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MINI REVIEW

Complexity in O-linked oligosaccharide biosynthesis engendered by multiple polypeptide *N*-acetylgalactosaminyltransferases

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Linkage of *N*-acetylgalactosamine (GalNAc) to the hydroxyl group of serine and threonine in a peptide context initiates the production of vertebrate O-glycosylation, although the identity, specificity and location of components involved in this posttranslational modification have been debated. Studies by Robert Hill and others have found that O-glycosylation begins in one or more compartments of the Golgi apparatus (Hanover *et al.*, 1982; Elhammer and Kornfeld, 1984; Roth, 1984; Abeijon and Hirschberg, 1987; Deschuyteneer *et al.*, 1988; Piller *et al.*, 1990; Roth *et al.*, 1994; Schweizer *et al.*, 1994). While various cell types were used, others have indicated that the endoplasmic reticulum is the site of O-glycan biosynthesis (Strous, 1979; Cummings *et al.*, 1983; Patzelt and Weber, 1986; Perez-Vilar *et al.*, 1991; Ellinger and Pavelka, 1992). Although it is possible that a significant number of laboratories are in error, alternatively, all may be correct with these differing results due to a source of complexity in O-glycan biosynthesis not previously defined.

In efforts to determine a sequence motif directing O-linked oligosaccharide formation, no consensus signal has emerged, although a negative influence of adjacent charged residues has been noted as well as the enhanced frequency of adjacent proline, serine, and threonine residues (Hill *et al.*, 1977; O'Connell *et al.*, 1991, 1992; Wilson *et al.*, 1991; Wang *et al.*, 1992, 1993; Elhammer *et al.*, 1993; Nehrke *et al.*, 1996). However, some surveys of O-glycan sequences have led to the reported prediction of O-glycosylation sites with approximately 78–88% success (Elhammer *et al.*, 1993; Chou *et al.*, 1995). Purification and characterization of polypeptide (pp) GalNAc-transferase activity from vertebrate sources has provided material to address such issues (Hagopian and Eylar, 1969; Sugiura *et al.*, 1982; Elhammer and Kornfeld, 1986; Wang *et al.*, 1992), and recent studies *in vitro* have indicated a surprisingly strong preference for threonine residues using highly purified bovine GalNAc transferase (O'Connell *et al.*, 1992; Wang *et al.*, 1992, 1993). These data have supported the view proposed by Robert Hill and others that multiple ppGalNAc-transferases may exist that might act independently to control O-glycan production in vertebrate cells. Recent experiments described below have now confirmed this biochemical prediction, revealing that multiple ppGalNAc-transferases are encoded within the vertebrate genome and thus indicating a complexity in the mechanisms governing O-glycan biosynthesis and function *in vivo*.

Molecular cloning and characterization of a gene encoding ppGalNAc-transferase activity was first reported from bovine tissues (Hagen *et al.*, 1993; Homa *et al.*, 1993). cDNA sequence comparisons indicated that both groups had isolated the same gene. The deduced amino acid sequence of bovine ppGalNAc-transferase did not exhibit much homology to other characterized glycosyltransferases although the enzyme also appeared to be a Type II transmembrane protein with a small cytoplasmic-oriented sequence. Production of active recombinant ppGalNAc-transferase and enzymatic characterizations *in vitro* yielded similar data obtained from purified biochemical preparations, showing a strong preference for GalNAc transfer to threonine approximately 50-fold over serine (Hagen *et al.*, 1993), a result that has not been reported thus far *in vivo* (Nehrke *et al.*, 1996). Studies of ppGalNAc-transferase expression at the RNA level indicated widespread and abundant levels in the human (Homa *et al.*, 1993) and the mouse (Figure 1), as would be expected considering the ubiquitous presence of O-glycans among vertebrate cell types. RNA analysis in one study showed somewhat varied expression among rat tissues, with lower levels in spleen and testis and apparently little or no expression in lung (Hagen *et al.*, 1995), indicating that species-specific differences may occur. Two RNA transcripts have been consistently observed to accumulate in all tissues studied so far. Although the molecular definition of each transcript awaits resolution, alternative RNA splicing or differential usage of polyadenylation signals are explanations that would not invoke the production of different polypeptides.

