

Chapter 14

Immunoglobulin A Glycosylation and Its Role in Disease



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Abstract Human IgA is comprised of two subclasses, IgA1 and IgA2. Monomeric IgA (mIgA), polymeric IgA (pIgA), and secretory IgA (SIgA) are the main molecular forms of IgA. The production of IgA rivals all other immunoglobulin isotypes. The large quantities of IgA reflect the fundamental roles it plays in immune defense, protecting vulnerable mucosal surfaces against invading pathogens. SIgA dominates mucosal surfaces, whereas IgA in circulation is predominately monomeric. All forms of IgA are glycosylated, and the glycans significantly influence its various roles, including antigen binding and the antibody effector functions, mediated by the Fab and Fc portions, respectively. In contrast to its protective role, the aberrant glycosylation of IgA1 has been implicated in the pathogenesis of autoimmune diseases, such as IgA nephropathy (IgAN) and IgA vasculitis with nephritis (IgAVN). Furthermore, detailed characterization of IgA glycosylation, including its diverse range of heterogeneity, is of emerging interest. We provide an overview of the glycosylation observed for each subclass and molecular form of IgA as well as the range of heterogeneity for each site of glycosylation. In many ways, the role of IgA glycosylation is in its early stages of being elucidated. This chapter provides an overview of the current knowledge and research directions.

Keywords IgA glycosylation · IgA receptors · IgA1 · IgA2 · *N*-glycosylation · *O*-glycosylation · Mass spectrometry

Abbreviations

C1GalT1	Core 1 β 1,3-galactosyltransferase
dIgA	Dimeric IgA
EBV	Epstein–Barr virus
ECD	Electron capture dissociation
ETD	Electron transfer dissociation
FcRL4	Fc receptor-like 4
Gal	Galactose
GalNAc	<i>N</i> -acetylgalactosamine
GalNAc-T	GalNAc-transferase
Gd-IgA1	Galactose-deficient IgA1

HC	Heavy chain
HR	Hinge region
Ig	Immunoglobulin
IgAN	IgA nephropathy
IgAV	IgA vasculitis
IgAVN	IgA vasculitis with nephritis
LC	Light chain
MBL	Mannose-binding lectin
mIgA	Monomeric IgA
MS	Mass spectrometry
pIgA	Polymeric IgA
RA	Rheumatoid arthritis
SA	Sialic acid
SC	Secretory component
SIgA	Secretory IgA
SLE	Systemic lupus erythematosus
TG2	Transglutaminase 2
V	Variable

14.1 Introduction

Immunoglobulin A (IgA) is one of the five primary immunoglobulins and its production in humans is greater than that of all other immunoglobulins. Humans expend a considerable amount of energy to produce IgA, as ~70 mg of IgA per kilogram of body weight is synthesized daily (Mestecky et al. 1986; Conley and Delacroix 1987). IgA is secreted by IgA-producing cells in two main molecular forms: monomeric IgA (mIgA) and dimeric IgA (dIgA), the latter having a joining chain (J chain) to bind two mIgA molecules. Furthermore, secretory IgA (SIgA) is the main form of IgA found on mucosal surfaces. SIgA has an additional protein chain attached, a secretory component, which is derived from polymeric immunoglobulin (Ig) receptor during transcytosis of dIgA through mucosal epithelial cells. IgA is the second most abundant Ig in the circulation, predominantly as mIgA, (~2 mg/mL), after IgG (~12 mg/mL); however, IgA is the most abundant antibody in external secretions (tears, saliva, colostrum, milk, nasal fluid, gallbladder bile, and intestinal fluid) of mucosal surfaces, secreted locally as SIgA (Jackson et al. 2005). The abundance of IgA highlights the important roles it plays in immune defense processes. Specifically, mucosal surfaces (i.e., respiratory, gastrointestinal, and genitourinary tracts) are often exposed to invading pathogens and IgA acts to protect such vulnerable areas from infection. Equipped with unique structural attributes of its heavy chain and its ability to form monomeric, dimeric, and higher polymeric forms (i.e., three or more mIgA molecules associated with J chain), both circulatory IgA and secretory forms of IgA are capable of neutralizing and removing pathogens

by activating innate and acquired immune functions—blocking pathogens by antigen-specific and nonspecific binding (Russell 2007; Renegar and Small 1994; Phalipon et al. 2002).

Serum IgA is produced predominantly by plasma cells in the bone marrow. It is primarily a monomeric protein with a quaternary structure consisting of two heavy chains (HC) and two light chains (LC) linked by disulfide bonds. In contrast, mucosal IgA, specifically dIgA linked together by the J chain, is produced close to the epithelium by plasma cells in the lamina propria of mucosal surfaces (Koshland 1985). This IgA becomes SIgA when, during transcytosis, the secretory component (SC) is attached. Although most IgA-producing cells are localized in the gastrointestinal system, SIgA is also one of the major antibodies in human tears, saliva, milk, and colostrum (Underdown and Mestecky 1994). Maternal milk is essential for the passive immunity of infants (Newburg et al. 2005; Hanson 1998; Peterson et al. 2013), protecting babies from a variety of infections that otherwise may lead to sepsis and meningitis (Schroten et al. 1998). The diversity of IgA post-translational modifications, due to variable glycosylation, contributes to the effector functions of IgA.

Regardless of the source of IgA, heavy chains of all molecular forms of IgA, as well as the J chain and SC, are glycosylated. In general, glycosylation is the result of a non-template, multi-enzymatic multistep process that ultimately results in a range of glycan heterogeneity. Thus, the post-translationally modified IgA proteoforms are cell type- and lineage-specific. Overall, glycans associated with Igs have been shown to influence antibody functions. Specifically, glycosylation patterns differentially affect the effector functions of Igs (Lin et al. 2015; Li et al. 2017) depending on the type, branching, and modifications of *N*-glycans and/or the terminal sugars of *N*- and/or *O*-glycans, which include galactose and sialic acid (Steffen et al. 2020). In fact, Ig glycosylation can determine whether an antibody glycoform is pro-inflammatory or anti-inflammatory (Li et al. 2017; Pagan et al. 2018). The multiple biological roles of IgA glycans include glycan-mediated antigen-nonspecific binding to bacteria and viruses. The complexity is enhanced by the diverse glycan structures that create a range of heterogeneity.

Herein, we will focus on the glycosylation of human IgA. Specifically, we discuss the location and observed structures at individual amino-acid sites and/or individual clusters of sites. We include a summary of the assessment of native populations of IgA glycans, the range of glycans observed at each site, and how changes in the glycans at a given site have been connected to biological activity and/or diseases.

14.2 IgA Subtypes and Overall Structure

Human IgA exists in two subclasses: IgA1 and IgA2 (occurring in two allotypes—IgA2m(1) and IgA2m(2)) (Fig. 14.1). Each Ig comprises two HCs and two LCs, each with one variable (V) and one (LC) or three (HC) constant domains. Each domain is approximately 110–130 amino acids, averaging 12–13 kDa. Functionally, the

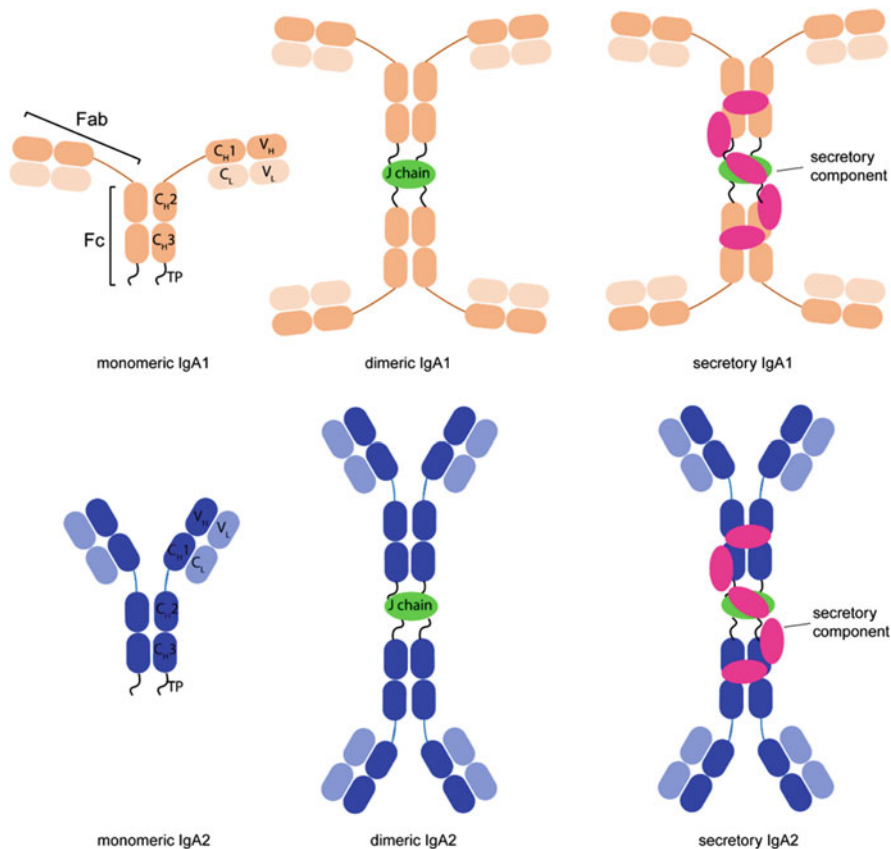


Fig. 14.1 Human IgA. Schematic diagram of IgA1 (top) and IgA2 (bottom) in several molecular forms (monomeric, dimeric, and secretory IgA). Although secretory IgA is depicted as a dimer, larger polymers such as trimers and tetramers can be formed. Heavy chains (V_H, C_{H1}, C_{H2}, and C_{H3}) are shown in orange (IgA1) and blue (IgA2), and light chains (V_L and C_L) are shown in light orange (IgA1) and light blue (IgA2). The tailpiece (TP) is portion of the C_{H3} domain that is depicted as an extension. The J chain (green) is present in dimeric and secretory IgA, the secretory component (pink) is a component of secretory IgA

variable regions of HC and LC are responsible for antigen binding, while the constant domains are important for structure/function and defining/modifying the effector functions. Each of the two identical Fab regions of IgA consists of V_H and V_L and C_{H1} and C_L, with C_L being either kappa or lambda. HCs differ within their constant regions encoded by the C_α genes, C_{α1} and 2. Each chain begins at its *N*-terminus with the variable region (V_H), followed by the constant region, C_{H1}, 2, and 3. C_{H1} is connected by the hinge region (HR) to the Fc region, consisting of C_{H2} and 3. Interchain disulfide bridges connect HC and LC of the Fab region and the two HC C_{H2} domains of the Fc region. Pairings between the collapsed β-barrel domains of adjacent chains (V_H with V_L, C_{H1} with C_L, and both C_{H3} domains) are

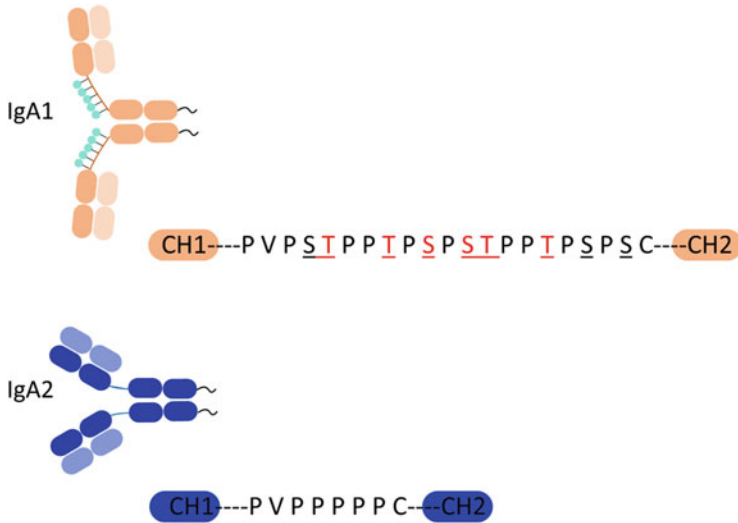


Fig. 14.2 IgA1 and IgA2 hinge regions. Comparison of amino-acid sequences of human IgA1 (top) and IgA2 (bottom) hinge regions. Human IgA1 has nine Ser (S) and Thr (T) amino-acid residues (underlined) in the hinge region segment (between constant regions C_{H1} and C_{H2} of the heavy chains). Usually, three to six clustered *O*-glycans are attached per hinge region (shown in red). IgA2 hinge region is shorter compared to that of IgA1, does not have Ser and Thr residues and, thus, IgA2 does not have *O*-glycans

mediated through non-covalent interactions, primarily hydrogen bonds and van der Waals contacts. A conserved 18-amino-acid extension of the CH3 heavy chain C-terminus is known as the tailpiece. This feature is unique for IgA and IgM and plays a critical role in dimer and pentamer formation, respectively, though binding with the J chain (Atkin et al. 1996; Yoo et al. 1999).

