. It has been proposed that glycans located on exposed protein loops, rather than sequestered within β-sheeted pleats, will be more highly processed, because they are more accessible for enzymatic modification (10). However, additional factors such as primary sequence information, which might regulate the degree of processing, remain unclear at this time.

The murine anti-α(1→6) dextran V_H gene 14.6b.1, used in previous investigations of V region glycosylation, was found to contain a complex N-linked carbohydrate at Asn^58 in CDR2 (9). The V_H gene from another hybridoma, 19.22.1, is identical except that it contains Asn at position 60 instead of Thr and lacks the glycosylation sequon. Site-directed mutagenesis of CDR2 (Lys^62→Thr) of 19.22.1, resulted in a glycosylation site at Asn^60 (11). Interestingly, the carbohydrate attached at Asn^60 was high mannose. Asn^58 and Asn^60 are near each other on the exposed loop of CDR2, and consequently, the glycans attached to both would be expected to be complex. That they differ suggests that other factors in addition to surface exposure play a role in determining the extent of processing of carbohydrate precursors.

Using the V region gene of the 19.22.1 mutant as a model (referred to here as Asn^60 V_H), we addressed the question of whether processing of the glycan attached to Asn^60 is influenced by its context. The environment was altered in several ways. The Asn^60 V_H H chain was expressed with three anti-dextran L chains containing carbohydrate in their CDRs, and in three different cell lines. The CDR2 sequence of the Asn^60 V_H was also used to replace

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4 Abbreviations used in this paper: CDR, complementarity-determining region; ER, endoplasmic reticulum; GlcNAc, N-acetylglucosamine; Tn, tunicamycin; TG, Tris-glycine; MERG, major ER glycoprotein; β-COP, β-coatomer protein.

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CDR2 of a murine V region gene 27.44 (12) specific for the hapten dansyl, to determine whether the V region context would influence the processing of the carbohydrate within V{subscript}H CDR2. We found that the carbohydrate attached to Asn{superscript}60 within the anti-dextran V region was, regardless of cell type or surrounding environment, processed only to the high mannose form. Surprisingly, we found that placing the CDR2 from the Asn{superscript}60 C{subscript}H4 in the anti-dansyl C{subscript}H4 resulted in an Ab that was not secreted. This was shown to be a consequence of the presence of the V region carbohydrate, because aglycosylated Abs produced in the presence of tunicamycin (Tm) were secreted.

Materials and Methods

Mutagenesis and cloning of anti-dextran Abs

The murine anti-(1-6)-dextran V{subscript}H chain gene obtained from hybridoma 19.22.1, mutated to bear carbohydrate at Asn{superscript}60 (11), was amplified by PCR as a NotI-NheI insert to be ligated with the NheI-NorI backbone of a pdCNA3.1(−) expression vector (Invitrogen, Carlsbad, CA) containing the human IgG1 H chain gene. The oligonucleotide primers used were as follows: forward 5'-GGGGCGGCGCCACCATGGAGACGCGG-3' and 5'-CCCGGCTAGCTCGAGACATGGTGACCGAG-3', encoding NorI and NheI sites in bold, respectively. All primers used in this study were prepared by Life Technologies/BRL (Grand Island, NY).

