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The Role of Mucin-type O-glycans in Eukaryotic Development

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Abstract

Newly emerging genetic studies have revealed that a subset of the family of glycosyltransferases responsible for the formation of mucin-type O glycans is essential for normal development. As additional genetic, biochemical and physical tools are developed to interrogate the complex structure and surface location of this under-studied class of carbohydrate, no doubt additional roles will be elucidated.

1. Introduction: “The sweet husk”

Almost 50 years ago, H. Stanley Bennett, speaking before the Mexican Anatomical Society, proposed that all cell surfaces were enveloped in a sugar coat, that he termed the “glycocalyx” or sweet husk. While early studies (1,2) emphasized the filtration, barrier and supportive functions of this sugar structure, over time, compelling evidence that cell surface carbohydrates mediate specific cell adhesions to either each other or to the underlying extracellular matrix accumulated (reviewed in 3–6). In particular, the ability of surface carbohydrates to form a “polyvalent array” of low affinity binding sites affords a mechanism whereby cells may sample each other and their environment before entering into specific, long-term relationships via high affinity interactions with either carbohydrates and/or proteins.

Mucin-type glycoproteins (mucins) are heavily decorated with carbohydrate side-chains, termed O-glycans, which are linked (via an O-glycosidic bond) to serine (Ser) or threonine (Thr) residues of the protein backbone. One unique feature of O-glycans is that they often cluster within repeating amino acids sequences of the protein, termed tandem repeats, which are enriched in Ser/Thr residues (7–9). Many glycoproteins contain one or more mucin-like domain, typically enriched in Pro, Ser and Thr residues, yielding a discrete region(s) within the overall molecule that are heavily decorated with O-glycans (10).

Mucins or proteins containing mucin-like domains, are expressed in two flavors – membrane bound molecules that contribute directly to the composition of the cellular glycocalyx (5,10–12) and secreted forms that contribute either to the formation of the extracellular matrix (13,14) or to the gel-like mucus coat that envelopes mucosal surfaces of the body thereby forming the most exterior face of the innate immune system (7,15). In terms of location therefore, both surface bound mucins within in the glycocalyx and secreted mucins contributing to the formation of the extracellular matrix are well positioned to influence development.

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2. Biosynthesis and structure of mucin-type O-glycans: Advances and challenges

The step-wise synthesis of O-glycans begins with the transfer of N-acetylgalactosamine (GalNAc) from the sugar donor, UDP-GalNAc, to selected Ser/Thr residues of the protein backbone yielding Tn antigen (GalNAc- α -1-O-Ser/Thr) (8,9,16–18). This key first step is catalyzed by a multi-gene family of enzymes (E.C. 2.4.1.41) termed UDP-GalNAc:polypeptide α -N-acetylgalactosaminyltransferases (ppGalNAcTs) (19). Most recent data indicates that there are 20 human and 18 mouse genes (*galnts*) with evidence for additional diversity in the form of splicing variants (20) and as many as 12 genes in *Drosophila* (*pgants*) (21).

Why nature requires so many ppGalNAcTs to perform a seemingly simple reaction has been the subject of intense investigation. Transcripts encoding individual ppGalNAcTs display unique temporal and spatial patterns of expression during development (21–23) and so part of the need for the large number of isoforms relates to which isoform is expressed when and where. Moreover, unique substrate preferences have been demonstrated for some ppGalNAcTs (17,19 and references cited therein) using in vitro assays and recently we have demonstrated isoform-specific O-glycosylation of two bone proteins in vivo (24).

Although many studies have probed the question, no consensus amino acid sequence has emerged that is both necessary and sufficient for mucin-type O-glycosylation (ppGalNAcT review). However two classes of ppGalNAcTs have been identified. One class of ppGalNAcTs glycosylates peptides (“naked” or unglycosylated peptide). The other type of ppGalNAcT will only act on glycopeptide substrates (peptides previously decorated with GalNAc residues). This strongly implies that there is a hierarchical acquisition of GalNAc by specific Ser/Thr residues among clusters of hydroxyamino acids found in the tandem repeats (17,19,25–28). Despite the large number of genes that encode ppGalNAcTs, there is remarkable conservation of functional specificity across phyla (29), suggesting an evolutionary requirement to decorate proteins with this class of glycans in a highly specific manner. Collectively, the available evidence strongly supports the view that it does matter which Ser or Thr is, or is not, glycosylated *in vivo*.