The HR between the Fab and Fc portions is another feature distinguishing each IgA isotype. IgA1, unlike IgA2, has an HR segment consisting of two octapeptide repeats rich in Ser, Thr, and Pro residues with nine potential *O*-glycosylation sites (Fig. 14.2). Usually, three to six of these sites are *O*-glycosylated (Novak et al. 2012, 2018). In contrast, the IgA2 HR lacks potential *O*-glycosylation sites. Unlike in IgG, IgA HR amino-acid sequence allows for greater flexibility, particularly for IgA1 with its extended sequence. Although this structural characteristic may enhance antigen binding by IgA, it also provides a target for bacterial IgA-specific proteases.

14.3 IgA Glycosylation Sites

The *N*-glycans at individual sites create a range of heterogeneity for both IgA isotypes, as they do for most Igs. Both IgA subclasses carry *N*-linked oligosaccharides. IgA1 and IgA2 have two and four conserved sites of *N*-glycosylation,

respectively, all located in constant regions of the heavy chains. *N*-glycosylation significantly contributes to the total molecular mass of human IgA, accounting for 6–7% of the mass of IgA1, and 8–10% of the mass of IgA2 (Tomana et al. 1976). The IgA subclasses differ from other Igs in both the attachment sites and the overall positions of the *N*-linked glycans and the proximal disulfide bridges. IgA1 and IgA2 possess two similarly located conserved sites of *N*-linked glycosylation. The first site is at Asn144 (IgA1) and Asn131 (IgA2) in the CH2 domain of the heavy chain (the *N*-glycosylation sites herein are indicated by the residue number based on UniProt numbering, IgA1: P01876; IgA2: P01877) (UniProt Consortium 2017). The glycopeptide resulting from trypsin proteolytic digestion, as is conventional for analysis by mass spectrometry (MS), has an identical amino-acid sequence for both isotypes. Thus, if the two isotypes are not separated initially, these two *N*-glycosylation sites are observed as a mixture in standard MS glycosylation analysis (Steffen et al. 2020; Plomp et al. 2018). This is also the case for the second conserved site at Asn340 (IgA1) and Asn327 (IgA2) in the tailpiece. IgA2 has two additional sites of *N*-glycosylation, Asn47 located in the CH1 domain and in the CH2 domain at N205. All adjacent domains (VH with VL, CH1 with CL, and CH3 with CH3) are paired in close proximity to each other, except for the neighboring chains of the CH2 domains, which are not closely aligned. Such non-pairing is a feature also observed in IgG (CH2) and IgE (CH3). The potential solvent exposure, a consequence of the distance between the two CH2 domains, seems to be limited by the presence of *N*-glycans (located at Asn144 in IgA1 and Asn131 and Asn205 in IgA2) which shield the outer surface of the domain. The CH2 domain of IgA1 and IgA2 is stabilized by inter-chain disulfide bonds formed between three or four cysteine residues (Cys241, Cys242, Cys299, and Cys301) in the upper region of the domain. This feature is in contrast to IgG which instead has several disulfide connections located in the HR (2 HR disulfide bonds for IgG1 and IgG4, 4 for IgG2, and 11 for IgG3) (Liu and May 2012).

The tailpiece extension of the CH3 domain, of both IgA1 and IgA2, has a conserved *N*-glycan located at Asn340 and Asn327, respectively. The tailpiece, similar to the one found in IgM, contains a cysteine residue responsible for polymerization with the J chain. Previous studies have indicated that the tailpiece *N*-glycan of IgA plays an important role in J chain incorporation (Atkin et al. 1996; Sørensen et al. 2000). In plant-produced IgA proteins, the tailpiece is incompletely glycosylated and this deficiency may be the reason for the observed inefficient dimer formation (Göritzer et al. 2017, 2020; Westerhof et al. 2015; Castilho et al. 2018). Recently, it has been demonstrated that the tailpiece of IgA provides an innate line of defense against viruses, with the *N*-glycan mediating such activity (Maurer et al. 2018).

For IgA1, further glycosylation diversity arises through the clustered *O*-glycans attached to the HR. The glycosylated HR is a unique feature to Igs, shared by IgA1 and IgD, and in some forms of IgG3. In IgA1, these clustered *O*-linked glycans are composed of core 1 glycans or terminal or sialylated *N*-acetylgalactosamine (GalNAc), often attached to Thr225, Thr228, Ser230, Ser232, Thr233, and/or Thr236 (amino-acid numbering is based on conventionally used nomenclature for

IgA1 HR). Each Ser/Thr residue can be modified by a GalNAc residue that can be further extended by the addition of galactose (Gal) through a β 1,3 glycosidic bond. Up to two sialic acid (SA) residues can be added, one attached to the GalNAc through an α 2,6-linkage and the other to Gal by an α 2,3-linkage.

14.4 Molecular Forms of IgA and Their Distributions (Monomeric and Polymeric IgA, SIgA, J Chain, Secretory Component)

Unlike most other Ig isotypes, IgA exists in multiple molecular forms (Fig. 14.1). IgA in the circulation is produced mainly in the bone marrow and to a lesser extent in the spleen and lymph nodes. The predominant form of serum IgA is monomeric. IgA destined for mucosal surfaces is produced locally by plasma cells in a polymeric form (pIgA), predominantly dimeric. The ~16-kDa J chain is an additional polypeptide that connects together two or more monomers of IgA via the Fc region tailpiece through disulfide bonds, forming pIgA (Fig. 14.1). Two of the J chain's eight cysteine residues are involved in covalent binding to IgA's tailpiece and the additional six cysteine residues form intramolecular disulfide bridges (Cys12-Cys100, Cys17-Cys91, and Cys108-Cys133) (Bastian et al. 1992). The J chain has a single *N*-linked glycan attached at Asn48. pIgA is formed after glycosylation occurs. Glycosylation is required for dimer assembly, as drastically reduced dimer formation was observed when Asn340 was substituted with alanine to prevent the attachment of *N*-glycans to the tailpiece (Atkin et al. 1996).

SIgA is a multi-polypeptide complex produced in mucosal tissues from pIgA, produced by local plasma cells, undergoing transcytosis through mucosal epithelial cells (Norderhaug et al. 1999). SIgA consists of an SC covalently attached to pIgA (i.e., IgA plus J chain). SIgA is mainly dimeric, although higher oligomers, including trimers and tetramers, are also present. The ~80-kDa SC is comprised of five immunoglobulin-like domains. To form SIgA, a polymeric immunoglobulin receptor-mediated pathway transports pIgA by transcytosis into the mucosal secretions. On reaching the mucosal surface, the polymeric immunoglobulin receptor (pIgR) that binds IgA is cleaved to release SIgA with bound SC which is a proteolytic cleavage product of the pIgR. SC is highly glycosylated by seven *N*-glycans, which contribute up to 25% of its molecular mass (Norderhaug et al. 1999). The SC glycans have several functions, in addition to protecting the SC and the SIgA from proteases (Crottet and Corthésy 1998), they can interact with adhesins and lectins. SC has been shown to bind to a range of bacteria via its glycans (Schroten et al. 1998; Borén et al. 1993; Wold et al. 1990), thereby inhibiting attachment and the subsequent infection of epithelial surfaces. The SC glycans are also involved in the localization of SIgA by anchoring the SIgA to the mucus lining the epithelial surface through its carbohydrate residues.

Circulating IgA is predominantly monomeric and predominantly of IgA1 subclass (~84% IgA1 and ~16% IgA2) (Mestecky et al. 1986), with a small proportion of pIgA (~5–8%) (Delacroix et al. 1982). Dimeric SIgA represents the dominant IgA at almost all mucosal surfaces, although the levels and subclass distributions among the different compartments of the mucosal immune system can vary considerably. For example, in saliva, 90–95% of total IgA is SIgA with a subclass distribution of ~65% IgA1 and ~35% IgA2. This is different for hepatic bile which has a much lower abundance of SIgA (~65%) and a subclass distribution of ~75% IgA1 and ~25% IgA2 (Delacroix et al. 1982). However, even the same compartment, sampled via different methods or times, can provide varying levels of isotype distribution and molecular forms. Furthermore, these distinct differences reflect the dominant Ig source (local production in mucosal tissue versus plasma), the expression of isotype-specific receptors that transport Igs, and the effects of specific regulatory mechanisms (e.g., cytokines and hormones) that influence Ig distribution.

14.5 IgA1 O-Glycosylation Pathways

The IgA1 HR with clustered sites of mucin-like *O*-glycosylation represents a unique acceptor site for a post-translational modification process that differs from most other Igs (only IgD has clustered *O*-glycans in its HR). Of the nine potential *O*-glycosylation sites in the HR of human IgA1, usually three to six are *O*-glycosylated (Baenziger and Kornfeld 1974; Mattu et al. 1998; Novak et al. 2000; Renfrow et al. 2007; Takahashi et al. 2010; Hiki et al. 1998; Iwase et al. 1996a; Royle et al. 2003; Franc et al. 2013; Wada et al. 2010a), although glycoforms with up to seven *O*-glycans have been observed in serum IgA1 (Pouria et al. 2004). These clustered *O*-glycans of serum IgA1 are usually core 1 glycan (Field et al. 1989; Iwase et al. 1996b). The GalNAc-Gal disaccharide may be sialylated on GalNAc, Gal, or both sugars (Field et al. 1989; Takahashi et al. 2012). *N*-acetylneuraminic acid can be attached to GalNAc through an α 2,6-linkage or to Gal by an α 2,3-linkage. In addition to the core 1 glycans, some glycans may remain without Gal, i.e., as terminal GalNAc or sialylated GalNAc (Ohyama et al. 2020a, b). Unlike in the process of *N*-glycan biosynthesis, there is no control system for *O*-glycan biosynthesis, and *O*-glycosylation is also not required for folding or export of IgA1 (Gala and Morrison 2002).