A hybrid V{subscript}H was made by preparing two cassettes, which together encoded a V{subscript}H anti-dansyl gene with a CDR2 sequence from the anti-dextran Asn{superscript}60 V{subscript}H. The murine 27.44 anti-dansyl V region gene (12) was used as a PCR template, and each product was cloned into the TA Cloning Vector (Invitrogen). The first was an EcoRV-Scal fragment and the second was a Scal-NheI fragment, and the hybrid V{subscript}H was assembled by cutting with Scal and ligating the two V{subscript}H fragments. The hybrid V{subscript}H was then placed as an EcoRV-NheI fragment in the human IgG1 expression vector described above. The primers used were as follows: 5'-GGGGATCTACCATGC TACTTGGGAGCTGAA-3' and 5'-GGGAGTACTACACTCAG GAAATTTCAGCAACACCACCTCAAG-3'; and 5'-GGTAGCTACTA ACTACAAAGGACGTTCAAAGGGATGACATCCTCAAGA-3' and 5'-GGGCTTCTGACGACATGACGACAGG-3', with EcoRV, Scal, and NheI sites in bold. Thus, the following amino acid sequence (from the anti-dextran Asn{superscript}60 V{subscript}H) replaced a 50-65 of the anti-dansyl V{subscript}H: EILPGSSTYNETF, with the N-linked glycosylation sequence underlined.

A murine/human chimeric anti-dansyl κ L chain expression vector was constructed by first removing the hybridoma 27.44 κ C region gene from vector pS184/H3Ho-κH3 (13) and placing it into pdCNA3.1(−). PCR using the template 27.44 V{subscript}κ gene as template yielded most of the V{subscript}κ region and the beginning of the C region as an EcoRV-BbsI fragment. This partial V{subscript}κ C region product was joined to the remainder of the C{subscript}κ gene in pdCNA3.1(−). The PCR primers used were as follows: 5'-GGGCTAGT CATT-3' and 5'-ACTGTCGAGCTCAGCTCAGCTC-3', with EcoRV and BbsI sites, respectively, in bold (the EcoRV site is complete when joined with a cytosine in the template).

Previously engineered mutants of the V{subscript}κ anti-dextran, coding for N-linked glycan addition in each of the CDRs (8), were inserted as EcoRV-HindIII fragments into the pdCNA3.1(−) vector containing the κ V{subscript}κ HindIII gene.

Gene transfection

Electroporation of murine Sp2/0 cells was performed as previously described (8) with 10 μg each of H and L chain expression vectors, and 10 μg of pSV2hs for selection, all linearized using PvuII. Selection was performed with 5 mM histidinol, and clones were screened for Ab expression by ELISA, as described (8, 14).

Lipofection was performed as described previously (15) with 10 μg of uncut H and/or L chain vector per tissue culture dish containing ~5 × 10{superscript}5 Chinese hamster ovary (CHO) Pro-5 cells. After transfection, the cells were plated into six 96-well plates in 125 μl/well of IMDM with 10% FBS and 2% clonal selection for the L chain vector, or in 1 ml/well for selection from the C region vector (Invitrogen) for selection on the H chain vector. Culture supernatants from surviving clones were screened by ELISA, using dansyl-BSA-coated plates and goat anti-human κ conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO). If Abs were not secreted, 24 clones were randomly selected and grown to 1 × 10{superscript}6 cells for screening by biosynthetic labeling and immunoprecipitation of cell lysates, followed by SDS-PAGE. Cell populations selected by either ELISA or SDS-PAGE were subcloned by limiting dilution, and the clone producing the highest amount of Ab was selected.

Transfected human kidney epithelial 293T cells was performed using reagents from 5 Prime-3 Prime (Boulder, CO) or from Edge Biosystems (Gaithersburg, MD) following the manufacturers’ protocols. Briefly, 10{superscript}6 cells were grown to 60% confluence in 100-mm tissue culture dishes, and the reagent mix containing 3 μg each of H and L chain vectors was added to cells in 10 ml of fresh IMDM plus 5% calf serum. After a minimum of 4 h of incubation, cells were washed, and then incubated in IMDM plus 5% calf serum overnight before biosynthetic labeling.

