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Genetics

T-cell-specific deletion of a polypeptide *N*-acetylgalactosaminyl-transferase gene by site-directed recombination

(O-glycosylation/gene targeting/T-cell differentiation)

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ABSTRACT **UDP-*N*-acetylgalactosamine (GalNAc): polypeptide *N*-acetylgalactosaminyltransferase (polypeptide GalNAc-T) catalyzes transfer of the monosaccharide GalNAc to serine and threonine residues, thereby initiating O-linked oligosaccharide biosynthesis. Previous studies have suggested the possibility of multiple polypeptide GalNAc-Ts, although attachment of saccharide units to polypeptide or lipid in generating oligosaccharide structures in vertebrates has been dependent upon the activity of single gene products. To address this issue and to determine the relevance of O-glycosylation variation in T-cell ontogeny, we have directed Cre/*loxP* mutagenic recombination to the polypeptide GalNAc-T locus in gene-targeted mice. Resulting deletion in the catalytic region of polypeptide GalNAc-T occurred to completion on both alleles in thymocytes and was found in peripheral T cells, but not among other cell types. Thymocyte O-linked oligosaccharide formation persisted in the absence of a functional targeted polypeptide GalNAc-T allele as determined by O-glycan-specific lectin binding. T-cell development and colonization of secondary lymphoid organs were also normal. These results indicate a complexity in vertebrate O-glycan biosynthesis that involves multiple polypeptide GalNAc-Ts. We infer the potential for protein-specific O-glycan formation governed by distinct polypeptide GalNAc-Ts.**

UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (polypeptide GalNAc-T) initiates O-linked oligosaccharide biosynthesis by transferring GalNAc to serine and threonine residues on proteins located in the cis-Golgi apparatus (1, 2). A member of the glycosyltransferase gene superfamily, polypeptide GalNAc-T is a type II transmembrane protein that exhibits a stem region and C-terminal catalytic domain on the luminal side of the Golgi compartment (3). In the thymic milieu, the O-linked oligosaccharide repertoire is differentially regulated in cortical and medullary regions, as detected by binding to the lectin peanut agglutinin (PNA; ref. 4). Additionally, certain mucins are found on the thymocyte and T-cell surface, including CD43, CD44, and CD45 (5), the latter of which is involved in T-cell development (6, 7). The function of mammalian O-glycosylation is generally not well resolved, although the sialyl Lewis X structure is a selectin ligand crucial for leukocyte extravasation during inflammatory responses (8).

A gene encoding polypeptide GalNAc-T activity has recently been isolated (9, 10). Polypeptide GalNAc-T RNA is found in a wide range of vertebrate tissues and appears to be ubiquitously expressed in the mouse, consistent with its key role in O-glycan formation (ref. 10 and unpublished observations). Inactivation of polypeptide GalNAc-T function *in vivo* might reveal roles of O-linked oligosaccharides roles in T-cell differentiation and perhaps mechanisms regulating peripheral T-lymphocyte colonization of secondary lymphoid organs, including the spleen and lymph nodes. However, such an approach could be problematic, since a possible requirement for polypeptide GalNAc-T function in early embryogenesis might lead to lethality in mutant embryos and preclude the development of T lymphocytes.

The bacteriophage P1 Cre/*loxP* recombination system has been shown to excise *loxP*-flanked genomic DNA in cells of transgenic mice (11–13). *In vivo* applications of this activity include a method for mammalian cell fate mapping and conditional gene inactivation. Such methods are attractive because of the possible pleiotropic nature of results derived from systemic gene-targeting studies and should allow distinctions to be made between cell-autonomous and environmentally derived phenotypes. However, these applications can depend upon achieving a high efficiency of recombination. The efficacy of Cre action *in vivo* may be enhanced by the presence of Cre sequence modifications involving translational and nuclear localization signals (14, 15) or may in part depend upon chromatin structure that encompasses *loxP*-flanked DNA (12, 16).

With these considerations in mind, we generated polypeptide GalNAc-T gene-targeted and Cre transgenic mice harboring a gene deletion in polypeptide GalNAc-T alleles in a systemic manner and at high efficiency specifically in the T-cell lineage. We detail the impact of this mutation in analyses of T-cell development and peripheral T-cell colonization of secondary lymphoid organs.