Biosynthesis of IgA1 *O*-glycans occurs in the Golgi apparatus of IgA1-producing cells. As for other proteins with clustered *O*-glycans, it is a stepwise process that is not template-driven but rather controlled by expression, activity, and localization of different glycosyltransferases catalyzing the sequential addition of monosaccharides to the acceptor (glyco)protein during its transition through the Golgi apparatus (Reily et al. 2019). The clustered nature of the potential sites of *O*-glycosylation in the IgA1 HR creates a unique amino-acid synthesis template where each step of monosaccharide addition changes the template and has implications for the subsequent steps of monosaccharide addition at adjacent sites. This effect occurs due to

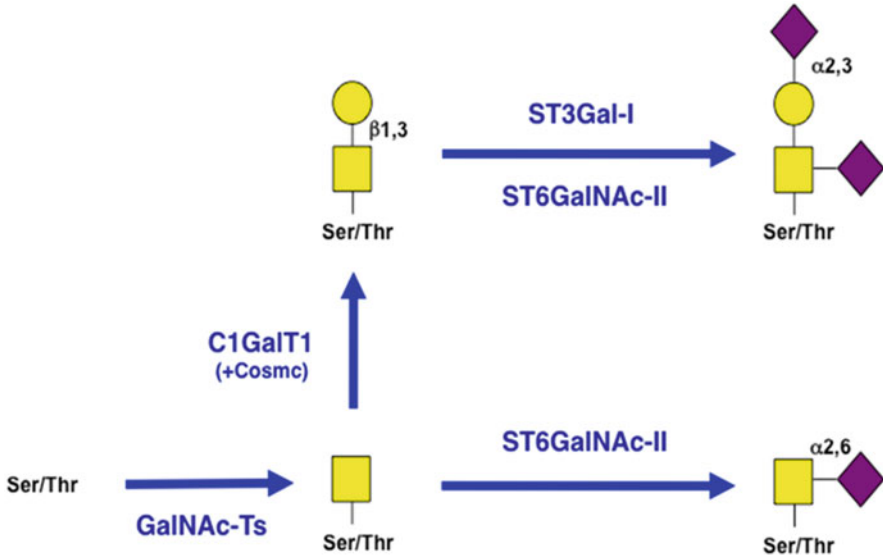


Fig. 14.3 IgA1 *O*-glycosylation pathways. Biosynthesis of IgA1 *O*-glycans occurs in the Golgi apparatus of IgA1-producing cells. The stepwise process begins with the attachment of GalNAc to some of the Ser/Thr residues in the hinge region catalyzed by GalNAc-transferases (GalNAc-Ts). The attached GalNAc residues can be then modified by addition of Gal, mediated by core 1 β 1,3-galactosyltransferase (C1GalT1). Production of the active C1GalT1 enzyme depends on its chaperone (C1GalT1C1, Cosmc). The core 1 structures (GalNAc-Gal) of IgA1 can be further modified by sialyltransferases that attach sialic acid to Gal (mediated by an ST3Gal enzyme, e.g., ST3Gal-I) and/or GalNAc residues. Sialylation of GalNAc is mediated by ST6GalNAc-II, as the usual ST6GalNAc-I is not expressed in IgA1-producing cells. Conversely, if terminal GalNAc is sialylated by ST6GalNAc-II, this structure cannot be further modified

some of the glycosyltransferases having glycan-recognizing lectin domains that strongly influence their activity (Stewart et al. 2019, 2021). Also, as more *O*-glycan chains are added and extended in the confined space of the HR, there is a steric hindrance that precludes or inhibits further addition or extension of the clustered *O*-glycans. Still, these physical constraints on the HR create a consistent fidelity of the range and distribution of *O*-glycans that are attached. This is similar to the clustered *O*-glycosylation sites of mucins that have multiple amino-acid tandem repeats as opposed to the two found in IgA1.

IgA1 *O*-glycosylation begins with the attachment of GalNAc to some of the Ser/Thr residues in the HR. In humans, some members of a family of 20 enzymes, GalNAc-transferases (GalNAc-Ts), are involved in this process (Daniel et al. 2020) (Fig. 14.3). It has been proposed that GalNAc-T2 is the main GalNAc-T enzyme responsible for the initiation of *O*-glycosylation of IgA1 (Iwasaki et al. 2003), although other GalNAc-Ts may contribute to the process, including GalNAc-T1, -T11, and -T14 (Stewart et al. 2021; Daniel et al. 2020; Wandall et al. 2007).

GalNAc-Ts determine not only the sites of GalNAc attachment but also the final glycan density, as there are three to six sites per IgA1 HR that are typically

glycosylated. Most GalNAc-T enzymes have two domains, a catalytic domain and lectin domain. Much of our understanding of GalNAc-T enzyme activities comes from studies of purified enzymes, synthetic peptides, glycopeptides, and glycoprotein substrates (Stewart et al. 2019, 2021; de Las et al. 2017). Data from multiple studies point to distinct and complementary roles of catalytic and lectin domains of GalNAc-Ts in the biosynthesis of clustered *O*-glycans of IgA1. Each monosaccharide addition can generate multiple isomers due to the selection of a specific attachment site. Whereas the selection of the first site is driven by the catalytic domain of the GalNAc-T enzyme, the subsequent site selection is substantially impacted by the lectin domain. The glycan density, i.e., the number of GalNAc residues per HR glycopeptide, depends on the expression and activity of GalNAc-Ts isoenzymes that can act on IgA1 and on the selection of the initiation and follow-up sites in the HR. Despite the variability of this step of *O*-glycan biosynthesis, most IgA1 HR glycoforms have three to six *O*-glycans.

The attached GalNAc residues can be modified by addition of Gal, mediated by core 1 β 1,3-galactosyltransferase (C1GalT1) (Stewart et al. 2021; Ju et al. 2002, 2006; Aryal et al. 2012). The production of the active enzyme depends on its chaperone (C1GalT1C1, Cosmc) (Ju and Cummings 2002) (Fig. 14.3).

The core 1 structures of IgA1 may be further modified by sialyltransferases that attach sialic acid to Gal (mediated by a ST3Gal enzyme) and/or GalNAc residues (Stewart et al. 2021; Takahashi et al. 2014). Sialylation of GalNAc is mediated by ST6GalNAc-II, as the usual ST6GalNAc-I is not expressed in IgA1-producing cells (Takahashi et al. 2014; Raska et al. 2007; Stuchlova Horynova et al. 2015; Suzuki et al. 2008) (Fig. 14.3). Notably, sialylation of GalNAc on IgA1 by ST6GalNAc-II prevents subsequent galactosylation (Stewart et al. 2021; Stuchlova Horynova et al. 2015; Suzuki et al. 2014). Furthermore, studies with purified enzymes ST6GalNAc-II and ST3Gal-I and partially Gal-deficient IgA1 substrate revealed that prior sialylation by either enzyme influences the activity of the second enzyme (Stewart et al. 2021). These data suggest that the extent of sialylation of the clustered IgA1 HR segment is not only a net result of the enzyme activity but also involves steric hindrances of the clustered *O*-glycans (Novak et al. 2018; Stewart et al. 2021).

Additional knowledge about IgA1 *O*-glycan biosynthesis was obtained from genetic and genomic studies as well as studies of Epstein–Barr virus (EBV)-immortalized IgA1-secreting cells derived from the cells in human peripheral blood (Suzuki et al. 2008). EBV-immortalized IgA1-secreting cell lines derived from healthy individuals and patients with IgA nephropathy (IgAN) provided a new tool for comparative studies of IgA1 *O*-glycosylation in health and disease states. These cell lines produce IgA1 that mimics glycosylation of serum IgA1 of the respective donors (Suzuki et al. 2008). As detailed elsewhere, patients with IgAN have IgA1 in the glomerular immunodeposits and in the circulation enriched for glycoforms with some *O*-glycans deficient in Gal (Novak et al. 2018). Studies of EBV-immortalized IgA1-secreting cell lines from healthy individuals and patients IgAN revealed that dysregulation of expression and activity of several key enzymes is associated with elevated production of Gal-deficient IgA1 (Suzuki et al. 2008, 2014). Specifically, reduced expression/activity of C1GalT1 and its chaperone

Cosmc and elevated expression/activity of ST6GalNAc-II in the cells from IgAN patients are associated with reduced Gal content in the secreted IgA1 (Suzuki et al. 2008).

Studies of familial and sporadic IgAN cohorts revealed heritability of serum levels of Gal-deficient IgA1 (Gharavi et al. 2008; Hastings et al. 2010; Kiryluk et al. 2011). Genome-wide association studies (GWAS) revealed single nucleotide polymorphisms (SNPs) in the noncoding region of C1GALT1, the gene encoding the C1GalT1 galactosyltransferase, associated with serum levels of Gal-deficient IgA1 (Kiryluk et al. 2017; Gale et al. 2017). One of these studies also found associations between SNPs in the noncoding region of C1GALT1C1 (Cosmc), the gene encoding the C1GalT1-specific chaperone, and serum levels of Gal-deficient IgA1 (Kiryluk et al. 2017). siRNA knock-down experiments using immortalized IgA1-secreting cell lines further validated these findings (Kiryluk et al. 2017).

Furthermore, the genetically co-determined Gal content can be further influenced by some cytokines and growth factors, such as interleukin 6 (IL-6), IL-4, and leukemia inhibitory factor (LIF) (Suzuki et al. 2014; Yamada et al. 2010, 2020). This cytokine-mediated overproduction of Gal-deficient IgA1 is due to further dysregulation of expression/activity of specific enzymes (C1GalT1, Cosmc, ST6GalNAc-II) (Suzuki et al. 2014). This process, uniquely enhanced in the IgA1-producing cells from patients with IgAN, is associated with the enhanced and prolonged cytokine signaling, likely due to an aberrant regulation of cellular signaling in JAK-STAT pathways that are engaged by IL-6 or LIF in the cells derived from IgAN patients (Yamada et al. 2017, 2020).

These observed genetic and genomic differences provide points of investigation as to how the final fidelity of IgA1 clustered *O*-glycans can be altered and lead to differences in observed distributions of IgA1 glycosylated proteoforms (IgA1 *O*-glycoforms).

14.6 Assessing Heterogeneity: Analysis of Glycan-Attachment Sites and Range of Heterogeneity

Analysis of IgA1 and IgA2 glycosylation has proven technically challenging due to the multiple molecular forms of IgA, the overall heterogeneity of *N*- and *O*-glycosylation, and the nature of the IgA1 HR where *O*-glycans are attached. Historically, several strategies have been used, including monosaccharide compositional analysis by gas-liquid chromatography (Renfrow et al. 2005) or high-performance anion-exchange chromatography with pulsed-amperometric detection (HPEAC-PAD), Edman sequencing (Baenziger and Kornfeld 1974; Mattu et al. 1998), glycan-specific lectin blotting and ELISA (Moldoveanu et al. 2007), and more recently liquid chromatography-mass spectrometry (LC-MS) (Takahashi et al. 2010). For IgA1 *O*-glycosylation, lectin ELISA has become a standard means to allow high-

throughput analyses in a quantitative manner for serum-based studies (Moldoveanu et al. 2007; Gomes et al. 2010; Moore et al. 2007); however, limitations for several of these methodologies include lack of individual glycoform specificity and the inability to provide information on the sites of attachment and heterogeneity in the context of amino-acid sequence. As a result, much of the standard analysis of IgA glycosylation has moved toward LC-MS.

MS-based techniques utilized to profile glycans and address glycan heterogeneity have become the standard tool for the structural characterization of carbohydrates. Over the last two decades, our understanding of the structures and diversity of glycans in biological samples has increased dramatically. This is the result of the significant improvements in the sensitivity and variety of mass spectrometry approaches. With the help of chromatographic separation, glycan derivatization to improve ionization, and tandem MS fragmentation techniques, oligosaccharide structures and sites of attachment can be elucidated (de Haan et al. 2019). Due to the increased use of IgG as a therapeutic, the majority of analytical development regarding glycosylation has focused on exploring *N*-glycan structures and heterogeneity. While the enzymatic release of *N*-glycans is still a common tool for global *N*-glycan analysis (glycomics), for Ig *N*-glycosylation, amino-acid site-specific analysis has become standard as well as MS analysis of intact proteins to assess the composition and distribution of *N*-glycan heterogeneity (de Haan et al. 2019). Such methodologies that have become standard for the analysis of IgG *N*-glycans are easily applicable to IgA *N*-glycans. As we will discuss more below, that trend has occurred but comparatively there are considerably more studies of *N*-glycans of IgG than IgA. For analysis of IgA *N*-glycans, as discussed above, nearly all sources of IgA in the body have some level of mixture of isotypes and molecular forms. Thus, many of the currently reported analyses of IgA glycosylation are mixtures of IgA produced from multiple sources, however, some groups have begun to make distinctions between various molecular forms.

The features of IgA1 clustered *O*-glycans create a unique analytical challenge. The *O*-glycans are often referred to as “mucin-like” given they mimic *O*-glycans found on the heavily *O*-glycosylated mucin family of proteins that line most epithelial surfaces. Mucins have a large number of tandem repeats that are *O*-glycosylated. The IgA1 HR has only two tandem repeats making it somewhat more amenable to heterogeneity analysis. The *O*-glycans of IgA1 have been implicated in the pathogenesis of IgAN (Tomana et al. 1997, 1999; Novak et al. 2005), and the closely related IgA vasculitis with nephritis (IgAVN) (Allen et al. 1998; Levinsky and Barratt 1979). The goal of identifying differences in IgA1 HR *O*-glycan heterogeneity and composition between patients and normal healthy individuals has driven the development of methodologies. This includes determining the range of IgA1 *O*-glycoforms present and the sites of *O*-glycan attachment defined in the context of adjacent sites as well as the heterogeneity at each individual site (Novak et al. 2018).