To study the roles of O-linked oligosaccharides in ontogeny and physiology, this laboratory previously initiated studies to inactivate the mouse ppGalNAc-transferase gene by using homologous recombination in embryonic stem cells and subsequently generating mice harboring this mutation in the germline. We reasoned that such an approach could also confirm whether multiple ppGalNAc-transferases are encoded within the vertebrate genome, should O-glycosylation continue in the absence of a functional targeted ppGalNAc-transferase allele. A similar approach previously revealed that complex asparagine-linked oligosaccharides are necessary for mouse embryogenesis during postimplantation development, as embryos lacking these structures died at embryonic day 9 (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). Considering the widespread expression of O-glycans in early mouse development as well as in adult tissues, it seemed possible that inactivation of the ppGalNAc-transferase gene might also preclude normal embryonic development, leaving little possibility for analyses of cell-type-specific O-glycan function. A recently developed method to achieve tissue- and site-specific gene inactivation

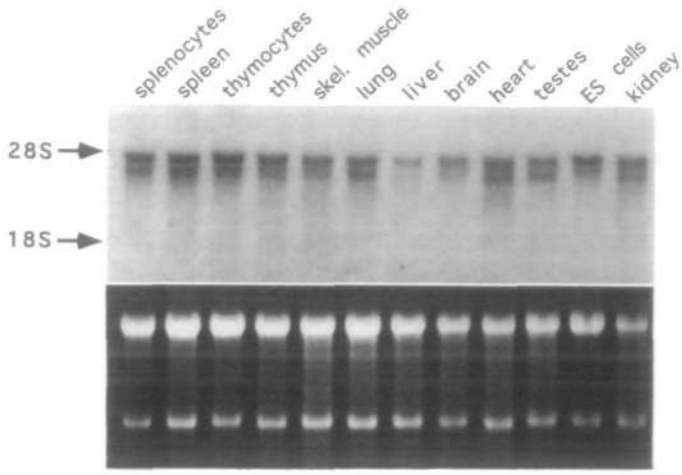

Fig. 1. RNA expression of ppGalNAc-T1 in normal mouse tissues. Using techniques previously published (Pownall *et al.*, 1992), levels of RNA hybridizing to the bovine ppGalNAc-T1 cDNA were assessed among a variety of mouse tissues using 10 μg of total RNA. In all tissues surveyed thus far, significant RNA expression has been observed with two transcripts appearing at approximately 4.5 and 4.0 kilobases. Lower panel is an ethidium-stained pattern of duplicate samples for assessing loading accuracy.

using the Cre-*loxP* recombination system of bacteriophage P1 was therefore employed (reviewed in Marth, 1996). With this approach, control over inactivation of both alleles of the ppGalNAc-transferase gene could be obtained by restricting expression of the Cre recombinase transgene to specific cell types.

Among the many cell types that regulate the production and diversification of oligosaccharide structures, T lymphocytes have been relatively well characterized. During T cell development in the thymus, expression patterns of Core 2 GlcNAc-transferase and certain sialyltransferases differ dramatically between cortical and medullary compartments, indicating differentiation stage-specific O-linked oligosaccharide changes that are also visualized using the lectin peanut agglutinin (PNA) (Gillespie *et al.*, 1993; Baum *et al.*, 1996). In mature thymic and peripheral T lymphocytes, high level expression of the ST6Gal I sialyltransferase is observed, as is the resulting Siaα2–6Galβ1–4GlcNAc termini, an oligosaccharide structure that is recognized by the lectin CD22 on B cells (Powell *et al.*, 1993; Sgroi *et al.*, 1993). Additionally, T lymphocyte trafficking to sites of inflammation is known to be dependent in part upon selectin binding to oligosaccharides bearing the sialyl Lewis x structure on human cells as produced by multiple glycosyltransferases, including certain fucosyltransferases (Lowe *et al.*, 1990; Maly *et al.*, 1996).