Biosynthetic labeling and immunoprecipitations

Transfected cells were washed twice with sterile PBS and grown overnight at ~10{superscript}5 cells/ml in DMEM lacking methionine and cysteine (Life Technologies/BRL), supplemented with 1× Glutamax glutamine analogue (Life Technologies/BRL) and EasyTag [35]methioninel/[35]cysteine mix (Amersham, Arlington Heights, IL) at 25 μCi/ml. Supernatants were collected and cytoplasmic lysates were prepared and immunoprecipitated, as previously described (16), using rabbit anti-human Fab (both κ and λ chain specific) and rabbit anti-human IgG, both prepared in the laboratory, or rabbit anti-human Fab and rabbit anti-human IgA (Sigma-Aldrich), followed by a 10% Staph A solution (IgGor; The Enzyme Center, Malden, MA). Samples were then analyzed by SDS-PAGE under nonreducing conditions on 5% phosphate gels or under reducing conditions on 12.5% Tris-glycine (TG) gels, as described (16). Samples prepared for Endoglycosidase H (Endo H) analysis were resuspended in Endo H buffer (see Endo H hydrolysis) rather than electrophoresis sample buffer.

Tm treatment of transfectants

Cells were preincubated with fresh IMDM containing 5% calf serum and 8 μg/ml Tm for ~3 h. Cells were washed and then resuspended in labeling medium, as above, with Tm at 8 μg/ml. Supernatants were collected for immunoprecipitation after overnight incubation. For cytoplasmic lysates, cells were preincubated with Tm for 30 min, washed, incubated in labeling medium with Tm for 3 h, and then lysates were prepared.

Endo H hydrolysis

After immunoprecipitation, Staph A pellets with bound, labeled Abs were resuspended in 100 μl of Endo H buffer containing 50 mM sodium citrate (pH 5.5), 2 mM PMSF, and 100 mM 2-ME. A volume of 25–40 μl of each sample, prepared from the supernatants or cell lysates of ~1–2 × 10{superscript}6 cells, was incubated overnight at 37°C with 6–9 U of Endo H, which cleaves the bond between the two proximal N-acetylgalosamine (GlNaC) residues, but not complex carbohydrates (17). Duplicate control samples were incubated without enzyme. Sample buffer (5×) (125 mM Tris [pH 6.7], 1.5% SDS, 50% glycerol, and 20 μg/ml bromophenol blue) was added to each tube, and samples were placed in a boiling water bath for 3 min to elute labeled molecules before analysis by SDS-PAGE. Ten to 20% gradient TG gels were purchased from BioWhittaker Molecular Applications (Rockland, ME).

Immunofluorescent staining and confocal microscopy

The wells of eight-chamber Permanox slides (Nalge Nunc, Naperville, IL) were seeded with adherent cells in IMDM containing 5% FBS and incubated overnight. Cells were gently washed with PBS and then fixed in a freshly prepared solution of 0.01 M sodium metaperiodate, 0.075 M lysine, 0.0375 M NaH{subscript}2PO{subscript}4 (pH 7.4), and 2% paraformaldehyde for 2–3 h at room temperature. Wells were washed three times with a solution of 0.5% OVA (Sigma-Aldrich) in PBS (pH 7.4) (buffer A) for 10 min. Cells were then permeabilized by incubation with buffer A containing 0.05% saponin (Sigma-Aldrich) (buffer B) for 5 min at room temperature. Primary Abs were diluted in buffer B: rabbit anti-major ER glycoprotein (MERG; 1/30 dilution; a generous gift from Dr. D. Meyer (University of California, Los Angeles, CA)), rabbit anti-Golgi β-coatomer protein (β-COP; 1/75 dilution; Affinity Bioreagents, Golden, CO), and goat anti-human IgG (1/75 dilution; Zymed, South San Francisco, CA). Abs were added to relevant wells; slides were incubated with shaking overnight at 4°C, and the cells were later washed three times with buffer B. Texas Red-conjugated swine anti-goat IgG (Vector Laboratories, San Mateo, CA) and FITC-conjugated swine anti-rabbit IgG (Nord Immunology, Tilburg, The Netherlands) were diluted in buffer B at 1/25 and 1/100, respectively, and centrifuged to remove Ab aggregates, which bind to cells directly, increasing background fluorescence. The cells were stained with secondary Ab at room temperature for 1 h, washed three times with buffer B and one time with PBS, and chambers were removed from each slide. A drop of Prolong (Molecular Probes, Eugene, OR) was placed over each group of cells to reduce

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quenching of dyes, and then a coverslip was overlaid and sealed to the slide. Data were collected using a Nikon (Melville, NY) Eclipse E800 system with a Bio-Rad (Hercules, CA) MRC1024ES confocal microscope equipped with a krypton/argon laser.