As stated, investigators in the IgAN field drove the initial progress in assessing IgA1 *O*-glycosylation. In 1996, Iwase et al. identified two IgA1 HR glycopeptides containing four or five *O*-glycan chains by use of MALDI-TOF mass spectrometry

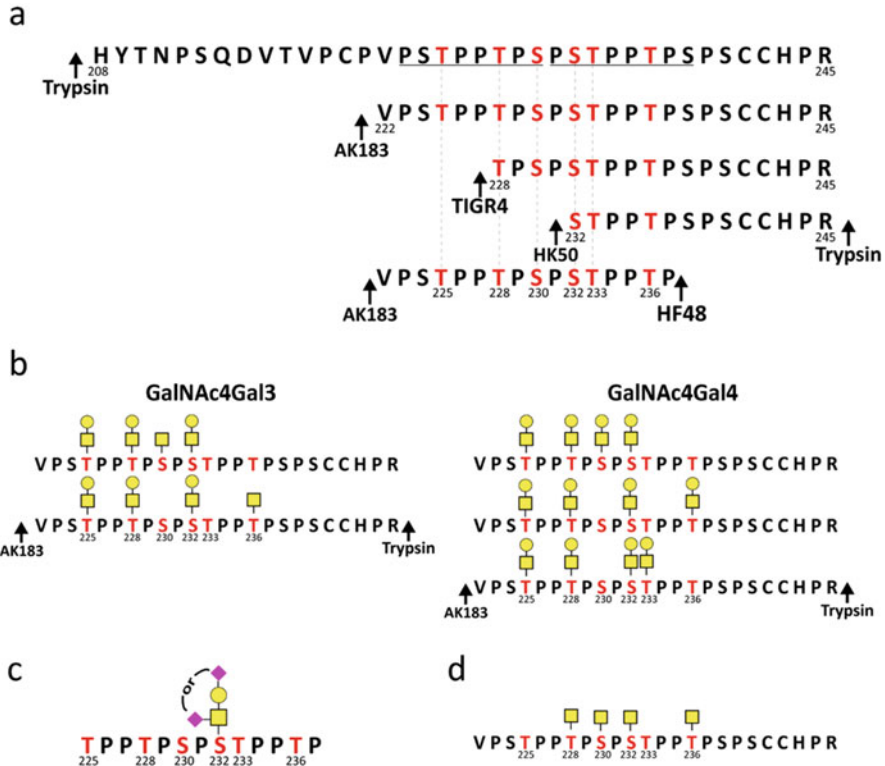


Fig. 14.4 Heterogeneity of IgA1 hinge-region *O*-glycosylation. (a) Amino-acid sequence of the hinge region of human IgA1 showing fragments cleaved by IgA-specific proteases (see Fig. 14.6 legend). The six commonly utilized *O*-glycosylation sites are highlighted in red and marked by the numbers below Ser/Thr residues for the AK183-HF48 fragment. The two tandem repeats are underlined in the trypsin fragment (top). (b) Examples of two identified IgA1 HR *O*-glycopeptide positional isomers, GalNAc4Gal3 and GalNAc4Gal4 produced by trypsin + AK183 IgA-specific protease. (c) Illustration of the potential for sialic acid to be attached to Gal and/or GalNAc residues creating isomers at a single site. (d) Four initial sites of GalNAc addition observed by *in vitro* GalNAc-T2 reactions (Stewart et al. 2019)

(Iwase et al. 1996a). These trypsin-released *O*-glycopeptides were confirmed as the IgA1 HR by sequential glycosidase treatment (neuraminidase, galactosidase, and *N*-acetylgalactosaminidase) and the spectra showed a shift in the peak distribution toward lower masses as carbohydrate residues of the *O*-glycan chains were removed. This promising beginning led to several reports of IgA1 *O*-glycopeptides isolated from serum (Iwase et al. 1998, 1999) and pooled sera of patients with IgAN (Odani et al. 2000), IgA1 isolated from pooled renal biopsies (Hiki et al. 2001), and tonsillar IgA1 (Horie et al. 2003). In 2000, Novak et al. reported the use of IgA-specific proteases that released distinct IgA1 HR fragments (Fig. 14.4) and provided novel means of generating IgA1 HR *O*-glycopeptides for analysis by mass spectrometry and lectins (Novak et al. 2000). This preparation was unique because one of the

IgA-specific proteases (Haemophilus HK50) cleaved between two sites of *O*-glycan attachment (cleavage after Pro231 between Ser230 and Ser232) and therefore provided a general localization of specific *O*-glycan chains N- or C-terminal to the cleavage site.

In terms of IgA1 *O*-glycan site localization, two groups initially identified the predominant sites of *O*-glycan attachment to include Thr225, Thr228, Ser230, Ser232, and Thr236 by use of modified N-terminal sequencing methods (Baenziger and Kornfeld 1974; Mattu et al. 1998). However, these reports analyzed the IgA1 population as a whole with no separation of individual IgA1 *O*-glycopeptides. Later, the introduction of electron radical fragmentation (electron capture dissociation, ECD, and electron transfer dissociation, ETD) in tandem MS lead to the direct assessment of individual IgA1 HR *O*-glycopeptide ions released by trypsin and other proteases, including IgA-specific proteases (Fig. 14.4a). A series of reports by the Renfrow group demonstrated the direct assessment of sites of *O*-glycan attachment for several IgA1 *O*-glycopeptide forms ranging from three to five glycans and having one or two sites comprised of only terminal GalNAc (i.e., galactose-deficient sites-containing IgA1; Gd-IgA1). In the 2012 paper, Takahashi et al. also reported the presence of several IgA1 *O*-glycopeptides that were comprised of isomeric mixtures of equal *O*-glycan chain compositions that were attached at alternate amino-acid sites in the IgA1 HR (Fig. 14.4b) (Takahashi et al. 2012). They also identified a predominant sixth site of *O*-glycan attachment at Thr233. The three attachment sites at Ser230, Thr236, and Thr233 were identified as the predominant sites with galactose-deficient IgA1 (Gd-IgA1) glycans in the IgA1 myeloma protein that was studied as well as two samples of serum IgA1 isolated from healthy individuals. Interestingly, it was the combination of the IgA-specific proteases (Fig. 14.4) plus the novel tandem MS fragmentation that allowed elucidation of many of the details of *O*-glycan attachment that are now considered standard in our understanding of what the heterogeneity of IgA1 *O*-glycans includes. Concurrent with these studies, a study comparing methodologies for assessing *O*-glycans was reported in 2010 using an IgA1 myeloma protein as the reference standard analyzed by fifteen different laboratories (Wada et al. 2010a). While none of the participating laboratories included amino-acid site-specific analysis of IgA1 myeloma reference standard, the study demonstrated that the range of IgA1 *O*-glycopeptides observed was consistent across MS platforms and that there was also a level of consistency in the relative abundance of individual IgA1 *O*-glycopeptides. Since these studies, there has been a steady increase in the use of MS and LC-MS methodologies to assess the distribution of IgA1 hinge-region *O*-glycosylated forms.

One area that remains a challenge is the assessment of IgA1 *O*-glycan sialylation. IgA1 *O*-glycans can be α 2,6 sialylated or α 2,3 sialylated at any given position. With anywhere from three to six *O*-glycans per hinge region, sialylation significantly increases the number of isomeric mixtures in a population of IgA1 *O*-glycosylated forms (Fig. 14.4c). Takahashi et al. demonstrated the potential for sialic acid isomers at a single site (Thr232). As such, many of the LC-MS analyses have used sialidases to remove the sialic acid residues and reduce the complexity of the IgA1 *O*-glycopeptide mixtures (Takahashi et al. 2010, 2012; Moldoveanu et al. 2007).

The goal of understanding the difference in IgA *O*-glycans in patients with IgAN vs. healthy individuals has led to the development of lectin-based quantitative ELISA tests (Moldoveanu et al. 2007; Zhao et al. 2012). Lectins recognize specific glycan structures and in the case of IgAN, several lectins specific for terminal GalNAc have been utilized in ELISA to provide an IgA1-specific assessment of the extent of Gal-deficient IgA1 in different cohorts. This is done by using IgA from serum or tissue samples and then probing for lectin reactivity (using lectins from *Helix pomatia* agglutinin, HPA or *Helix aspersa* agglutinin, HAA) compared to a reference standard. Through this analysis, investigators have been able to establish the range of Gd-IgA1 levels (based on standardized lectin reactivity) that exists in patients with IgAN, even to the point of utilizing Gd-IgA1 as a marker for IgAN disease progression (Zhao et al. 2012; Maixnerova et al. 2019; Reily et al. 2018). It is possible that LC-MS methodologies may at some point be able to provide a similar output, but currently, these lectin-based ELISA tests have been successfully implemented for larger-cohort studies.

14.7 Observed IgA *N*-Glycosylation Heterogeneity

The characterization and analysis of IgA *N*-glycosylation is a systematic process of localizing the composition and range of glycans linked to asparagine at the glycosylation sites. Assessing Ig *N*-glycosylation has become a common methodology in recent years due to the advent of therapeutic antibodies. This is more the case for IgG, but the principles of systematic mapping and assessment of the range of *N*-glycans at a given site by the use of mass spectrometry have become a tool utilized in the analysis of Igs. For example, these techniques can assess how glycosylation of IgG is affected in different forms, sources, and in responses to specific antigens/vaccines. In the case of IgA, there is the added complexity of assessing the J chain and SC sites of *N*-glycosylation as well as the *O*-glycosylated IgA1 HR (discussed below). To date, comprehensive studies have been performed for mIgA and SIgA from plasma, saliva, and colostrum samples and, in a few cases, these studies have included separation of IgA isotypes. Several different structures of *N*-glycans have been observed, contributing to heterogeneity for both IgA subclasses (Fig. 14.5). Glycan heterogeneity for mIgA and all potential SIgA *N*-glycosylation sites, including the seven on the SC, two on IgA1, four on IgA2, and one on the J chain, are detailed below. Overall, several sources identified IgA *N*-glycans to be mainly of the biantennary complex type, with various levels of galactosylation, sialylation, bisecting GlcNAc, and fucosylation (Steffen et al. 2020; Plomp et al. 2018; Goonatilleke et al. 2019; Bondt et al. 2016, 2017). The following text provides details of what has been observed to date, but continued experimental analysis of IgA glycan heterogeneity will ultimately aid in understanding the complex biological roles of IgA and SIgA (including SC and J chain) in health and disease.