MATERIALS AND METHODS

Polypeptide GalNAc-T Gene Targeting and Chimeric Mouse Production. A portion of a mouse polypeptide GalNAc-T gene was cloned from a bacteriophage library of Pcc4 genomic DNA (Stratagene) by using the bovine polypeptide GalNAc-T cDNA as a probe (9). Analysis revealed the following exon (uppercase) surrounded by intron consensus sequences: ttcttctccccag-ATC-TGG-CAA-TGT-GGA-GGT-TCT-TTG-GAG-ATT-GTG-ACT-TGC-TCT-CAT-GTC-GGT-CAT-GTT-TTT-CGA-AAG-GCA-ACT-CCA-TAC-ACA-TTC-CCT-GGT-GGC-ACT-GGC-CAT-GTC-ATT-AAC-AAG-AAC-AAC-AGA-AGA-CTG-GCA-GAA-GTG-TGG-ATG-GAT-GAA-TTC-AAA-GAT-TTC-TTC-TAT-ATC-ATA-TCC-CCA-G-gtatgcag. A gene-targeting vector was constructed by subcloning a 2.6-kb *Stu* I–*Xba* I genomic fragment containing this polypeptide GalNAc-T exon into the *Bam*HI site of pLox²Neo, a plasmid derived by substitution of the neomycin phosphotransferase (Neo) gene from pMC-Neo (minus a polyadenylylation signal) for the *Sma* I–*Sma* I fragment of pLox² (12). Adjacent intronic fragments of 1.3 kb

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Abbreviations: polypeptide GalNAc-T, UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferase; PNA, peanut agglutinin; Neo, neomycin phosphotransferase; ES cells, embryonic stem cells.

(*Stu* I–*Nhe* I) and 8.7 kb (*Xba* I–*Xba* I) were then inserted into the *Kpn* I and *Xba* I sites of pLox²Neo, respectively. Ten micrograms of the *Sal* I-linearized targeting vector was then introduced into embryonic stem (ES) cells by electroporation (R1 cells; ref. 17). ES cells were cultured for 10 days in medium containing the neomycin analogue G418 (Life Technologies, Grand Island, NY) at 120 μg/ml. Cell clones that had undergone homologous recombination were initially identified by PCR using thymidine kinase promoter- and polypeptide GalNAc-T allele-specific oligonucleotide primers (5′-GAGAGGCTTTTTGCTTCCTCTTGC-3′ and 5′-ATTTTAGGCACGCATGTATTTCC-3′, respectively). Homologous recombination was confirmed by genomic Southern blotting using a 0.7-kb *Nhe* I–*Xba* I genomic probe adjacent to the targeted sequences. Cells retaining *loxP* sites were microinjected into 3.5-day C57BL/6 embryos. Chimeric mice were also generated from ES cells (3M3) bearing GalNAc-T deletion following electroporation of Cre expression plasmid pMC-Cre (15) and clonal isolation.

Cre Transgenic Mice. The Cre coding sequence derived from pMC-Cre was excised as a 1.1-kb *Mlu* I–*Mlu* I fragment and cloned into the *Sal* I site of p1017 (18) by blunt-end ligation. The transgene was isolated from the bacterial plasmid after *Not* I digestion and was microinjected into the male pronuclei of (C57BL/6 × CBA)F_2 zygotes prior to implantation into CD-1 foster mothers. Resulting transgenic offspring were identified by hybridization of tail DNA hybridization to Cre DNA.

DNA Preparation. Cells and tissues were lysed overnight at 55°C in 50 mM Tris·HCl, pH 8.0/50 mM EDTA, pH 8.0/0.5% SDS containing proteinase K (100 μg/ml; Boehringer Mannheim). DNA was purified by two phenol/chloroform extractions, one chloroform extraction, and ethanol precipitation.

Polypeptide GalNAc-T Assay. Linkage of [^{14}C]GalNAc to the peptide substrates PTTDSTTPAPTTK, PPDAATAAPLR, PPDAASAAPLR, and PTSTNSANSSSSTTTSST was measured in Golgi apparatus/microsomal fractions of cells. Specific activity was determined in cell extracts as described (9).