Subsequent elongation of the Tn structure yields an array of 8 distinct “Core” structures that in turn may be further modified by many of the glycosyltransferases resident in the Golgi yielding an extraordinary array of O-glycan structures (9,16–18,30). The most common structure, Core 1 or “T-antigen” (Gal β 1,3GalNAc α 1-O-Ser/Thr) is formed by addition of galactose (Gal) under the direction of the Core β 1,3 galactosyltransferase. In turn, the transfer of an N-acetylglucosamine (GlcNAc) onto the Core 1 structure, catalyzed by Core 2 β 1,6-N acetylglucosaminyltransferase, results in the formation of the Core 2 structure (Gal β 1,3[GlcNAc β 1,6] α 1-O-GalNAc-Ser/Thr). Additional peripheral sugars can be assembled into unique structures that may impart biological specificity including those observed for the blood group antigens (31), selectin-ligands involved in cell trafficking (32,33) and counter receptors for microbial adhesins (34,35).

Structurally, the presence of O-glycans leads to local stiffening of the protein backbone, an effect potentiated by the presence of repeating clusters of sugar afforded by the tandem repeat motif. The result is a less flexible “rod-like” configuration (36) which can “telescope” the business end of a membrane receptor away from the cell surface. Counter to intuition, longer and/or more complex O-glycan structures do not impact the shape of the mucin greater than simple carbohydrate side chains; rather, considerable expansion occurs with just the core GalNAc present with maximum expansion occurring with only a disaccharide structure (36). When multiple O-glycans are more evenly distributed about the peptide

backbone, the resultant structure tends to resist proteolysis and assume an expanded and highly hydrated “jello-like”, form (7–9,11,15,36).

3. Implications of multivalency and the glycoside cluster effect for the role of mucin-type O-glycans in eukaryotic development

Affinities for carbohydrates are usually weak – with μM - mM affinity constants being typical (37). However as the density of target carbohydrate is increased, the affinity can increase dramatically. For example, the carbohydrate binding protein soy bean agglutinin binds to a single chain of Tn antigen with an affinity of $\sim 170\mu\text{M}$; in contrast, a fragment of porcine mucin containing ~ 2300 O-glycans, binds to this same carbohydrate binding protein with an approximately 100 fold increase in affinity (38). Therefore, the densities of sugars that make up either the glycocalyx or the extracellular matrix could have profound functional importance during development. Since the collective action of ppGalNAcTs regulate the density pattern of O-glycan acquisition, the substrate specificity and expression of specific ppGalNAcTs may underlie a here-to-for unrecognized regulatory system. As will be outlined subsequently, a new generation of reagents will be required to facilitate detection of changes in glycan composition, structure and density in real time.

4. Current approaches to studying glycan function in development

Unlike proteins, carbohydrates cannot be “mutated” directly using genetic approaches. Therefore, one strategy to ascertain function has been to alter the carbohydrate using either chemical and/or enzymatic treatment (39). Antibodies (40) and carbohydrate-binding proteins (lectins) (41,42) have also been used to bind and “block” carbohydrate function and inhibition assays employing excess concentrations of glycans have also been performed (43). Small molecule inhibitors of biosynthetic intermediates have also been used widely (44–46) although the complexities of delivering perturbants with precision, at the appropriate temporal moment and spatial location, and in sufficient quantities to yield an effect, have rendered these approaches as most useful for in vitro studies.

The general in vivo strategy to study glycan function has been to either alter or ablate the expression and/or activity of the relevant proteins responsible for the assembly of the carbohydrate side-chain genetically. Targets include both the glycosyltransferases (47) and the protein transporters that are required to form the various sugar donors (48). Given the large number of genes that encode the family of ppGalNAcTs, and the overlap of expression patterns observed, this approach has limitations that must be addressed. These include difficulties in identifying subtle phenotypes and partial penetrance of phenotypes.