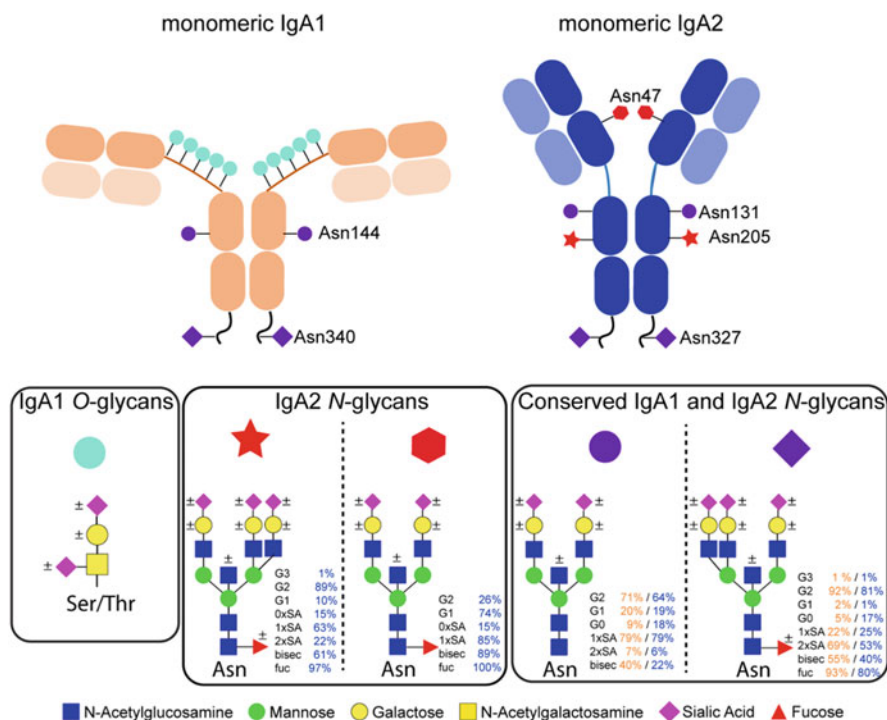


Fig. 14.5 Sites of IgA glycosylation. Illustration of IgA1 and IgA2 and their respective glycosylation sites. IgA1 has three to six observed sites of *O*-glycosylation (shown in cyan) and two IgA1 *N*-glycosylation sites (Asn144 and Asn340, shown in purple). IgA2 has four *N*-glycosylation sites (Asn47 and Asn205, shown in red; and Asn131 and Asn327, shown in purple). The sites in purple are conserved in both IgA subclasses. The outlined area contain representations of the possible *O*- and *N*-glycan structures reported at each site (Steffen et al. 2020; Plomp et al. 2018; Goonatilleke et al. 2019; Bondt et al. 2016, 2017; Deshpande et al. 2010; Huang et al. 2015; Gomes et al. 2008). The \pm symbol indicates the variable presence of each monosaccharide. The relative abundance values (%) are as reported by Steffen et al., who performed site-specific MS quantification of glycans from isolated serum IgA1 and IgA2 (Steffen et al. 2020). Values in orange correspond to IgA1 and values in blue correspond to IgA2

14.7.1 IgA1/IgA2 Asn340/327 *N*-Glycosylation

The *N*-glycosylation sites Asn340 and Asn327 on the tailpiece of IgA1 and IgA2, respectively, are located on tryptic peptides common to both IgA subclasses. Therefore, unless IgA1 and IgA2 are separated (often via jacalin agarose that binds only IgA1), MS analysis will not distinguish between subclasses and information on site-specific glycosylation is gathered for total IgA. At Asn340/327, two different tryptic peptide sequences are commonly identified, the expected LAGKPTHVNVSVVMAEVDGTCY and the truncated LAGKPTHVNVSVVMAEVDGTC. The abundance of each peptide seems to

depend on the origin of IgA, specifically IgA from plasma displays a higher abundance of the truncated peptide while IgA derived from saliva includes higher amounts of the peptide with the C-terminal tyrosine (Plomp et al. 2018). The glycans at this location are primarily biantennary complex-type; however, a low abundance of triantennary glycans (~5%) on the truncated peptide has been reported (Bondt et al. 2016). Additionally, high-mannose and hybrid glycans have been detected in low amounts on the non-truncated peptide (<5% IgA1; <20% IgA2) (Steffen et al. 2020). The relative abundance of bisection, galactosylation, sialylation, and fucosylation are similar for both the non-truncated and truncated peptide. Over 89% galactosylation, sialylation, and fucosylation has been observed for the *N*-glycans of IgA1. Sialylation of IgA1 non-truncated peptide was slightly higher compared to the truncated peptide (89% and 95%, respectively) (Bondt et al. 2016). When observed and reported, the non-truncated peptide on IgA2 displayed slightly lower bisection (<60%) compared to IgA1 (<40%). Fucosylation was also lower in IgA2 (80% IgA2 versus 95% IgA1) (Bondt et al. 2016). In SIgA versus mIgA, Plomp et al. (2018) noted lower fucosylation (1.4-times), galactosylation (2-times), and sialylation (5-times) and higher content of bisecting GlcNAc (1.7-times) in salivary compared to plasma IgA. High-mannose glycans were significantly higher in salivary IgA.

14.7.2 IgA1/IgA2 Asn144/131 N-Glycosylation

IgA1 Asn144 and IgA2 Asn131 also share the same tryptic amino-acid sequence LSLHRPALEDLLLSEANLTCTLTGLR, located in the CH2 domain. Analysis has shown that this site is primarily composed of complex-type *N*-glycans, and Plomp et al. (2018) identified approximately 20% high mannose-type glycans and up to 5% hybrid-type/mono-antennary glycans. Steffen et al. (2020) isolated IgA1 and IgA2, specifically identifying non-complex glycan abundance of each subclass at 5% and 20%, respectively. For IgA1, complete galactosylation and approximately 60% sialylation was observed. There was variation in the reported bisecting glycans of Asn144 on IgA1. For example, Bondt et al. reported approximately 25% bisecting glycans which increased to approximately 28% during pregnancy (Bondt et al. 2016). Other studies found it to be higher—between 40 and 50% for IgA1 (Steffen et al. 2020; Plomp et al. 2018). Furthermore, it has been well documented that glycans at Asn144/131 are almost entirely afucosylated (<1%), in contrast to Asn340/327 (Plomp et al. 2018; Bondt et al. 2016). Notable differences in plasma versus salivary IgA were seen for high-mannose glycans, bisecting GlcNAc, galactose, and sialylation (18-times, 1.5-times, 3-times, and 5-times higher in plasma IgA, respectively). This is an interesting finding, as a higher abundance of unprocessed glycans usually reflects rapid processing through the cellular machinery, differential expression of glycosylation enzymes, or restricted access to a specific glycosylation site.

14.7.3 *IgA2 N-Glycosylation*

For the glycosylation sites Asn47 in the CH1 domain and Asn205 in the CH2 domain, unique to IgA2, *N*-glycans were observed by Plomp et al. on the peptides SESGQNV^TAR and TPLTANITK, respectively. The glycans at these sites were mostly complex biantennary (>98% of total abundance) (Plomp et al. 2018). For plasma IgA2, Steffen et al. observed that both glycan sites were mainly monosialylated, had a high level of bisecting GlcNAc, and were almost always fucosylated (Steffen et al. 2020). Differences in galactosylation were observed as Asn47 was mainly monogalactosylated while the antennae of Asn205 were often fully galactosylated. Overall, two- to fivefold differences were observed in the relative abundances of the different glycan types present on plasma vs. salivary IgA2. Specifically, salivary *N*-glycans at both Asn47 and Asn205 showed a higher degree of bisecting GlcNAc and a lower degree of galactosylation, sialylation, and sialylation per galactose compared to plasma *N*-glycans (Steffen et al. 2020; Plomp et al. 2018; Bondt et al. 2016).

14.7.4 *Joining Chain (J Chain) N-Glycosylation*

The single *N*-glycosylation site Asn71 on the J chain has been observed as two tryptic peptides: EN₇₁ISDPTSPLR (JC Asn71) and the miscleaved IIVPLNNREN₇₁ISDPTSPLR (JC Asn71m). Plomp et al. (2018) determined that this site contained between 20 and 50% monoantennary and hybrid-type glycans, which is significantly higher than the IgA constant-domain *N*-glycosylation sites. However, differences in glycans in salivary and plasma IgA J chain are consistent with observations for the IgA1 and IgA2 heavy chain *N*-glycosylation sites, namely a higher bisection (3.2 times higher) and lower galactosylation (1.1 times lower) and sialylation (1.7 times lower) in the saliva-derived samples. Surprisingly, the two glycopeptides, JC Asn71 and JC Asn71m, exhibited different glycoprofiles. The miscleaved glycopeptides were more abundant in fucosylation as compared to the expected tryptic glycopeptides (3.2-times higher in plasma and 2.9-times higher in saliva) (Plomp et al. 2018). This observation was previously observed of colostrum, as 10% tryptic and 67% miscleaved glycopeptides were fucosylated (Deshpande et al. 2010). The range of glycan heterogeneity at this site is enhanced as the tryptic glycopeptide exhibits partial or full sialylation, which is uncommon for the miscleaved glycopeptide.

14.7.5 Secretory Component N-Glycosylation

Seven *N*-glycosylation sites are located on six SC tryptic glycopeptides. *N*-glycans at Asn135, Asn186, Asn421, and Asn469 were determined to be complex-type and biantennary with antennae fully galactosylated and partially sialylated (Plomp et al. 2018; Deshpande et al. 2010). However, other reports showed that sialylation was uncommon for Asn135, Asn186, Asn469, and Asn499 and highest for Asn421 and that Asn135 was abundantly tetraantennary (Huang et al. 2015). Mono-antennary species were identified on Asn499 and the observed glycoforms carried zero to five fucose residues, contributing to both the core and antennary fucosylation. On Asn135, Asn469, and Asn499 between 1 and 4% bisection was observed. All glycosylation sites contained at least five galactose residues and one to three fucose residues.

Glycans Asn83 and Asn90 are located on the same tryptic peptide, complicating site-specific glycosylation analysis. This can be remedied by employing additional proteases. Furthermore, the joint glycan composition $H_{10}N_8F_{2-8}S_{0-3}$ at Asn83 and Asn90, reported by Plomp et al., indicates a similar composition to other SC glycans (Plomp et al. 2018).

14.8 IgA Receptors and Role of Glycans

As detailed above, IgA subclasses are quite diverse in the origin, molecular form, and site-specific glycosylation profiles. The isotype, glycosylation, and molecular form of IgA can impact interactions with various types of IgA receptors. In humans, these receptors include Fc receptors (Fc α -receptor I [Fc α RI; CD89] and Fc α / μ receptor [CD351]), polymeric immunoglobulin receptor, transferrin receptor (CD71), lectins (e.g., asialoglycoprotein receptor on hepatocytes), cell-surface galactosyltransferase (e.g., β 1,4-galactosyltransferase), and Fc receptor-like 4 (FcRL4, CD307d) (de Sousa-Pereira and Woof 2019; Monteiro and Van De Winkel 2003; Breedveld and van Egmond 2019; Cho et al. 2006; Aleyd et al. 2015; Honda et al. 2016; Yang et al. 2013; Tomana et al. 1993). Fc α RI is specific for IgA1 and IgA2 and FcRL4 binds polymeric IgA and IgA found in immune complexes, whereas other receptors can bind other ligands in addition to IgA. Below, we discuss several IgA receptors, focusing on details with respect to glycosylation and molecular forms of IgA.

Fc α RI is expressed by myeloid cells, such as monocytes, neutrophils, and some subsets of macrophages and dendritic cells (van Egmond et al. 2001). Serum IgA, predominantly monomeric IgA1, can bind to Fc α RI (CD89) (Monteiro et al. 1990; Herr et al. 2003). This receptor plays the main role in the IgA-mediated clearance of pathogens and cancer cells (Woof and Kerr 2006; de Tymowski et al. 2019; Brandsma et al. 2019; Hansen et al. 2018, 2019; Heemskerk and van Egmond 2018). Both the ligand (IgA1 and IgA2) and the receptor are glycosylated (Mattu

et al. 1998; Royle et al. 2003; de Sousa-Pereira and Woof 2019; Aleyd et al. 2015). Whereas IgA *N*-glycans affect IgA thermal stability but not receptor binding (Gomes et al. 2008; Göritzer et al. 2019), *N*-glycans of Fc α RI significantly modulate binding affinity to IgA (Göritzer et al. 2019). Furthermore, the binding of IgA1 to Fc α RI induces long-range conformational changes in IgA1, propagating up to the *O*-glycosylated HR (Posgai et al. 2018).

Fc α RI does not contain any signaling motifs in its cytoplasmic tail and Fc α RI-mediated signaling depends on the associated Fc receptor γ chain and its immunoreceptor tyrosine-based activation motifs. Fc α RI-mediated activation can induce degranulation, phagocytosis, chemotaxis, and antibody-dependent cellular cytotoxicity. Furthermore, when IgA-Fc α RI signaling is combined with activation through pattern recognition receptors (e.g., Toll-like receptors), cytokine production can be induced in antigen-presenting cells. This process is essential for controlling inflammation and inducing both innate and adaptive immune responses. Depending on whether IgA binds to Fc α RI in a soluble or aggregated form, it can induce immunosuppressive or pro-inflammatory responses. Soluble forms of IgA in the circulation, monomers and dimers, have low affinity for Fc α RI and bind only transiently, thus mediating inhibitory signaling under homeostatic conditions (Hansen et al. 2019). Similar inhibitory effects can be exerted by peptidomimetics and such approaches may be useful to prevent undesirable inflammatory conditions triggered by abnormal IgA-containing immune complexes, such as in IgA-mediated blistering skin diseases (Breedveld and van Egmond 2019; Heineke et al. 2017; Ben Mkaddem et al. 2019).