Tissue Fractionation and Flow Cytometry. Single cells were isolated from thymus, spleen, and lymph nodes by dissociation through wire-mesh grids and were cleared of large debris by passage through screens (Falcon; Becton Dickinson). Isolated cells were labeled with fluorescein isothiocyanate-coupled jacalin lectin, fluorescein isothiocyanate-coupled PNA lectin (Vector Laboratories), phycoerythrin-conjugated anti-CD4 antibody and biotinylated anti-CD8 antibody (PharMingen), and Peridinin-chlorophyll protein-streptavidin (Becton Dickinson). Labeled cell populations were analyzed on a FACScan flow cytometer (Becton Dickinson). In studies using jacalin lectin and PNA, cells were subjected to lectin binding and subsequent washes prior to labeling with antibodies.

Cell Sorting. Cells were isolated from splenocytes and labeled with biotinylated anti-CD4 antibody (PharMingen) followed by streptavidin-Cychrome (PharMingen), and CD4$^+$ cells were sorted with a FACStar (Becton Dickinson).

RESULTS

The polypeptide GalNAc-T gene-targeting vector was constructed to encompass an exon encoding almost 61 aa within the catalytic domain, ≈172 aa from the C terminus (Fig. 1*A* and unpublished results). From sequence analysis, this exon maintains >93% amino acid identity with the corresponding catalytic region of bovine polypeptide GalNAc-T and contains 5′ and 3′ boundaries at the +1 and +2 codon positions, respectively; hence, deletion would result in missense and nonsense mutations. From studies of bovine GalNAc-T, less severe deletions involving the C-terminal 19 and 36 aa resulted in loss of detectable polypeptide GalNAc-T activity in recombinant cell extracts (S. Wragg, F.K.H., and L.A.T., unpublished observations).

Homologous recombination between the wild-type allele (GT^{WT}) and the targeting vector would create a *loxP*-flanked exon, with two 34-bp *loxP* sites residing in adjacent introns (GT^F; Fig. 1*A*). A third *loxP* site, included to excise the Neo gene prior to chimeric mouse formation (13), was left out of vector construction in this instance. We did not observe a deleterious effect from Neo inclusion on GalNAc-T RNA expression, and Neo expression itself depended upon the GalNAc-T polyadenylylation signal (unpublished observations). Following gene transfer in ES cells, PCR screening and genomic Southern blotting were used to confirm vector integration by homologous recombination (Fig. 1*B Upper*). Hybridization with a *loxP* probe revealed that two of four clones (31.7 and 32.4) had not retained the 5′ *loxP* site (Fig. 1*B Lower*), suggesting a crossover during homologous recombination within the *loxP*-flanked sequence.

ES cell subclones that harbored a polypeptide GalNAc-T deletion (GT^-) were isolated following Cre expression (Fig. 1*C*). Polypeptide GalNAc-T activity in the presence of the GT^{WT}/GT^- genotype was found to be decreased by ≈50% in ES cell extracts, consistent with the presence of a null allele (Fig. 1*D*).

The GT^F allele (ES cells 21.4 and 31.2) and the GT^- allele (3M3 ES cells) (Fig. 1 *B* and *C*) were separately introduced into the mouse germline after microinjection into C57BL/6 blastocyst-stage embryos. Independently, the Cre recombinase gene, which contained Kozak translational consensus and nuclear localization signals, was inserted into the p1017 vector to generate transgenic mice with thymocyte-specific Cre expression (12, 18). GT^F/GT^F;Cre transgenic offspring were derived from parental mice that were heterozygous for the GT^F allele and transgenic for Cre. Total thymocyte numbers among these offspring were normal, and polypeptide GalNAc-T genomic structure was determined in tail and thymocyte DNA. Tail DNA retained the *loxP*-flanked germline configuration in either heterozygous or homozygous state (Fig. 2 *Upper*). In comparison, thymocyte DNA from these mice exhibited GT^- allelic structure (Fig. 2 *Lower*). Deletion was Cre-dependent, generating thymocyte GT^-/GT^- genotypes at high efficiency in a GT^F/GT^F germline configuration in all samples from >18 GT^F/GT^F;Cre transgenic offspring.