**Results**

Portions of the V regions used for the present studies are depicted in Tables I and II. The different V<sub>H</sub> genes include the murine anti-α(1–6) dextran gene 19.22.1, a 19.22.1 mutant with a Lys<sup>62</sup>Thr substitution within CDR2 (referred to as Asn<sup>60</sup> V<sub>H</sub>), the anti-dansyl gene 27.44, and 27.44 with the CDR2 sequence from Asn<sup>60</sup> V<sub>H</sub>. This CDR2 was exchanged in place of CDR2 in the anti-dansyl V<sub>H</sub> by PCR mutagenesis and cloning, creating a hybrid V<sub>H</sub>. All V<sub>H</sub> were joined to human IgG1 and expressed in human kidney epithelium 293T cells, murine myeloma Sp2/0 cells, and CHO cells. Transfectants were expressed in human kidney epithelium 293T cells, murine myeloma Sp2/0 cells, and CHO cells. Transfectants were biosynthesized by guest on April 8, 2017 http://www.jimmunol.org/ Downloaded from

| Table I. Anti-dextran V region CDR sequences with carbohydrate addition sites introduced by mutation<sup>a</sup> |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sequence | 50 | 51 | 52 | 52A | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 |
| Anti-dextran V<sub>H</sub> | | | | | | | | | | | | | | | | | |
| Asn<sup>60</sup> V<sub>H</sub> CDR2 | Glu | Ile | Leu | Pro | Gly | Ser | Gly | Ser | Thr | Asn | Tyr | Asn | Glu | Lys | Phe | Lys | Gly |
| Anti-dextran V<sub>L</sub> | | | | | | | | | | | | | | | | | |
| wt CDR1 | | | | | | | | | | | | | | | | | |
| wt CDR2 | | | | | | | | | | | | | | | | | |
| wt CDR3 | | | | | | | | | | | | | | | | | |
| Asn<sup>91</sup> V<sub>L</sub> | | | | | | | | | | | | | | | | | |

<sup>a</sup> CDR sequences are shown. Carbohydrate addition sequons are underlined. Amino acid substitutions are shown in boldface. wt, Wild type.

| Table II. Anti-dansyl V<sub>H</sub> sequence with CDR2 from anti-dextran<sup>a</sup> |
|---|---|---|---|---|---|---|---|---|
| Sequence | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 |
| Anti-dansyl V<sub>H</sub> | Leu | Glu | Trp | Val | Ala | / | Glu | Ile | Arg | Lys | Ala | Asn | Asn | His | Ala |
| Anti-dextran | FR2 | / | Glu | Ile | Leu | Pro | --- | Gly | Ser | Gly | Ser | Thr | |
| / CDR2 | |
| Anti-dansyl V<sub>L</sub> | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 |
| Anti-dansyl | Tyr | Tyr | Ala | Glu | Ser | Val | Lys | Gly | / | Arg | Phe | Thr | Ile | Ser |
| Anti-dextran | Asn | Tyr | Asn | Glu | Thr | Phe | Lys | Gly | / | |