In a standard molecular cloning approach, hybridization of bovine ppGalNAc-transferase cDNA sequence to a mouse 129/SvJ genomic library resulted in the isolation of a clone bearing an exon utilizing the same splice junction sequences as in the rat and human ppGalNAc-T1 gene (Meurer *et al.*, 1996; F.Hagen and L.Tabak, personal communication) and exhibiting over 93% amino acid identity to the corresponding bovine ppGalNAc-T1 catalytic domain region (Hennet *et al.*, 1995). This level of conservation indicated that this clone likely represented the murine homolog of the bovine GalNAc-T1 enzyme. To inactivate the enzyme *in vivo* in mice, *loxP* sites were placed to flank the exon in embryonic stem cells while the neomycin phosphotransferase (Neo) gene expression cassette was placed adjacent to one *loxP* site within an intron. Recombination by the Cre recombinase resulted in excision of the exon, leading to a frameshift mutation that should inactivate the enzyme, considering that less severe C-terminal truncations ablated recombinant bovine ppGalNAc-T1 activity toward various peptide acceptors *in vitro*. While T cell specific deletion of the exon was achieved *in vivo*, mice bearing this null mutation in all cell types were also found to be unaffected and reproduced normally (Hennet *et al.*, 1995). O-Glycosylation continued unimpeded as judged in part by jacalin and PNA lectin binding on thymocytes and other cell types.

Direct evidence of a second mammalian ppGalNAc-transferase came from the laboratory of Henrik Clausen as molecular cloning and characterization of an isozyme isolated from human placenta revealed that it was derived from a distinct gene (ppGalNAc-T2; Sorenson *et al.*, 1995; White *et al.*, 1995). In other studies, analysis of Expressed Sequence Tag databases (EST) revealed multiple sequence entries that were highly homologous to bovine ppGalNAc-T1, providing opportunities to rapidly isolate novel isoform candidates. Research underway by Fred Hagen and others in the laboratory of Lawrence Tabak recently succeeded in cloning a distinct cDNA from human and mouse sources representing another ppGalNAc-transferase isozyme (ppGalNAc-T3), a third member of what now appears to be a growing family of genes closely related to ppGalNAc-T1 (J.Zara, F.Hagen, K.T.Hagen, and L.Tabak, personal communication).

In recent sequence comparisons of these gene products from multiple mammalian species, homologs exhibit between 97% and 100% amino acid identity spanning the enzyme, of approximately 560–570 amino acids (Figure 2; Hagen *et al.*, 1993; Homa *et al.*, 1993; White *et al.*, 1995; and data not shown). Nucleic acid homology is obviously substantial among homologs with approximately 90% identity within the presumptive coding sequence. In isozyme comparisons among human sequences, ppGalNAc-T2 and ppGalNAc-T3 display 60% and 67% amino acid identity, respectively, to ppGalNAc-T1 in a region of the catalytic domain (Figure 2), but only 44% and 49% identity, respectively, throughout the entire amino acid sequence (J.Zara, F.Hagen, K.T.Hagen, and L.Tabak, personal communication). Currently, the gene most closely related to ppGalNAc-T1 is ppGalNAc-T8, originally cloned from the mouse by cross-hybridization to the bovine ppGalNAc-T1 cDNA and previously thought to be the mouse ppGalNAc-T1 homolog as it contains over 93% amino acid identity with bovine ppGalNAc-T1 (Figure 2 and Hennet *et al.*, 1995). Additionally, two processed ppGalNAc-T1 pseudogenes have been found thus far, one in human DNA (Meurer *et al.*, 1996) and a structurally distinct pseudogene in the mouse germline that harbors various mutations including frameshifts and premature translational termination signals (K.Marek and J.Marth, unpublished observations).