Genomic DNAs from various tissues of GT^F/GT^F; Cre transgenic mice were analyzed for the presence of recombination. As depicted for a representative study, production of the GT^-/GT^- genotype was restricted to thymocytes (Fig. 3*A*). With the exception of spleen, other tissues did not exhibit GalNAc-T gene deletion. GT^- alleles in the spleen most likely reflect the presence of thymus-derived T cells in this lymphoid organ (ref. 12; see below).

To compare polypeptide GalNAc-T genomic structure among thymic and peripheral T-cell populations, single cells residing in thymus, spleen, and lymph nodes were isolated from Cre transgenic mice bearing a germline GT^F/GT^F genotype. Cell numbers in each of these organs were similar to numbers found among control mice (data not shown). GT^- alleles were observed among isolated cells derived from the thymus, spleen, and lymph nodes (Fig. 3*B*). The degree of recombination correlated with the percentage of T cells generally found in each tissue, implying that recombination was possibly complete among all GT^F alleles in the peripheral T-cell population.

Splenic CD4$^+$ cells were isolated by flow cytometry from Cre transgenic mice bearing GT^{WT}/GT^F or GT^F/GT^F germline genotypes and also exhibiting GT^{WT}/GT^- or GT^-/GT^- thymocyte genotypes, respectively. CD4$^+$ splenic cell DNA from two GT^{WT}/GT^F germline heterozygotes displayed the GT^{WT}/GT^- genotype (Fig. 3*C*). In comparison, CD4$^+$ splenic cells from two GT^F/GT^F homozygotes did not exhibit similarly