<sup>a</sup> CDR2 sequence of anti-dansyl V<sub>H</sub> is shown with some of flanking framework regions. Below is the CDR2 of anti-dextran (including the N-linked glycosylation sequon shown underlined). The anti-dansyl CDR2 was replaced with the anti-dextran sequence. FR, Framework region.
IgG lacking V region carbohydrate produced in Sp2/0 cells was included as a marker of H chain size and as a negative control for Endo H digestion. The size of the aglycosylated H chains produced in the presence of Tm (Fig. 2, lanes 3 and 8) can be compared with that of H chains with both V_{\text{H}} and Fc glycans (lanes 4, 6, and 9) and with only one glycan in the Fc region after Endo H treatment (lanes 5, 7, and 10). In every case, the H chain carbohydrate at Asn^{60} V_{\text{H}} is susceptible to Endo H digestion, indicating that it is high mannose in form. Thus, there do not appear to be differences in oligosaccharide processing of this glycoprotein in these cell lines.

The presence of CDR2 from Asn^{60} V_{\text{H}} in an anti-dansyl V_{\text{H}} inhibits Ab secretion

It is unclear why the exposed V_{\text{H}} carbohydrate at position 60 in CDR2 remains in the high mannose form, whereas a carbohydrate attached nearby at position 58 is processed to complex form. To further explore the contribution of surrounding sequences to glycosylation, we created a hybrid V_{\text{H}} in which CDR2 of the Asn^{60} V_{\text{H}} was exchanged with the CDR2 of the anti-dansyl V_{\text{H}}, which bears no glycan (see Table II). A Pro-5 cell line stably expressing anti-dansyl V_{\text{H}} was produced in Pro-5 cells and labeled in the presence of Tm indicate the size of the aglycosylated H chain, which was not secreted (Fig. 2, lane 3). The intracellular H chain is identical in size to secreted Asn^{60} V_{\text{H}} IgG1 H chain, which bears carbohydrates in the V region and in the Fc (Fig. 2, lane 10). Therefore, it seems that the presence of V region glycan in the hybrid Ab inhibits its ability to be secreted.

Hybrid V_{\text{H}} IgG1 H chains do not traffic to the Golgi apparatus

To determine the intracellular location of hybrid V_{\text{H}} Abs, transfectants expressing either the V_{\text{H}} region hybrid or wild-type anti-dansyl Abs were examined using confocal microscopy. Cells grown on microscope slides were stained with goat anti-IgG and rabbit antiserum specific for either an ER glycoprotein or the Golgi β-COP, followed by secondary Abs conjugated with either Texas Red or FITC (Fig. 4). Wild-type IgG1 colocalized with the ER (Fig. 4C) and with the Golgi apparatus (F), whereas the hybrid Ab localized to the ER (I), but not to the Golgi apparatus (L). As has been observed with other misfolded proteins, it appears that the hybrid Ab is withheld in the ER and does not proceed any further along the secretory pathway.

Discussion

Previous studies from this laboratory using a murine V region specific for α(1-6) dextran have shown a role for V region glycosylation in Ab affinity, half-life, and immunogenicity (8, 9, 11). Abs differing in the location of a glycan addition site within CDR2

FIGURE 2. The cell line used for expression does not influence the processing of carbohydrate at Asn^{60} V_{\text{H}}. Asn^{60} V_{\text{H}} IgG1 was expressed with κ L chain in 293T, Pro-5, and Sp2/0 cell lines. Anti-dansyl IgG1, which contains no V region carbohydrate, was produced in Sp2/0 transfectants. Cells were biosynthetically labeled by overnight growth in medium containing [\textsuperscript{35}S]Met and [\textsuperscript{35}S]Cys. Anti-dansyl IgG1 and anti-dextran IgG1 produced in Pro-5 cells and labeled in the presence of Tm indicate the size of aglycosylated protein (lanes 3 and 8). Abs were immunoprecipitated with rabbit anti-human IgG Fc and Fab, followed by Staph A. Samples were resuspended in Endo H buffer, incubated at 37°C overnight with or without Endo H, and analyzed on a 12.5% TG gel.