Adding to the experimental genetics approach is the phenotypic characterization of patients with congenital disorders of glycosylation that have increased our understanding of the functional roles played by glycans (49 and see article by _____ in this issue).

5. Mucin-type O-glycans matter in eukaryotic development

Recent studies in model organisms provide the clearest and most compelling evidence for the importance of mucin type O-glycans in development. A gene encoding a member of the *Drosophila* *pgant* family (50), *pgant 35A*, is recessive lethal (51,52). Subsequently it was determined that the underlying developmental defect was aberrant tracheal tube formation leading to a respiratory system that lacked an intact diffusion barrier (53). The loss of *pgant 35A* leads to a marked diminution in the level of apical and luminal O-glycans and specifically the apical protein Crbs, known to play a role in formation of cell shape and polarity, suggesting that *pgant 35A* mediated O-glycosylation is required for the proper

sorting of apical proteins in the developing *Drosophila* tracheal system (53). Ablation of the enzyme (C1GalTA) that catalyzes the addition of Gal to the GalNAc to yield the core 1 T antigen is lethal in flies – presumably due to aberration in CNS morphogenesis (54) by an as yet unknown mechanism.

Parallel observations have been made in mice. Ablation of the single mammalian gene (*C1galt1*) that encodes T-synthase leads to embryonic hemorrhage and death. In particular developing embryos display aberrant microvasculature in the brain, pointing to an essential role for the mucin-type O-glycans in angiogenesis (55). Partial inactivation of T-synthase (via an N-ethyl-N-nitrosourea mediated mutagenesis of *C1galt1*) allows animals to reach adulthood. These animals develop both thrombocytopenia and kidney pathology; the kidneys displayed glomerular lesions and fatty degeneration of corresponding proximal tubules (56). The authors suggest podocalyxin as the affected substrate and hypothesize that proper negatively charged O-glycans are responsible for proper formation of filtration slits in kidney. Targeted deletion of *C1galt1* from only endothelial and hematopoietic cells results in abnormal connections between the circulatory and lymphatic systems (57). Using antibody probes to detect glycoproteins involved in angiogenesis, it was ascertained that podoplanin fails to acquire the requisite complement of O-glycans in these mice. Further, podoplanin nulls (*Pdpn* $-/-$) phenocopy aspects of the *C1galt1* nulls suggesting that this O-glycosylated protein plays a role in keeping blood vessels separate from the lymphatic system (57).

Drosophila pgant3 has been demonstrated to be required for proper integrin-mediated cell-cell adhesion in the developing fly wing (58; personal communication, Kelly Ten Hagen). A decrease in O-glycans was observed along the basal surfaces of columnar epithelial cells in wing discs derived from *pgant3* homozygous mutant flies. It is the basal surface of the epithelium that forms the contact interface in wing formation. Using a combination of bioinformatics and proteomic approaches, candidate substrates within the wing epithelium were identified and challenged for their ability to act as acceptor molecules. The extracellular matrix protein tigrin was shown to be a PGANT3-specific target that fails to acquire O-glycans in a *pgant3* null background. Genetic interaction experiments with *pgant3* and tigrin mutant flies demonstrated that these genes act to exacerbate the same phenotype (58). This work is a major step forward in that it is the first to experimentally deduce the substrate implicated in an O-glycan-dependent phenotype.

Gain and loss of function experiments in *Xenopus*, demonstrate that a frog homologue for mammalian ppGalNAc-“like”T-1 (*Galnt1*) negatively regulates TGF β -signaling and is required for proper formation of both the neural crest and the neural tube (59) underscoring that mucin-type O-glycans have both structural and signaling roles during development.

To date ablation of ppGalNAcTs in mice (ppGalNAcT-1 [33], T-3 [60], T-4[19], T-5[19], T-10 [61], T-13[62] and T-4/T-5 [Ten Hagen and Tabak, unpublished]) does not result in lethality, although several interesting phenotypes have been described (33). Recent work from our group has shown variable occurrence of cardiac abnormalities in the *Galnt1*-null mice (submitted); both heart valve thickening and ventricular septal defects are observed suggesting primary defects in valvulogenesis. The incomplete penetrance may result from compensatory effects of other ppGalNAcT-1 family members and underscores the difficulties of working with this complex gene family in mice.