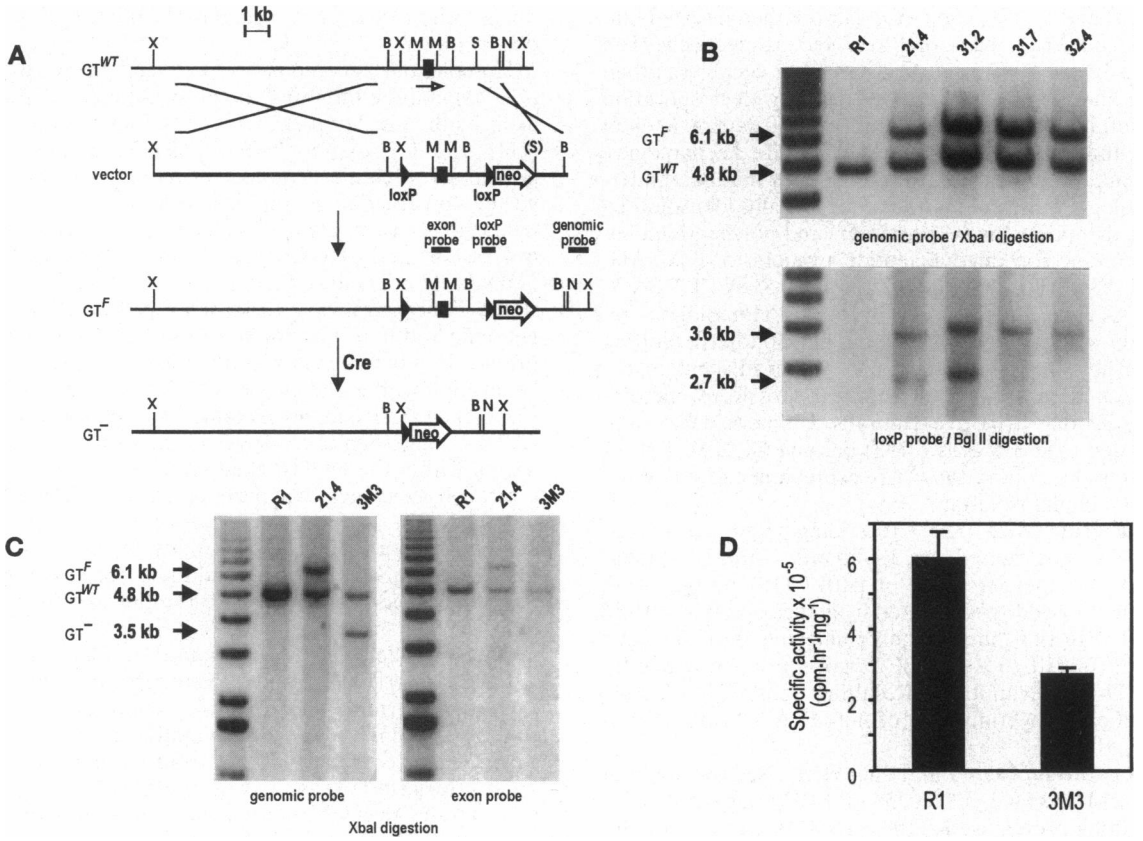

FIG. 1. Production of conditional and systemic null mutations in the mouse polypeptide GalNAc-T gene. (*A*) Genomic structure of the mouse polypeptide GalNAc-T wild-type allele (GT^{WT}) and the GalNAc-T gene-targeting vector. The vector contains two 34-bp *loxP* sites (black triangles, not to scale) and the Neo gene (white arrow) in intronic sequences flanking the polypeptide GalNAc-T exon (black box, arrow below denotes transcription direction). Homologous recombination would generate the polypeptide GalNAc-T genomic structure shown (GT^F). Following Cre recombination, the exon is deleted, producing the null allele (GT^-). Restriction enzyme sites: B, *Bgl* II; M, *Msc* I; N, *Nhe* I; S, *Stu* I; X, *Xba* I. (*B*) Genomic Southern blot analyses of four ES cell clones (21.4, 31.2, 31.7, and 32.4) confirm homologous recombination by means of a polypeptide GalNAc-T genomic probe. A *loxP*-specific probe shows loss of one site during recombination in lines 31.7 and 32.4. Five micrograms of DNA was analyzed from each sample. (*C*) Cre recombinase-generated recombination and exon deletion in 3M3 ES cells derived from 21.4 ES cells following Cre recombination. Five micrograms of DNA was analyzed from each sample. (*D*) Polypeptide GalNAc-T specific activity assayed from extracts of R1 ES cells (GT^{WT}/GT^{WT}) and 3M3 ES cells (GT^{WT}/GT^-) with the acceptor substrate PTTDSTTPAPTTK. Reduction in enzymatic activity was also observed with the substrates defined in *Materials and Methods*.

complete recombination. Quantitation by phosphor-imager analyses revealed that 17% and 36% of GalNAc-T alleles retained the *loxP*-flanked configuration (Fig. 3*C* and data not shown).

T-cell development was assessed by analyzing thymocyte CD4 and CD8 expression profiles among samples derived from GT^{WT}/GT^{WT} mice and mice homozygous for GT^- allelic structure in a systemic manner. Systemic GT^-/GT^- mice were derived from 3M3 ES cells (Fig. 1) and were viable without apparent defects. The percentages of thymocytes residing within the four subpopulations of such GT^-/GT^- mice were identical to controls (Fig. 4*A*). As total thymocyte numbers were also similar, these data indicate that T-cell development was unaltered.

To determine the level of O-glycosylation at the thymocyte cell surface, flow cytometry was performed with jacalin and PNA lectins. These two lectins are unique in that they are O-glycosylation-specific and bind to many O-glycans containing the structure Galβ1,3GalNAcα, although PNA binding is abolished following sialic acid addition (19). No differences in lectin binding were observed between GT^{WT}/GT^{WT} and GT^-/GT^- cells among the four subpopulations of thymocytes (Fig. 4*B*), and thus O-glycan formation continues in the absence of a functional targeted polypeptide GalNAc-T allele. T-cell colonization of secondary lymphoid organs was also normal. In addition, polypeptide GalNAc-T enzyme activity in thymocytes from GT^{WT}/GT^{WT} and GT^-/GT^- mice was quantitated with the peptide substrate PTTDSTTPAPTTK. The specific activity of GalNAc transfer in GT^{WT}/GT^{WT} thymocytes (1.9 $\pm$ 0.5 units/mg) was similar to that in GT^-/GT^- thymocytes (2.1 $\pm$ 0.4 units/mg).