FIGURE 3. An IgG1 Ab with a hybrid anti-dansyl/anti-dextran Asn^{60} V_{\text{H}} was not secreted. 293T transfectants (lane 4) and CHO cell transfectants (all other lanes) were biosynthetically labeled overnight with [\textsuperscript{35}S]Met and [\textsuperscript{35}S]Cys, with or without Tm. Supernatants were collected, and cytoplasmic lysates were prepared. Abs were immunoprecipitated with rabbit anti-human IgG Fc and Fab and resuspended in 1× sample buffer with 2-ME. The samples were analyzed by SDS-PAGE on a 12.5% TG gel. L, Cell lysate; S, secretions.
were shown to contain carbohydrates that were processed differently. The V<sub>H</sub> carbohydrate at Asn<sup>58</sup> of hybridoma 14.6b.1, within the naturally occurring Asn<sup>58</sup>-Tyr<sup>59</sup>-Thr<sup>60</sup> sequon, is complex. This is consistent with the observation that carbohydrates located on exposed loops, such as CDR2, are accessible to glycosidases and glycosyltransferases as proteins migrate through the ER and Golgi apparatus, and therefore tend to be processed to a greater extent than those that are shielded or sequestered (10). Surprisingly, when a carbohydrate addition site was introduced into the same V<sub>H</sub> at position 60, the glycan was located on the surface of the molecule (11) but was found to remain high mannosyl. Therefore, stearic accessibility to processing enzymes during protein maturation (18) may not be solely responsible for the degree of glycan processing, and structural information near an addition site may influence enzyme activity. For example, differences in glycan processing between closely related strains of influenza virus (19, 20) and murine leukemia virus (21) have indicated that primary sequence may play a role in directing oligosaccharide processing. Local conformation and domain interactions have been reported to contribute to processing at individual glycosylation sites on soluble variants of rat and human CD4 expressed in CHO cells (22). In this study, we have investigated glycan processing by expressing the anti-dextran Asn<sup>60</sup> V<sub>H</sub> in different contexts.

Expression of carbohydrate-processing enzymes is species and tissue specific (23, 24). To address the issue of possible cell line and species-specific differences in glycan processing, anti-dextran  

Asn<sup>60</sup> V<sub>H</sub>-IgG1 was produced in the murine myeloma Sp2/0, in CHO cells, and in human kidney epithelial 293T cells. We found no evidence for either species-specific or cell type-specific differences in processing of the Asn<sup>60</sup> V<sub>H</sub> carbohydrate (Fig. 2).

It has long been known that not all N-linked carbohydrate addition sites are used (2, 25, 26), and that some sites may be variably occupied (27–29). It has also been observed that processing at one site may be affected by the presence of a glycan at another site. For example, tissue plasminogen activator is a protease glycoprotein consisting of five protein domains, with two main classes of glycosylation variants. Type I has three N-linked carbohydrates, at Asn<sup>117</sup>, Asn<sup>164</sup>, and Asn<sup>448</sup>, whereas type II has only two, at Asn<sup>117</sup> and Asn<sup>448</sup>. Fine structural analysis showed that the glycan at site 448 is different if glycan is present at site 184 (30). When we expressed the anti-dextran Asn<sup>60</sup> V<sub>H</sub> with the C region genes for human IgA1, which has two exposed N-linked glycans in the Fc and O-linked carbohydrates in the hinge, or with IgA1 mutated to lack N-linked Fc glycosylation sites, we found that both IgA1 Abs bore high-mannose V<sub>H</sub> carbohydrate (data not shown). This indicated that neither isotype sequence differences between IgA1 and IgG1 nor different Fc region glycans caused detectable differences in Asn<sup>60</sup> glycan processing. We also found that the isotype of the L chain (κ vs λ) did not influence the processing of the carbohydrate at Asn<sup>60</sup> (data not shown), although the L chain isotype has been found to influence some of the properties of IgG (31).