6. Congenital disorders of mucin-type O-glycosylation

A number of diseases are now known to be the result of aberrant O-glycosylation (described below) although no fatal congenital disorders of mucin-type O-glycosylation have been identified in mammals to date. This may indicate that this class of O-glycan is not essential

for mammalian development or that, in contrast to *Drosophila*, there is sufficient functional redundancy built into mammalian systems to ensure that mutation of multiple glycosyltransferases must occur before a lethal effect is observed. It may also be possible that lethality occurs at the very earliest stages of development and the underlying event has not yet been recognized.

Several congenital hematological conditions are associated with defects in mucin-type O-glycan biosynthesis. Tn syndrome is a rare autoimmune disorder characterized by a failure to biosynthetically extend the Tn antigen on the surface of blood cells into the T antigen. The typical clinical presentation is hemolysis or thrombocytopenia (63). The underlying defect is not with the T-synthase (*Clgalt1*) per se, but rather with the molecular chaperone of T-synthase, Cosmc, that is required for enzymatic activity of the glycosyltransferase (64).

Under galactosylation of O-glycans is observed in IgA1 mediated nephropathy (65). IgA is among the few serum proteins to be decorated with varying forms of mucin-type O-glycans within its “hinge region” (66). The heritability of the galactose-deficient form of IgA has been estimated at 0.54 using a lectin-based enzyme-linked immunoadsorbent assay suggesting that abnormally glycosylated IgA is an inherited rather than acquired condition (67,68). It is speculated that the under galactosylated form of IgA is recognized by antibodies that had previously been formed against microbial flora (69). While a recent case control association study has implicated genetic variants of the *ClGalT1* gene to be associated with some cases of IgA nephropathy (70), the specific genetic defect responsible for this disease has not yet been identified.

Hyperphosphatemic familial tumoral calcinosis is characterized by ectopic skin and subcutaneous calcifications and hyperphosphatemia resulting from mutations in one of three genes - *Galnt3*, which encodes ppGalNAc-T3, *fgf23*, which encodes fibroblast growth factor 23 (FGF23) and *KL*, encoding Klotho, the co-receptor for FGF23 (71). FGF23 is known to regulate blood phosphate levels and is inactivated by proteolytic cleavage. The acquisition of O-glycans (suggested to be mediated by ppGalNAcT-3, [72]) protects FGF23 from subtilisin-like proprotein convertase mediated proteolysis and thus is thought to maintain an active pool of FGF23. In humans, inactivating *Galnt3* result in increased proteolysis of FGF23 and increased levels of blood phosphate, leading to the suggestion that O-glycans added to FGF23 by *Galnt3* offer protection from proteolysis. *Galnt3* null mice also display hyperphosphatemia (60).

Several single nucleotide polymorphisms (SNPS) in *Galnts* have been demonstrated to be associated with several diseases and conditions. For example, *galnt1* shows association with ovarian cancer (73). Polymorphism in *galnt4* has been shown to associate with acute coronary syndrome (74). Recently inactivating germ-line and somatic mutations in *galnt12* have been demonstrated in individuals with colon cancer (75). Genetic ablation of *Muc2* in mice increases their susceptibility to colonic tumor formation (76), and mice lacking core 3 mucin-type O-glycans display increased susceptibility to colitis and colorectal tumors (77), supporting the view that dysregulation of mucin-type biosynthesis is associated with pathogenesis of gastrointestinal disease.