DISCUSSION

O-Linked Oligosaccharides and T-Cell Development. While the majority of glycosyltransferases exhibit substrate specificity for underlying oligosaccharides, some recognize peptide- or protein-specific conformations (20). Polypeptide GalNAc-T is an example of the latter and transfers a GalNAc monosaccharide to serine and threonine residues (9, 10). Some studies have suggested the possibility of multiple GalNAc-T enzymes (9, 21), although genetic evidence in favor of this has not been reported. O-linked oligosaccharides are found ubiquitously among mammalian cell types, and their production is altered during differentiation (22). In T-cell development, alterations in the cell surface oligosaccharide repertoire correlate with thymocyte colonization of cortical and medullary compartments (4); hence it is possible that O-glycans may participate in T-cell differentiation and perhaps recirculation among lymphoid organs. Numerous biologically significant structures are likely to be present within O-linked oligosaccharides one of which is sialyl Lewis X. This tetrasaccharide is a ligand for

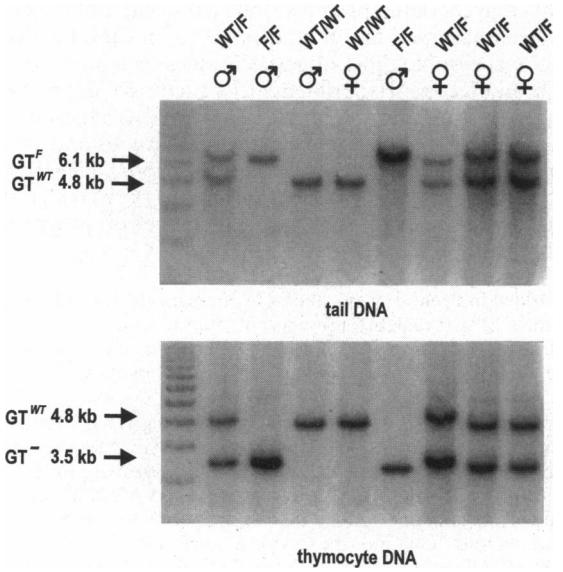

FIG. 2. *lck*–Cre recombination at polypeptide GalNAc-T alleles in DNA derived from thymocytes but not from tail tissue. Eight Cre transgenic offspring bearing the indicated GalNAc-T germline genotypes (top) were derived from Cre transgenic parents that were heterozygous in polypeptide GalNAc-T genomic structure (GT^{WT}/GT^{F}). DNAs from these offspring were subjected to Southern blot analysis with the polypeptide GalNAc-T genomic probe. Approximately 5 μg of DNA was digested with *Xba* I and analyzed from each sample.

selectin binding on vascular endothelium, and production of this structure can be dependent upon the activity of a fucosyltransferase (23, 24).

In experiments designed to further explore O-glycan function, we inactivated a polypeptide GalNAc-T gene by Cre

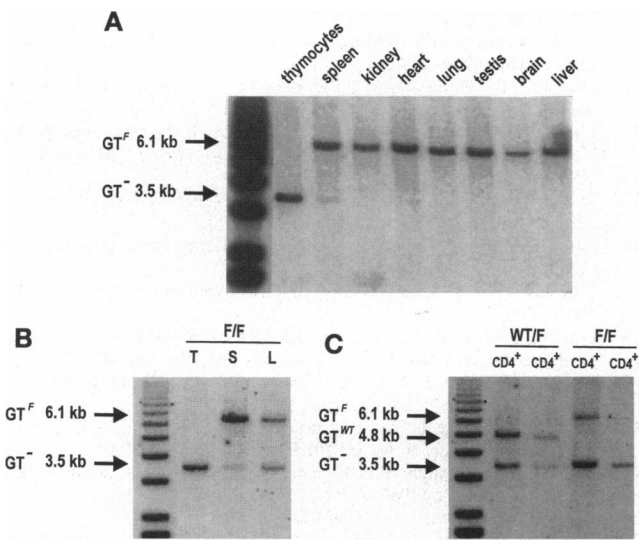

FIG. 3. Polypeptide GalNAc-T gene deletion occurs specifically in thymocytes, secondary lymphoid organs, and purified $CD4^+$ splenocytes, but not in other tissues. (*A*) *Xba* I-digested genomic DNA (5 μg) from indicated tissues of a Cre trangenic mouse bearing homozygous GT^F/GT^F germline polypeptide GalNAc-T alleles. (*B*) Polypeptide GalNAc-T gene structure in thymocytes (T), splenocytes (S), and lymph node cells (L) of a Cre transgenic mouse. (*C*) Cre transgenic $CD4^+$ splenic T cells from above mice containing the germline GT^F/GT^F genotype and the thymocyte GT^-/GT^- genotype were isolated by flow cytometry and analyzed for polypeptide GalNAc-T genomic structure. In *B* and *C*, structure of germline polypeptide GalNAc-T alleles are indicated above each lane. Approximately 5 μg of DNA was analyzed from each sample in *B* and *C*.