A recent study underlined differences in the effector functions of human serum IgA1 and IgA2 on myeloid cells. IgA proteins were heat-aggregated or immobilized to mimic immune complexes. Under these conditions, IgA2 was more effective than IgA1 in the induction of pro-inflammatory responses in neutrophils and macrophages (Steffen et al. 2020). However, these differences disappeared after the enzymatic removal of sialic acid or all *N*-glycans from IgA1. Thus, IgA effector functions depend on the subclass and glycosylation, as IgA1 and IgA2 have similar but different glycosylation profiles (IgA2 has 2 additional sites of *N*-glycosylation). Notably, IgA1 contains more sialic acid than IgA2, thus explaining the nature of distinct IgA subclass activities.

Asialoglycoprotein receptor on hepatocytes mediates clearance of IgA (and other asialoglycoproteins) from the circulation (Stockert et al. 1982; Baenziger and Maynard 1980; Baenziger and Fiete 1980; Rifai et al. 2000; Tomana et al. 1988). The binding of IgA is based on recognition of terminal Gal or GalNAc residues by the lectin receptor whereas sialic acid prevents this binding. The IgA molecules internalized by the asialoglycoprotein receptor are degraded and excreted into the bile. This catabolic pathway is thought to explain the relatively short half-life of IgA in the circulation (~5 days).

Polymeric immunoglobulin receptor mediates the specific transport of polymeric immunoglobulins (J-chain-containing polymeric IgA and IgM) across the mucosal epithelium into the secretions. The transport of dimeric IgA across the epithelium (transcytosis) begins with its binding to the polymeric immunoglobulin receptor at

the basolateral surface of the epithelial cell, which is followed by internalization and transport via vesicular compartments to the apical surface of the cell. During the process, the polymeric immunoglobulin receptor is cleaved to release the SC part of the receptor. Secretory component is attached through a disulfide bridge to dimeric IgA, forming secretory IgA released at the apical surface of mucosal epithelium. Notably, SIgA1 exhibits different *O*-glycosylation compared to circulatory IgA1. Circulatory IgA1 contains core 1 *O*-glycans (i.e., GalNAc-Gal that may be sialylated) whereas secretory IgA1 has also core 2 *O*-glycans with extended branches (Royle et al. 2003).

The transferrin receptor (CD71) binds IgA1, but not IgA2, and the IgA1 binding is inhibitable by transferrin (Moura et al. 2001). CD71 binds polymeric, but not monomeric IgA1, and the binding is dependent on glycosylation, namely *O*-glycosylation (Moura et al. 2004). CD71 is thought to participate in the binding of pathogenic IgA1-containing immune complexes by mesangial cells in IgAN (Moura et al. 2004).

FcRL4, expressed by a subset of memory B cells in the epithelia, recognizes polymeric IgA with J chain and heat-aggregated IgA but not secretory IgA (Wilson et al. 2012; Liu et al. 2020). FcRL4 is an inhibitory receptor for IgA. In addition to the four extracellular C2-type Ig domains and a transmembrane domain, it has a cytoplasmic domain that contains three immune-receptor tyrosine-based inhibitory motifs. FcRL4 is thought to regulate B cell responses to mucosal commensal antigens (Liu et al. 2018) and FcRL4-positive B cells have significantly increased usage of the IgA isotype (Amara et al. 2017). FcRL4-positive B cells are also enriched in the joints of patients with rheumatoid arthritis and it is thought that the production of cytokines with bone remodeling activity contributes to the disease pathology (Amara et al. 2017). It is currently not known whether glycosylation of IgA impacts recognition by FcRL4.

These several examples illustrate how glycosylation can impact interactions of different molecular forms of IgA with various types of IgA receptors. Consequently, these glycan-dependent interactions impact the effector function of specific IgA glycoproteoforms.

14.9 Observed IgA1 *O*-Glycosylation Heterogeneity

Based on the involvement of IgA1 in the pathogenesis of IgAN and other similar diseases, considerable progress has been made in the analysis of *O*-glycosylation of serum/plasma IgA1. Much of the current knowledge of IgA1 heterogeneity is based on the analysis of IgA1 myeloma proteins that have been extensively used for the development and comparisons of methodologies discussed above (Renfrow et al. 2005, 2007; Takahashi et al. 2010, 2012). These proteins have also served as model proteins for in vitro and in vivo experiments to understand the pathogenesis of IgAN (Takahashi et al. 2014; Novak et al. 2007, 2011a, b, 2015; Yanagihara et al. 2012; Knoppova et al. 2016; Suzuki et al. 2009, 2019; Rizk et al. 2019; Moldoveanu et al.

2021). Additionally, as the IgA1 HR has only two amino-acid tandem repeats rather than multiple repeats found in mucins, it has been used in many studies as a template for the understanding of clustered *O*-glycan synthesis by glycosyltransferases (Wandall et al. 2007; Ten Hagen et al. 2003; Fritz et al. 2004). This has also contributed to the understanding of what the final IgA1 *O*-glycan heterogeneity is and how it could be altered in a disease such as IgAN.

Nomenclature for IgA1 *O*-glycosylation forms core 1 *O*-glycans is based on glycan composition starting with the first monosaccharide attached, GalNAc, followed by Gal and then sialic acid (SA). A designation of GalNAc4Gal4 implies four *O*-glycan chains per heavy-chain HR comprised of GalNAc + Gal disaccharides. A designation of GalNAc5Gal4 implies five *O*-glycan chains comprised of four disaccharides and a single GalNAc monosaccharide. When sialic acid residues are assigned, such as GalNAc4Gal3SA1 or GalNAc5Gal5SA2, there is no assumption of where or how the SA is linked. IgA1 with at least one GalNAc without Gal in the HR *O*-glycans is often referred to as Gd-IgA1. Among the observed Gd-IgA1 glycoforms, there are variants with one to three Gal-deficient sites per HR. However, it should be noted that these forms are seen in IgA1 from healthy individuals as well as patients with IgAN. Still, most patients with IgAN exhibit elevated circulatory levels of Gd-IgA1.

Early studies of IgA1 from patients with IgAN and healthy controls observed the HR with three to six *O*-glycans (Baenziger and Kornfeld 1974; Mattu et al. 1998; Takahashi et al. 2010, 2012). This distribution has been consistent across many analyses by MS of serum and IgA1 myeloma proteins. There have been some examples of IgA1 myeloma proteins with as few as one to three *O*-glycans per HR, but these appear to be outside the norm (Renfrow et al. 2007). Consistently, the distribution of *O*-glycosylated forms appears to center around those with four (GalNAc4Gal4) and five (GalNAc5Gal4) *O*-glycans (Novak et al. 2000; Takahashi et al. 2010; Iwase et al. 1996a; Wada et al. 2010a; Renfrow et al. 2005). The 2010 comparison study (Wada et al. 2010a) across fifteen different labs confirmed that there was a clear distinction in the abundance of glycoforms no matter what MS technology was used. MS studies of site-specific protein and peptide glycosylation heterogeneity have made use of relative quantitation to reflect the consistent pattern and abundance of glycoform distributions that are observed. Experimentally manipulated changes in abundance are consistent and reflect that the ionization is driven by the peptide portion of the molecule (Stewart et al. 2019). Several groups have demonstrated this for both *N*- and *O*-glycosylation (Steffen et al. 2020; Plomp et al. 2018; Goonatilleke et al. 2019; Deshpande et al. 2010; Huang et al. 2015) as well as in the analysis of therapeutic glycoproteins (Yang et al. 2016). When IgA1 populations are analyzed with sialidase pretreatment, the distribution is dominated by GalNAc4Gal4 and GalNAc5Gal4 (usually >50% of total combined) followed by a second tier including GalNAc4Gal3, GalNAc5Gal3, GalNAc5Gal5, and GalNAc3Gal3 (usually 30–40% of total combined). The remaining distribution is comprised of GalNAc6Gal4, GalNAc6Gal3, GalNAc6Gal5, GalNAc3Gal2, GalNAc4Gal2, and GalNAc5Gal2 (each representing <5% of total) (Takahashi et al. 2012; Ohshima et al. 2020b). As discussed more below, most of these identified

IgA1 *O*-glycopeptides are mixtures of amino-acid positional isomers (i.e., glycans attached at variable sites) further complicating the complete assignment of the total distribution of clustered *O*-glycan heterogeneity (Fig. 14.4b).

When viewed in the context of the IgA1 structure, the distribution of *O*-glycosylated forms is a result of the number of available Ser and Thr in the two tandem repeat amino-acid sequence, the enzymatic activity of the glycosyltransferases, and the three-dimensional constraints of the clustered amino-acid sites and flanking cysteines that are involved in disulfide bonds as part of the overall IgA1 quaternary structure. A recent analysis of *in vitro* reactions of GalNAc-T2 enzyme with IgA1 HR acceptor peptide identified a fast-rate phase of clustered GalNAc addition. For the GalNAc-T2 clustered activity on the native IgA1 HR, the fast phase ends after three GalNAc residues are added, matching the lower end of the predominant IgA1 *O*-glycosylated forms observed in serum IgA1 (Stewart et al. 2019). Interestingly, the natural occurrence of proteoforms with up to six *O*-glycan chains observed for serum IgA1 clustered *O*-glycans is less than what is observed for *in vitro* synthesis reactions on IgA1 HR peptide substrates where seven and eight additions of GalNAc have been reported (Stewart et al. 2019, 2021). This is where the overall structure of the IgA1 heavy chain and the flanking cysteine residues likely play a role in limiting the final *O*-glycan density. It is also possible that the concurrent addition of Gal and SA to the existing GalNAc residues limits the final heterogeneity as well.

As mentioned above, within this range of three to six *O*-glycans per HR, it is also the presence of amino-acid positional isomers that were initially identified in serum IgA1 (Takahashi et al. 2012). These isomeric mixtures occur from the outset of the synthesis process as demonstrated by *in vitro* GalNAc-T2 reactions (Stewart et al. 2019). Instead of a strictly ordered process of preferred amino-acid site addition, Stewart et al. demonstrated that there were four alternative initial sites of GalNAc addition at Thr228, Thr236, Ser230, and Ser232 with Thr228 and Thr236 being favored over the other two (Fig. 14.4d) (Stewart et al. 2019). Each initial site of GalNAc addition led to a unique combination of possible second sites of GalNAc addition. While the sites of GalNAc addition were all consistent with those seen with serum IgA1, these experiments revealed multiple GalNAc-addition pathways and explained the biosynthetic origin of isomeric mixtures of IgA1 *O*-glycosylated forms. Additional types of isomers can then occur due to variable sites of the addition of Gal and SA residues. Despite these variable *O*-glycosylation pathways, there is a consistent fidelity of the final IgA1 *O*-glycosylation distribution. For example, Ser230, Thr236, and Thr233 are the sites that show the predominant mixtures of isomers at the disaccharide and monosaccharide levels (Takahashi et al. 2012). These same sites are the predominant sites of Gal deficiency in serum IgA1. A recent study of serum IgA1 used enzymatic removal of GalNAc-Gal disaccharides (Ohyama et al. 2020b). This process resulted in consolidated mixtures of Gd-IgA1 forms with one, two, or three Gal-deficient sites (Ohyama et al. 2020b). These results corroborated Ser230, Thr236, and Thr233 as the predominant sites with Gal-deficient glycans but also indicated low levels of Thr228 and Thr232 with a single GalNAc residue as well. This study thus correlates with the data from *in vitro*

studies of GalNAc-T2 that revealed multiple isomer possibilities. Since there are other GalNAc-Ts that have demonstrated activity on the IgA1 HR, it is possible that the distribution of IgA1 glyco-isomers is affected by expression patterns of GalNAc-Ts in specific IgA1-producing cells.