How many ppGalNAc-transferases are there? An estimate from cloning studies in the Tabak laboratory suggests the presence of at least eight genes in the mammalian genome that bear between 70–95% amino acid identity to the ppGalNAc-T1 homolog, making a present total of nine candidate isoforms (F.Hagen and L.Tabak, personal communication). Following recombinant production, GalNAc transfer activity toward peptide and protein substrates *in vitro* has been observed with all isoforms tested thus far, including ppGalNAc-T1, ppGalNAc-T2, and ppGalNAc-T3 (Hagen *et al.*, 1993; Homa *et al.*, 1993; White *et al.*, 1995; J.Zara, F.Hagen, K.T.Hagen, and L.Tabak,

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Bov ppGalNAc-T1     IWQCGGTLEIVTCSHVGHVFRKATPYTFPGGTGQIINKNNRRLAEVWMDEFKNFFYIISP  (327-386)

Rat ppGalNAc-T1     ............................................................
Hum ppGalNAc-T1     ............................................................
Mur ppGalNAc-T1     ............................................................
Hum ppGalNAc-T2     V.....S...IP..R.......QH.......S.TVFAR.T..A.......Y...Y.AAV.
Hum ppGalNAc-T3     V.....Q...MP..V......SKS.HS..K..-.V.AR.QV.........Y.EI..RRNT
Mur ppGalNAc-T3     V.....Q...MP..V......SKS.H...K..-.V.AR.QV.........Y.EI..RRNT
Mur ppGalNAc-T8     ......S.........................HV.................D.......
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Fig. 2. Amino acid comparison of mammalian ppGalNAc-T1 homologs and isoforms of bovine ppGalNAc-T1 sequence. Catalytic domain sequence of bovine ppGalNAc-T1 corresponding to exon 9 is compared with human, rat and mouse homologs, revealing 100% identity, while the human and mouse T3 homologs exhibit one conservative amino acid change. ppGalNAc-T2 and ppGalNAc-T3 homologs are displayed, exhibiting approximately 40% identity to human ppGalNAc-T1 among human isoforms ppGalNAc-T8 displays four differences, two of which are conservative, making this member most closely related to the ppGalNAc-T1 isoform.

personal communication). RNA expression data presently available indicates that ppGalNAc-T1 is found ubiquitously in the mouse (Figure 1), while ppGalNAc-T2 RNA is observed in only a subset of tissues (White *et al.*, 1995). Most other ppGalNAc-transferases are also expressed in a restricted manner as RNA appears in one or very few tissue types among those surveyed (unpublished observations; F.Hagen and L.Tabak, personal communication). The total number of other genes closely related to ppGalNAc-T1 in the mammalian genome may eventually be resolved by further cloning approaches using EST hybridization, low stringency cross-hybridization, and polymerase chain reaction.

Multiple ppGalNAc-transferases indicate a complexity in O-linked oligosaccharide biosynthesis that provides for several interesting hypotheses. It has been previously proposed by Robert Hill and others that different ppGalNAc-transferases may exist and each might harbor distinct substrate recognition profiles. The factual content of the first prediction is now clear and questions pertaining to function and substrate specificity emerge. Obviously, multiple enzymes bearing distinct substrate specificities can explain the lack of consensus motif for O-glycosylation, as exists for asparagine-linked oligosaccharide production. However, thus far only relatively minor variations in substrate preference have been reported and those have often been obtained in cell-free *in vitro* analyses. Still, such variations may turn out to be even more prominent in the context of the intracellular milieu *in vivo*. Alternatively, as ppGalNAc-transferase localization studies have produced opposing results, it seems worth investigating the possibility that ppGalNAc-transferases localize to different compartments among the endoplasmic reticulum and Golgi apparatus. If this were so, then substrate specificity might not be encoded for within the catalytic domain as assayed *in vitro*, but could be governed by sequences that exist to localize a given isoform to