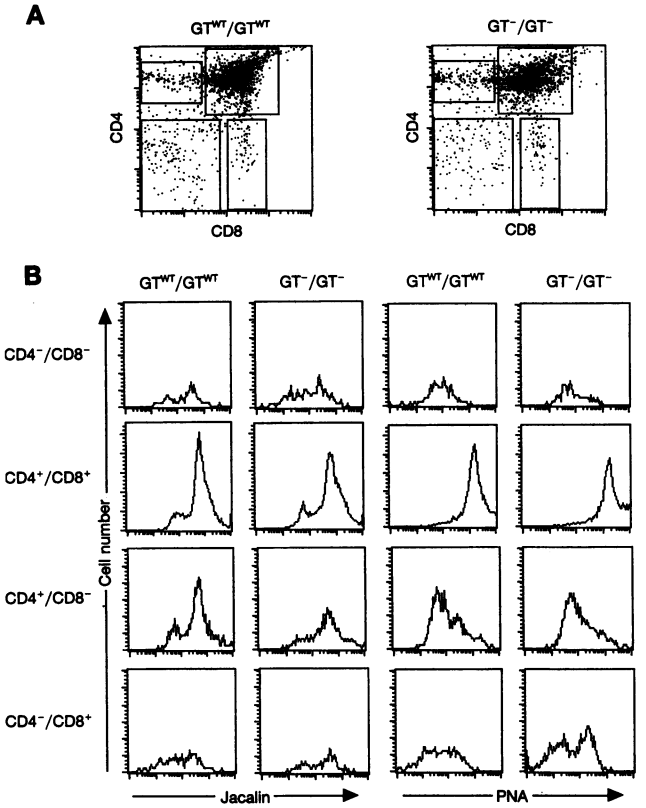

FIG. 4. Thymocytes homozygous for the systemic-null (GT^-) polypeptide GalNAc-T mutation exhibit normal development and O-glycan lectin binding. Mice heterozygous for the deleted GalNAc-T allele were produced from 3M3 ES cells and mated to generate homozygous offspring for these analyses. (*A*) Thymocytes were labeled with anti-CD4 and anti-CD8 antibodies prior to flow cytometric analysis. Fifteen thousand events were gathered and analyzed for each. (*B*) PNA and jacalin lectin binding were assessed on CD4/CD8 thymocyte subpopulations derived from *A*.

recombinase at high efficiency on both *loxP*-flanked alleles in a T-cell-specific manner *in vivo* (Figs. 2 and 3). However, PNA and jacalin lectin binding on the thymocyte cell surface revealed normal levels of O-glycans bearing structures that included Galβ1,3GalNAcα (see below). Although a long GalNAc-T half-life and incomplete recombination might provide sufficient enzyme for thymocyte O-glycan formation in this system, flow cytometric studies of peripheral T cells with PNA and jacalin lectins also revealed normal cell surface O-glycan levels (unpublished observations). Moreover, mice bearing the mutation systemically were viable and exhibited unaltered T-cell development, including normal cell surface O-glycan levels (Fig. 4). Enzymatic studies of thymocytes and other somatic cell types from such mice did not indicate significant loss of UDP-GalNAc transfer activity, as was the case in embryonic stem cells (Fig. 1*D* and unpublished observations). It is therefore likely that additional polypeptide GalNAc-T genes exist and are perhaps induced among somatic tissues in adult physiology (see further below).

Unlike results in thymocytes, $CD4^+$ splenic T cells harboring the germline GT^F/GT^F configuration and the Cre transgene exhibited a lower extent of recombination than their GT^{WT}/GT^F counterparts, perhaps reflecting an advantage among this heterogenic population for peripheral T cells that retain a functional targeted polypeptide GalNAc-T allele. Quantitation by phosphor-imager analyses revealed that in one sample 83% of alleles were GT^- among GT^F/GT^F;Cre transgenic mice (Fig. 3*C*), and thus the GT^-/GT^- population comprised from 66–83% of these splenic $CD4^+$ T cells. This situation

could arise if a small number of thymocytes retained a GT^F allele through differentiation and underwent clonal expansion in the periphery.