Our results showed that crowding of the V region did not alter carbohydrate processing. When Abs possessed oligosaccharides in the V region of both H and L chains, the H chain carbohydrates remained in the high mannose form, whereas L chain carbohydrates were processed almost exclusively to complex form (Fig. 1), consistent with results obtained in an earlier study (8). It is interesting that the presence of two glycans in close proximity did not alter their processing. The dimensions of an Ag-combining site are ~22 × 32 Å (32). N-Linked carbohydrates are very large, and the length of just their three sugar residues (GlcNAc, GlcNAc, and mannose) is 16 Å (33); therefore, two carbohydrates would occupy a relatively large area of the protein surface. It should be noted that this study merely addressed whether the carbohydrates were complex or high mannosyl. It is still possible that smaller structural differences exist between crowded and uncrowded states.

Anti-dansyl H chains do not normally bear V region glycans. When Asn was substituted for His<sup>55</sup> in an anti-dansyl V<sub>H</sub>, creating the consensus sequence Asn<sup>55</sup>-Ala<sup>56</sup>-Thr<sup>57</sup> in CDR2, the site was not used and Abs bearing only the Fc carbohydrate were secreted (11). In this study, we made a more radical change, replacing the whole CDR2 of the anti-dansyl V<sub>H</sub> with CDR2 of the anti-dextran V<sub>H</sub>. Our goal was to determine whether the environment of the carbohydrate addition sequence would influence either site use or carbohydrate processing. We found that, in the context of the anti-dansyl V<sub>H</sub> site was used, but that the hybrid V<sub>H</sub> Abs were not secreted (Fig. 3). Because Abs were secreted when transfectants were grown in the presence of Tm, it was evident that the carbohydrate and not the amino acid changes made with the CDR2 graft interfered with the trafficking of the Ab.

A model of the folded Fd (V<sub>H</sub> plus C<sub>H</sub>1) fragment of the hybrid V<sub>H</sub>, based on the murine anti-tumor Ab R24, which bears 84% identity to the hybrid V<sub>H</sub>, was obtained from SWISS-MODEL (data not shown); it places the Asn side chain nitrogen atom used for carbohydrate attachment on the surface of the molecule, where it should be readily available for glycosylation. Indeed, our results indicated that carbohydrate was attached to the hybrid V<sub>H</sub>; however, the presence of glycan appeared to impede secretion of the
Abs. It is known that newly made proteins associate with chaperones and other proteins in the ER, which assist the protein in proper folding (34–39). In particular, H chains transiently associate with the ER chaperone BiP/GRP78 during folding; BiP dissociates with L chain binding (40). If folding is incorrect after multiple attempts, the protein is eventually degraded by the proteosome after retrograde translocation into the cytosol from the ER (41, 42). It is possible that the carbohydrate in the hybrid VH of the H chain prevented association with the L chain so that BiP remained associated, and the H chains were held in the ER and, unlike the wild-type anti-dansyl IgG1, did not traffic to the Golgi apparatus (Fig. 4).

V-Linked carbohydrate addition sites can arise spontaneously during somatic hypermutation. In some cases, the introduction of V-linked carbohydrate addition sites in the V region results in an increase in affinity for Ag (11). Affinity may be raised either through direct hydrophobic interaction with Ag, especially a carbohydrate Ag, or through altering the combining site geometry for a better cognate fit. In other cases, introduction of a carbohydrate can result in lowered affinity for Ag or can entirely block its binding (8, 11). We have now shown that addition of carbohydrate may also result in an Ab that is not secreted. Therefore, generation of carbohydrate addition sites during somatic mutation may sometimes result in cells not capable of producing functional Ab. As we increase our knowledge of the factors influencing carbohydrate processing and our predictive ability regarding the effect of glycan positioning within the Ag combining site on assembly and function, we will be better equipped to more efficiently design Abs with the desired binding and functional capabilities.

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References