O-glycan heterogeneity of IgA1 can be impacted by additional constraints for the addition of Gal and SA. Based on the predominance of GalNAc4Gal4 and GalNAc5Gal4 HR glycoforms, it is logical to conclude the addition of up to four Gal residues is feasible for the C1GalT1 enzyme. The abundance of GalNAc5Gal5 HR glycoforms varies across some reports (~5–10%). This could reflect steric hindrances affecting the addition of a fifth Gal addition and/or a competition with SA addition occurring at the same time. The presence of SA on adjacent *O*-glycan chains has been demonstrated to inhibit C1GalT1 addition of Gal (Stewart et al. 2021; Takahashi et al. 2014; Suzuki et al. 2014). A similar inhibition has been demonstrated for SA addition by ST6GalNAc2 with prior addition of SA by ST3Gal1 and vice versa (Stewart et al. 2021; Suzuki et al. 2014). Interestingly, reports on *O*-glycan heterogeneity of serum IgA1 and IgA1 myeloma proteins have not found more than five SA residues for single IgA1 HR *O*-glycoforms despite there being two potential sites of addition on each glycan of core 1 disaccharide. This could be either the result of the steric hindrance of the clustered *O*-glycans or an artifact of analyzing IgA1 HR by positive-ion mode MS where SA residues add a negative charge. To date, it is unclear if a full accounting of the range of SA in IgA1 *O*-glycans has been accomplished.

In summary, while the generalized IgA1 *O*-glycan heterogeneity is consistent with three to six *O*-glycans per HR, there are underlying isomer mixtures that are likely cell-specific. Additionally, there is ample evidence from lectin ELISA studies that this complex heterogeneity is altered or shifted, reflecting elevated serum levels of Gd-IgA1 in patients with IgAN. The origin of these differences in IgA1 *O*-glycan heterogeneity is an active area of research.

14.10 IgA-Related Diseases

14.10.1 IgA Nephropathy

IgA nephropathy (IgAN) is an autoimmune disease characterized by the glomerular deposition of immune complexes containing galactose-deficient IgA1 (Gd-IgA1) (Knoppova et al. 2016; Rizk et al. 2019). This altered glycosylation results in the increased presence of terminal GalNAc or sialylated-GalNAc in the IgA1 HR. Gd-IgA1 in the glomerular immunodeposits is usually co-deposited with complement C3 and IgG autoantibodies specific to the abnormal glycosylation on the HR of Gd-IgA1. IgAN patients often have elevated levels of Gd-IgA1 and the corresponding IgG autoantibodies in the circulation, leading to the formation of Gd-IgA1-IgG circulating immune complexes. Circulatory levels of Gd-IgA1 are predictive of both disease progression and recurrence after transplantation (Berthoux

et al. 2017). The current hypothesis on the pathobiology of IgAN highlights a four-hit mechanism, where elevated levels of Gd-IgA1 in the circulation (1) coupled with the production of anti-Gd-IgA1 IgG autoantibodies (2) leads to the formation of circulating immune complexes (3) some of which deposit in the glomeruli (4) to induce renal injury (Novak et al. 2008).

The galactose deficiency in IgAN affects HR glycans of the IgA1 and, thus, represents a change in *O*-glycosylation (Fig. 14.3). Gd-IgA1 in the circulation is predominantly in the polymeric (dimeric) form, although monomeric IgA1 is the predominant molecular form in the circulation; mechanisms leading to circulation of this molecular-form-specific effect are not known. In addition to genetically determined serum levels of Gd-IgA1, several studies show that some cytokines can elicit increased Gd-IgA1 production in IgA1-producing cells from IgAN patients. This effect is mediated by reduced expression of the galactosyltransferase encoded by the *C1GALT1* gene, C1GalT1, an enzyme that is responsible for addition of galactose to the GalNAc residues in the HR of IgA1 (Suzuki et al. 2014; Yamada et al. 2017, 2020). Two GWAS studies have found association between IgAN and SNPs in the *C1GALT1* locus, as well as its chaperone protein Cosmc encoded by *C1GALT1C1* (Kiryluk et al. 2017).

The glycosylation of Gd-IgA1 in IgAN exhibits microheterogeneity patterns, both at the level of site attachments and specific glycan composition at each site (Novak et al. 2018; Franc et al. 2013; Ohyama et al. 2020a; Moore et al. 2007). The implication for this variability in glycosylation motifs suggests a semi-stochastic process based on enzyme activities and locations in the Golgi apparatus. Further research is needed to ascertain details of the mechanisms affecting the levels of Gd-IgA1 in the circulation of IgAN patients.

14.10.2 IgA Vasculitis with Nephritis (Formerly Known as Henoch–Schönlein Purpura Nephritis)

IgA vasculitis (IgAV) with nephritis (IgAVN) exhibits kidney-pathology features similar to IgAN, including IgA1 immunodeposits in the mesangium (Selewski et al. 2018). Notable, some patients with IgAVN progress to IgAN. As in patients with IgAN, IgAVN patients have elevated circulating levels of Gd-IgA1 (Kiryluk et al. 2011; Suzuki et al. 2019; Lau et al. 2007; Nakazawa et al. 2019; Pillebout et al. 2017) that form pathogenic immune complexes (Suzuki et al. 2019; Novak et al. 2007). The Gd-IgA1 immune complexes in IgAV typically deposit in the small vessels, leading to systemic vasculitis. In patients with IgAV, only a small fraction develops nephritis, 4–6 weeks from the disease onset, with mesangial proliferation found upon renal biopsy (Lau et al. 2010; Boyd and Barratt 2011). These biopsies show deposition of Gd-IgA1 in a fashion similar to IgAN (Zhao et al. 2020; Sugiyama et al. 2020). This, along with genetic studies showing heritability of circulatory Gd-IgA1 levels in patients with IgAN and IgAVN, indicates a close

relationship between IgAVN and IgAN (Kirylyuk et al. 2011; Suzuki et al. 2018; Hastings et al. 2021).

14.10.3 Crohn's Disease

Crohn's disease is a subset of inflammatory bowel diseases, which affects approximately 1% of the US population, and is characterized by inflammation of the gastrointestinal tract (Hanauer 2006). Serological predictors of this disease have been difficult to determine, but recent work on IgA1 HR glycoforms found that decreased number of GalNAc residues per HR was associated with progression vs. recovery in Crohn's patients. Additionally, IgA1 with reduced content of *O*-glycans was found in the inflamed sections of intestinal biopsies from Crohn's patients (Inoue et al. 2012).

14.10.4 Sjögren's Syndrome

Sjögren's syndrome is an autoimmune disease that can affect the tear and salivary glands, with a minority of patients developing other rheumatoid complications, such as systemic lupus erythematosus and rheumatoid arthritis. Patients with Sjögren's syndrome have increased sialic acid content and decreased galactose content on both IgA1 and IgA2 *N*-glycans (Dueymes 1995). Additionally, Sjögren's syndrome patients also have elevated serum levels of IgA1, and follow-up analysis showed that IgA1 was predominantly oversialylated in *N*-glycans (Basset et al. 2000; Levy et al. 1994).

14.10.5 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease where the immune system targets multiple tissues and organs, such as the brain, lung, kidneys, joints, and vasculature. Most SLE patients have a positive anti-nuclear antibody test, but additional tests for anti-ds DNA antibodies, anti-Smith antibodies, and anti-U1RP antibodies can be more specific (Mummert et al. 2018; Olsen et al. 2017). Elevated circulating levels of IgA, 4–6-times higher than in healthy individuals, and abnormal glycosylation of IgA were observed in SLE patients. IgA isolated from female SLE patients showed decreased levels of unbisected biantennary, and tri- and tetraantennary oligosaccharides. Additionally, decreased galactosylation of the HR of IgA1 was found in the same female SLE patients (Matei and Matei 2000).

14.10.6 Rheumatoid Arthritis

The clinical manifestations of rheumatoid arthritis (RA) are swelling and pain in the joints, bone, and cartilage due to inflammation from inappropriate targeting by the immune system. The standard test for RA is a blood analysis of IgG/IgA/IgM antibodies against IgG (RF; rheumatoid factor) and ACPA (anti-citrullinated protein antibodies) (Westra et al. 2021; Kurowska et al. 2017). Mass spectrometry analysis of IgA1 isolated from the circulation of RA patients showed decreased content of GalNAc in the HR of IgA1 (Wada et al. 2010b). Additionally, both IgG and IgA from synovial fluid showed differential galactosylation and sialylation at early and late stages of RA. Terminal α 2,6 sialic acid on IgA was lower in early RA compared to advanced RA, while terminal Gal on IgA was lower at early stages of RA and normalized later in the disease (Kratz et al. 2010).

14.10.7 IgA Myeloma

IgA myeloma is a clonal expansion of some IgA-producing cells that undergo genetic mutations leading to uncontrolled proliferation and increased serum levels of IgA (ranging from <30 g/L to >30 g/L) as the disease develops and progresses. Patients with IgA myeloma can develop multiple complications affecting the kidneys and bone and can exhibit various hematological pathologies. Production of IgA from clonal expansion in myeloma was found to include IgA glycoforms that were hyposialylated, which can affect Fc α R1 binding (Basset et al. 1999; Bosseboeuf et al. 2020). Studies of the HR *O*-glycans from different IgA1 myeloma proteins have shown ranges of *O*-glycans outside the norm of three to six glycans observed in normal human serum (Renfrow et al. 2007). IgA1 deposition in the kidneys of some patients with IgA myeloma has been found concomitantly with under-galactosylated HRs, similar to the abnormality common to IgAN (Zickerman et al. 2000).

14.10.8 Celiac Disease

Celiac disease is an inflammatory intestinal condition brought on by antibodies targeting the transglutaminase 2 (TG2) protein and gluten-derived TG2-deamidated gliadin peptides. In patients with celiac disease, circulating IgA that targets TG2 has been found to be under-galactosylated, in addition to nonTG2 targeting IgA in circulation (Lindfors et al. 2011; Abbad et al. 2020). CD89 may be involved (Papista et al. 2015) and the interaction of multiple proteins, including CD71, was proposed (Lebreton et al. 2012; Papista et al. 2012).

14.10.9 Complement and IgA

IgA has been considered an anti-inflammatory immunoglobulin (Monteiro 2014; Diana et al. 2013; Ben Mkaddem et al. 2013). Unlike IgG, IgA-mediated activation of complement is not well understood, but it is generally accepted that IgA does not activate the classical complement pathway. However, IgA is thought to be able to activate alternative and lectin pathways of complement, under some circumstances. For example, the less abundant monomeric IgA2 was found to bind mannose-binding lectin (MBL) in ELISA. Additionally, when IgA1 or IgA2, both monomeric and polymeric, were treated with a galactosidase to remove galactose, their binding to MBL increased by over 10-fold (Terai et al. 2006). This would presumably be due to reducing the heterogeneity complexity of *N*-glycans in favor of high mannose glycans such as those observed at Asn340/327 sites. Additionally, IgA1 *N*-glycosylation is critical for binding C3, as evidenced by using point-mutation deletions in critical Asn sites in the Fc region (Chuang and Morrison 1997). This observation was further validated by inhibiting *N*-glycosylation of IgA using tunicamycin and testing for C3 binding (Zhang and Lachmann 1994). Interestingly, sialic acid content was also found to be critical for both IgA1 and IgA2 to fix C3b (Nikolova et al. 1994). In IgAN, where Gd-IgA1 is found in the polymeric form, the polymeric IgA1 is found to be able to bind MBL, resulting in C4 mesangial deposition (Oortwijn et al. 2006; Roos et al. 2001). Of note, complement activation resulting from abnormal IgA1 glycosylation can be found indirectly via anti-Gd-IgA1 IgG autoantibodies in IgAN, where the IgG presumably activates C3 when bound in an immune-complex with Gd-IgA1 (Novak et al. 2015; Knoppova et al. 2016; Rizk et al. 2019; Maillard et al. 2015).

14.11 Infectious Diseases

Some bacteria that can cause human infections utilize tools that negatively affect the defense mechanisms of the host. These tools include IgA-specific proteases and various glycosidases. Whereas the former act exclusively on IgA, the latter affect multiple glycoproteins at the mucosal surfaces. Below, we focus on several aspects of these bacterial virulence factors most relevant to IgA structural integrity and function.