7. Where do we go from here?

7.1 Defining the mucin-type O-glycome

While advances in analytical methods (notably mass spectrometry) are making it easier to identify proteins are O-glycosylated (e.g. 78), we still are unable to predict with 100% certainty which proteins will become O-glycosylated by the family of ppGalNAcTs, although continued refinement of predictive algorithms continues (79, NetOGlyc

[<http://www.cbs.dtu.dk/services/NetOGlyc/>], OGPET [<http://ogpet.utep.edu/OGPET/index.php>] and see 80–82). This highlights the need for a better understanding of the activity and site preferences of each individual transferase family member. These efforts are greatly informed by systematic in vitro analysis of ppGalNAcT substrate preferences using oriented random peptide (83) and glycopeptides libraries (28,84). Ultimately a model incorporating all of these preferences will need to be integrated and tested experimentally. Efforts would be greatly enhanced by the availability of a high-throughput, unbiased approach to screen all possible substrates with all possible ppGalNAcTs. Attempts to use yeast-two hybrid have not been informative (Ten Hagen and Tabak, unpublished results), likely do the relatively low affinity of the ppGalNAcTs for their substrates and/or the less than optimal conditions in the yeast nucleus for a Golgi reaction event. A system is currently in development to redress the later limitation (Dube & Kohler, D. Dube, personal communication, 2009).

Current in vivo experimental approaches to define ppGalNAcT substrates are laborious since we lack specific inhibitors of ppGalNAcTs that are stable under a broad range of physiological conditions. The availability of crystal structures for both peptide (85,86) and glycopeptide (87) ppGalNAcTs, together with new approaches to high through put screening (88) should facilitate identification for small molecule inhibitors for the ppGalNAcT family and individual isoforms.

Perturbation of ppGalNAcT activity in vivo is currently limited to either targeted gene ablation or RNAi mediated reduction. The expression of glycoproteins in the experimental background is compared to that observed for wild type to determine if there is any change (e.g. 24,58).

7.2 Mapping the location of mucin-type O-glycans

Since the density of O-glycans can play an a potentially important role in setting the affinity for carbohydrate binding proteins, it is insufficient to simply know if a protein is decorated with mucin-type O-glycans. Experimental mapping of O-glycans remains technically challenging with relatively few mucin-type O-glycans unambiguously assigned to specific Ser/Thr (89–91). While site directed mutagenesis could be employed to localize specific O-glycans this is not a practical approach for most mucin-type glycoproteins given the large number and clustered arrangement of O-glycans that decorate the protein backbone.

Solid-phase Edman degradation remains the most robust approach to site mapping for mucin-type O-glycans but unfortunately this method currently requires relatively large quantities of purified sample and requires highly specialized equipment (90,91). To add further complexity, mucin-type O-glycosylation sites may vary both in the degree that they are occupied by glycans (91) and in the “completeness” of the structure that is attached (e.g. 66,91). Collectively, this variation has been termed “microheterogeneity”. As analytical methods become more robust, we will be able to perform high-throughput analysis of mucin-type glycoproteins at the site-specific level (90,91).

7.3 Dynamic analysis of mucin-type O-glycans in vivo

Present analysis of glycans is largely static in nature; by interrogating a system at different time points, iterative assessment of the system dynamics can be inferred. However, in the future, it will become increasingly important to conduct real-time measurements of the specific glycans on the cell surfaces. This will be particularly important as more attention is played to the increasing number of mucin-type glycoproteins that are being identified that function as part of signaling pathways (59,92–95).

Once substrate-ppGalNAcT isoform pairs are identified, one could envision engineering mice that express, either conditionally or with on/off promoters, fluorescent reporter molecules (e.g. 96) that would be engineered to act as sentinels for the appearance (or disappearance) of key glycans (that are labeled, e.g. 97) in real time using principles of Fluorescence Resonance Energy Transfer. It may also be possible to employ NMR spectroscopy to monitor glycosylation changes as has recently been done for the intracellular O-N-Acetylglucosamine (98).

8. Concluding remarks

Despite the ubiquitous nature of mucin-type O-glycosylation, the limited number of tools to facilitate their *in vivo* study and a muted appreciation for glycobiology by the broad scientific research community (99) has made for slow progress in defining the precise roles played by O-glycans in development. Recent advances that have been made by exploiting model organisms have highlighted the significance of mucin-type O-glycans (100). This, coupled with advances in analytical methods has enabled remarkable progress to be made in a relatively short period of time. With this progress will no doubt come a wider appreciation of this molecular class, which in turn will lead to larger numbers of investigations. The future does indeed appear to be “sweet”!

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