Cre Recombination at the Targeted Polypeptide GalNAc-T Locus. Conditional gene-inactivation methods hold promise for developing previously intractable research areas. The Cre/*loxP* integrase recombination system derived from bacteriophage P1 has previously been successfully applied *in vivo* to delete the mouse DNA polymerase β gene specifically in T cells (13). Those results however did not reproduce the efficiency observed at a different chromosomal locus (12), implying a potential selection for T cells that retained a functional polymerase β gene and the possibility that chromatin structure may affect Cre access and function (16). In comparison, a highly related member of the integrase family, Flp (reviewed in ref. 25), which acts in a mechanistically identical manner, has not been reported to be capable of reproducing Cre recombination efficiencies in mammalian cells. Because of the need for conditional gene-inactivation approaches, it is worth considering a few possible explanations for these observed variations.

Both reinsertion and failure to excise may occur. However, degradation of excised DNA (12–15) suggests that in the continued presence of recombinase activity, reinsertion should lead to reexcision and, hence, to irreversible deletion via degradation. Modifications to recombinase coding sequences or target/DNA binding sites could influence recombinase efficacy. Unlike the *loxP* structure, the 13-base binding site for Flp, termed *frt*, is reiterated on one side of the asymmetric spacer. Although Flp works well in some systems (26), resolution of the Holliday recombination intermediate in some mammalian cells may be adversely affected by the presence of an additional DNA-bound Flp monomer at the site of synapse.

Enhanced Cre function could be mediated by incorporating some aspects of Flp structure, including eukaryotic translational and nuclear localization signals (14, 15). We compared nine *lck* Cre transgenic lines in the course of this study and found that Cre recombination at the targeted polypeptide GalNAc-T locus occurred in a transgene dose-responsive manner and was significantly increased in comparison with the wild-type version of Cre, which lacks both eukaryotic translational and nuclear localization signals (ref. 12 and unpublished observations).

Potential for Polypeptide-Specific O-Glycosylation. The GalNAc-T mutant phenotype detailed thus far exposes a complexity in the biosynthesis of O-linked oligosaccharides that can be explained by the presence of multiple polypeptide GalNAc-Ts. Previously, linkage of saccharides to polypeptide or lipid in generating the vertebrate oligosaccharide classes have been dependent upon the activity of single gene products (20). Nevertheless, purified and recombinant polypeptide GalNAc-T enzyme has displayed unexpected preference for threonine residues *in vitro*, and other data have also suggested the presence of additional enzymes with similar activity (5, 24). Recent submissions to the DNA databases include multiple sequences that are highly homologous to bovine polypeptide GalNAc-T among organisms including *Caenorhabditis elegans* and *Homo sapiens*. The amino acid sequence identity among multiple human entries is $\approx$60% in comparison to the human polypeptide GalNAc-T catalytic region, revealing the presence of at least one highly related gene (27). Moreover, this degree of identity is unusually high among comparisons of glycosyltransferase sequences, implying that such putative proteins will exhibit similar enzymatic activities.

While complete functional redundancy among multiple polypeptide GalNAc-transferases is possible, polypeptide substrate preference may variably overlap, thus resulting in the potential for protein-specific O-glycan formation. The absence of a polypeptide consensus motif for O-glycosylation, as exists for N-linked glycosylation, may thus reflect the presence of multiple GalNAc-Ts with varied polypeptide specificities. In this regard, diminution specifically among the repertoire of highly O-glycosylated proteins (mucins) would not necessarily have been observed in our studies, since mucin domains may render inaccessible those oligosaccharides recognized by PNA and jacalin lectins. Experiments to structurally define potentially minor alterations in the O-linked oligosaccharide repertoire among various cell types from homozygous and systemic polypeptide GalNAc-T-null mice are nontrivial. Nevertheless, such studies will be important, as they may reveal significant phenotypic properties and the existence of polypeptide-specific O-glycan biosynthetic pathways.

Note Added in Proof. Recent studies in our laboratories and those now published (28) reveal the presence of multiple human polypeptide GalNAc-T genes that encode GalNAc transferase activity and contain between 40% and 99% amino acid identity to bovine GalNAc-T1.

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