14.11.1 Bacterial IgA-Specific Proteases

Multiple bacterial species that colonize human mucosal surfaces in the oral cavity, digestive tract, respiratory tract, and genital tract produce IgA-specific proteases. These bacteria include streptococci (e.g., *Streptococcus pneumoniae*, *S. sanguinis*,

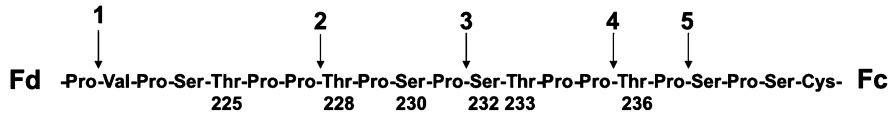


Fig. 14.6 Bacterial IgA-specific proteases target the hinge region of IgA1. Amino-acid sequence of the hinge region of human IgA1. Six commonly utilized *O*-glycosylation sites are marked by the numbers below Ser/Thr residues. Numbers 1–5 with arrows above the amino-acid sequence mark peptide bonds that are cleaved by IgA-specific proteases produced by the following species and strains shown as examples: 1, *Clostridium ramosum* AK183; 2, *Streptococcus pneumoniae* TIGR4; 3, *Haemophilus influenzae* HK50; 4, *Neisseria gonorrhoeae* HF13; and 5, *Neisseria gonorrhoeae* HF48

S. mitis, and *S. oralis*), *Prevotella* and *Capnocytophaga* species and several *Neisseria*, *Clostridium*, and *Haemophilus* species (e.g., *Clostridium ramosum*, *Neisseria gonorrhoeae*, *N. meningitidis*, *Haemophilus influenzae*) (Kilian and Holmgren 1981; Kilian et al. 1979, 1983; Senior et al. 2000; Frandsen et al. 1987, 1995, 1997; Reinholdt et al. 1990; Poulsen et al. 1989, 1996; Lomholt et al. 1992; Lomholt and Kilian 1994; Reinholdt and Kilian 1997; Kosowska et al. 2002; Wang et al. 2020; Chi et al. 2017; Murphy et al. 2015; Janoff et al. 2014; Johnson et al. 2009; Wani et al. 1996).

The bacterial IgA-specific proteases are post-proline endopeptidases that include metalloproteases, serine proteases, and cysteine proteases. These proteases recognize and cleave HR of IgA1, except the protease from *Clostridium ramosum* that can cleave both IgA1 and IgA2, with variable site-specific preferences (Senior et al. 2000; Kosowska et al. 2002; Batten et al. 2003; Mistry and Stockley 2011; Senior and Woof 2005). Figure 14.6 shows examples of commonly recognized sites in IgA1 HR by several representative IgA-specific proteases.

It is thought that bacterial IgA-specific proteases represent a virulence factor for many pathogenic bacteria. Cleavage of IgA1 breaks the Fc fragment from its antigen-binding Fab fragments. This process thus reduces the protective effects of IgA1, such as inhibition of bacterial adherence and antibody complement-dependent killing of bacteria by phagocytic cells. In vitro studies of these proteases have demonstrated that the clustered IgA1 HR *O*-glycans can inhibit cleavage of some proteases indicating a shielding effect of this susceptible region of IgA1 by the *O*-glycans. Various studies examined and confirmed these concepts and searched for IgA-specific protease inhibitors (Wang et al. 2020; Janoff et al. 2014; Weiser et al. 2003; Garner et al. 2013).

14.11.2 Bacterial Glycosidases and Metabolic Utilization of IgA Glycans

Various bacteria occupying mucosal surfaces produce hydrolytic enzymes, such as endo- and exo-glycosidases, able to degrade *O*- and *N*-glycans of mucosal

glycoproteins, including IgA. For example, in bacterial vaginosis, a common polymicrobial imbalance of the vaginal flora, IgA in the secretions is subjected to stepwise deglycosylation and subsequently enhanced proteolysis. These modifications are thought to compromise the ability of IgA to neutralize and eliminate pathogens (Lewis et al. 2012; Robinson et al. 2019; Govinden et al. 2018; Moncla et al. 2016; Smith and Ravel 2017). *Streptococcus pneumoniae* is a major airways pathogen that can produce multiple exo- and endoglycosidases to degrade *O*- and *N*-glycans of IgA and other glycoproteins (King et al. 2006; Marion et al. 2009; Syed et al. 2019; Robb et al. 2017; Blanchette et al. 2016; Kahya et al. 2017). For *S. pneumoniae* colonization of the upper airways, neuraminidase NanA is essential (Brittan et al. 2012). In some instances, a synergy between *S. pneumoniae* and influenza A sialidases can impact nasal colonization and middle ear infection by pneumococci (Wren et al. 2017). Conversely, IgA glycans, specifically sialic acid of the tail *N*-glycans, can inhibit influenza A and other enveloped viruses that use sialic acid as a receptor (Maurer et al. 2018). Another example of pathogenic bacteria utilizing glycosidases includes periodontitis and the associated bacteria, such as *Porphyromonas gingivalis* known to target host sialylated glycoproteins for immune dysregulation (Sudhakara et al. 2019). In the gastrointestinal system, IgA has multiple functions and glycans of secretory IgA1 (SIgA) are known to mediate the binding of SIgA to the microbiota. In fact, it has been proposed that IgA1 *N*- and *O*-glycans provide a link between innate and adaptive immune systems that sustain intestinal homeostasis (Royle et al. 2003; Reily et al. 2019; Mathias and Corthésy 2011a; Pabst and Slack 2020; Gupta et al. 2019; Nakajima et al. 2018; Corthésy 2013; Mathias and Corthésy 2011b). The net result of releasing the glycans is not only immune dysregulation and immune evasion but also a metabolic advantage. Some bacteria can metabolize the released glycans to support their growth (Perman and Modler 1982; Garbe and Collin 2012; Sjögren and Collin 2014).

14.11.3 IgA as a Potential Therapeutic Antibody

As outlined earlier, functions of human IgA subclasses, IgA1 and IgA2, are impacted by multiple aspects, including glycosylation, that directly and indirectly regulate IgA effector functions. For example, IgA1, but not IgA2, has *O*-glycans in addition to *N*-glycans and is more sialylated at the *N*-glycans. Consequently, IgA2 can induce pro-inflammatory responses in neutrophils and macrophages more effectively than IgA1 (Steffen et al. 2020). Conversely, polymeric IgA1, but not polymeric IgA2 or monomeric versions of either subclass, interacts with transferrin receptor CD71, apparently through IgA1 HR *O*-glycans (Moura et al. 2004). Furthermore, IgA glycans, specifically Gal and GalNAc, impact catabolism of IgA in the liver, a process that depends on the asialoglycoprotein receptor on hepatocytes and contributes to the short half-life of IgA in the circulation.

These aspects are at the forefront of recent research due to a growing interest in using IgA as a therapeutic antibody. The therapeutic applications of IgA may include

systemic and mucosal delivery. In either setting, a better understanding is needed about the impact of producing-cell-specific glycosylation on IgA effector functions, receptor interactions, and pharmacokinetics. Biotechnological systems for the production of recombinant IgA, as well as other recombinant Igs, can include plants (Göritzer et al. 2017, 2019, 2020; Strasser et al. 2021; Kallolimath et al. 2016, 2020; Montero-Morales et al. 2019; Montero-Morales and Steinkellner 2018; Kallolimath and Steinkellner 2015). A better understanding of the differential glycosylation of various molecular forms and subclasses of IgA is needed to inform design and production of recombinant therapeutic IgA for future preclinical and clinical trials.

14.12 Therapeutic IgA Antibodies

Monoclonal antibody therapeutics have gained popularity in the medical field to treat a large set of pathologies due to their intrinsic specificities to target antigens. These therapies have largely been using IgG, although some research has been performed in vivo and in vitro to assess the feasibility of IgA therapeutics. IgA can be both pro-inflammatory and anti-inflammatory depending on the structure of its glycans which can regulate its binding to the Fc α R1 and Fc α R2 receptors. For example, treatment with IgA monoclonal antibodies against human epidermal growth factor receptor 2 (HER2) and epidermal growth factor receptor (EGFR) enhanced antitumor activity in Fc α R1 transgenic mice (Boross et al. 2013; Meyer et al. 2016). This is thought to be done via activation of neutrophils and macrophages, which can be activated through IgA binding to Fc α R1 and activating the immunoreceptor tyrosine-based activation motifs (ITAMs) pathway (Heineke and van Egmond 2017).

Targeting Fc α R1 could prove to be an important clinical modality for IgA monoclonal treatments. However, a number of hurdles need to be overcome for IgA therapies to work. IgA is cleared more rapidly than IgG from the circulation in part due to asialoglycoprotein receptor expression in the liver. This receptor can bind multiple glycosylation sites on the IgA molecule, which can be inhibited by glycoengineering IgA to have increased terminal sialic acids (Rifai et al. 2000; Boross et al. 2013; Meyer et al. 2016). Interestingly, decreasing sialic acid content of IgA increases its pro-inflammatory capabilities (Steffen et al. 2020), leading to a trade-off between circulatory residence time (bioavailability) and the desired effector function of monoclonal IgA generated.

Production of properly folded and glycosylated IgA proteins can be difficult in mammalian systems, especially tuning the glycoengineering component to create the needed structures. This is in part due to the many different forms of *N*- and *O*-glycans that can be produced by mammalian cells, which makes it difficult to generate consistent glycoforms on a complex background (Montero-Morales and Steinkellner 2018). A more understudied way of generating consistent glycosylated proteins is to use modified plant cells that have undergone genetic modifications to specific glycosyltransferases involved in *N*- and *O*-glycosylation pathways. This approach was recently used successfully to generate IgG monoclonal therapies

against the Ebola virus outbreak in Africa (Qiu et al. 2014; Davey et al. 2016). The generation of mammalian-like *N*-glycosylation-competent plants for IgG antibodies was first done in the *Nicotiana benthamiana* species, using genetic manipulations to inactivate the endogenous β 1,2-xylosyltransferase (XylT) and α 1,3-fucosyltransferase (FucT) genes (Δ XT/FT). This produced a biologically competent anti-HIV monoclonal antibody, with the same glycosylation as those expressed in Chinese hamster ovary (CHO) cell lines (Strasser et al. 2008). These Δ XT/FT plants have now been deployed to produce normally *N*- and *O*-glycosylated IgA1. While the whole IgA1 protein was not reproduced in the Δ XT/FT variant, the researchers were able to overcome hydroxyproline formation at the HR by overexpressing GalNAc-T2. This produced a variant of the core-1 motif-containing galactose, and only modest sialylation (Dicker et al. 2016). These studies that generate the technology for robust manufacturing of glycosylation competent IgA will be able to open up a larger sector for IgA therapeutics.

14.13 Conclusions

Understanding IgA glycosylation and its role in disease is still a developing field of study. However, with the help of MS, and recent technological advances, substantial progress has been made. Although still in its early stages, it is becoming more feasible to evaluate and decipher glycan structures and range of heterogeneities, especially when the different IgA isotypes and molecular forms are separated. Our knowledge of the role of IgA glycosylation in various diseases is currently based on observational studies. The one exception is the role that IgA1 *O*-glycans have been shown to play in the pathogenesis of IgA nephropathy. As the role of IgA in the immune response is further delineated from other immunoglobulins, the role of the glycans, especially those unique to the various IgA isotypes and molecular forms will be better understood. This will likely lead to IgA being more frequently used as a therapeutic to target specific disease states and/or locations within the human body.

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Compliance with Ethical Standards

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Conflict of Interest MBR and JN are co-founders and co-owners of and consultants for Reliant Glycosciences, LLC. MBR and JN are co-inventors on US patent application 14/318,082 (assigned to UAB Research Foundation). ALH and CR declare no conflict of interest.

Ethical Approval Not applicable, as this is a review article and, thus, there are no human subjects recruited for this study or any animals used.

Informed Consent Not applicable, as this is a review article and, thus, there are no human subjects recruited for this study.

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