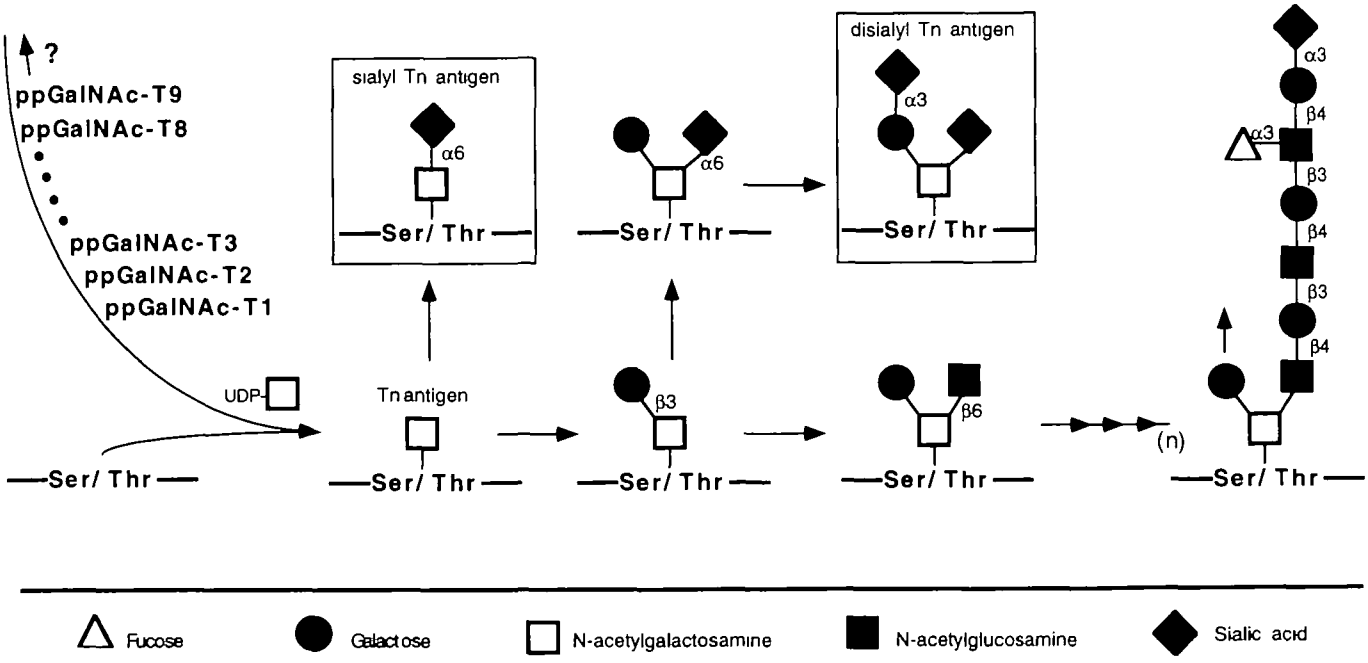

Fig. 3. O-Linked oligosaccharide biosynthesis can be independently initiated by multiple ppGalNAc-transferases. Nine or more related enzymes appear to exist to link UDP-*N*-acetylgalactosamine to peptide in protein, followed by possibly identical steps to produce Tn antigens, biosynthetic dead-end structures (boxed), and bi-antennary O-linked oligosaccharides including termini bearing the sialyl-Lewis x structure. Arrows represent enzymatic steps involving the addition of specific monosaccharides with the indicated linkage. n is defined by the number of monosaccharides in a given oligosaccharide.

a specific organelle compartment. Different compartments may then provide for O-glycan diversity based in part upon intracellular protein trafficking and the carbohydrate structural heterogeneity already present on any potential ppGalNAc-transferase substrate. These hypotheses will likely be tested in the future, bringing us closer to understanding why multicellular organisms have maintained multiple ppGalNAc-transferase enzymes encoded within their genomes.

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