cDNA Cloning and Expression of a Novel Human UDP-N-acetyl-α-D-galactosamine POLYPEPTIDE N-ACETYLGLACTOSAMINYLT RANSFERASE, GalNAc-T3*

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The glycosylation of serine and threonine residues during mucin-type O-linked protein glycosylation is carried out by a family of UDP-GalNAc-polypeptide N-acetylgalactosaminytransferases (GalNAc-transferase). Previously two members, GalNAc-T1 and -T2, have been isolated and characterized. Here we report the cDNA cloning and expression of a novel GalNAc-transferase termed GalNAc-T3. The gene was isolated and cloned based on the identification of a GalNAc-transferase motif (61 amino acids) that is shared between GalNAc-T1 and -T2 as well as a homologous Caenorhabditis elegans gene. The cDNA sequence has a G33-amino acid coding region indicating a protein of 72.5 kDa with a type II domain structure. The overall amino acid sequence similarity with GalNAc-T1 and -T2 is approximately 45%. 12 cysteine residues that are shared between GalNAc-T1 and -T2 are also found in GalNAc-T3. GalNAc-T3 was expressed as a soluble protein without the hydrophobic transmembrane domain in insect cells using a Baculo-virus vector, and the expressed GalNAc-transferase activity showed substrate specificity different from that previously reported for GalNAc-T1 and -T2. Northern analysis of human organs revealed a very restricted expression pattern of GalNAc-T3.

The glycosylation of serine and threonine residues during mucin-type O-linked protein glycosylation is carried out by a family of UDP-GalNAc-polypeptide N-acetylgalactosaminytransferases (GalNAc-transferase)1 (EC 2.4.1.41). Two distinct mucin-type O-linked protein glycosylation is carried out by a family of UDP-GalNAc-polypeptide N-acetylgalactosaminytransferases (GalNAc-transferase). Previously two members, GalNAc-T1 and -T2, have been isolated and cloned and characterized. Here we report the cDNA cloning and expression of a novel GalNAc-transferase termed GalNAc-T3. The gene was isolated and cloned based on the identification of a GalNAc-transferase motif (61 amino acids) that is shared between GalNAc-T1 and -T2 as well as a homologous Caenorhabditis elegans gene. The cDNA sequence has a G33-amino acid coding region indicating a protein of 72.5 kDa with a type II domain structure. The overall amino acid sequence similarity with GalNAc-T1 and -T2 is approximately 45%. 12 cysteine residues that are shared between GalNAc-T1 and -T2 are also found in GalNAc-T3. GalNAc-T3 was expressed as a soluble protein without the hydrophobic transmembrane domain in insect cells using a Baculo-virus vector, and the expressed GalNAc-transferase activity showed substrate specificity different from that previously reported for GalNAc-T1 and -T2. Northern analysis of human organs revealed a very restricted expression pattern of GalNAc-T3.

The human GalNAc-transferases T1 and T2 share a segment of 61 amino acids with 82% sequence similarity, and this segment is also found in a deduced homologue, ZK 688.8 (see Fig. 1), which has been observed to exhibit GalNAc-transferase activity (5). In the present study we have utilized this potential GalNAc-transferase motif to develop a PCR strategy that identified two novel cDNAs with sequence similarity. Here we report the cloning of a cDNA containing the complete coding sequence of one of these and show by expression that the gene encoded a GalNAc-transferase with an acceptor substrate specificity partly different from GalNAc-T1 and -T2. Northern analysis showed that the expression of GalNAc-T3 is highly tissue-restricted in contrast to GalNAc-T1 and -T2.

EXPERIMENTAL PROCEDURES

Identification of cDNA Homologous to GalNAc-T1 and -T2 by RT-PCR and Restriction Enzyme Analysis—Multiple sequence alignment analysis (DNASIS, Hitachi) of GalNAc-T1 and -T2 was applied to identify areas with highest degree of sequence similarity. Based upon a 61-amino acid segment shared by GalNAc-T1 and -T2 as well as a more recently reported sequence derived from a homologous Caenorhabditis elegans gene (5), a pair of sense and anti-sense primers (EBHC100, 5’-TTGTTGGGAGGAGARACCCTAGA-3’, and EBHC308, 5’-ATTCTCTCATCCATTCTTCT3’, respectively), was used in RT-PCR amplification of poly(A) RNA from several sources (see Figs. 1 and 2). The mRNA from human organs (liver, brain, and submaxillary gland) were obtained from Clontech, and mRNA from human cancer cell lines (MKN45, Colo205, and WI38) was prepared as reported previously (3).

A restriction enzyme search identified a common BstNI site within the expected 196-bp RT-PCR product of GalNAc-T1 and -T2, which would
produce two fragments of 128 and 68 bp. Novel DNA fragments representing putative additional GalNAc-transferases were identified by RT-PCR with EBHC100/EBHC106 primers. Six mRNA templates were analyzed after BstNI digestion in 2% agarose gels. Following reverse transcription using the EBHC106 primer, PCR was performed for 35 cycles of 95°C for 45 s, 53°C for 15 s, and 72°C for 15 s using Taq polymerase on a model 480 thermocycler (Perkin Elmer). Productswere sequenced. Two sets of sequences differing from GalNAc-T1 and -T2 but exhibiting a high degree of similarity were identified, and sequence information from one set of identical clones designated TE3 was used for the isolation of 5' and 3' sequences outside the GalNAc-transferase motif.

Cloning and Sequencing of GalNAcT3 by Rapid cDNA Library Screening—Rapid library screening was performed by diluting 1 × 10^6 pfu of human salivary gland cDNA library (Clontech) into 40 sublibraries (designated numbers 1-40), each possessing approximately 2.5 × 10^6 pfu. All sublibraries were subjected to phage amplification (approximately 40-fold) by liquid culture phage amplification (15), giving a sublibrary titer of 1 × 10^7 pfu. Phage amplification was performed in 1 ml of LB MgSO4 maltose medium in a shaking incubator at 37°C for 5 h. After amplification, 20 µl of chloroform was added to each sublibrary, cellular debris was pelleted, and the phage supernatants were titrated and used in subsequent screenings. All 40 sublibraries were screened to identify TE3 possessing phage clones. One µl of each sublibrary (approximately 10^4-10^5 pfu) was lysed in a µl of volume in the presence of 0.45% Nonidet P-40 and Tween 20, 100 µg/ml proteinase K at 56°C for 30 min. Proteinase K was heat-inactivated by boiling for 15 min, and 2 µl of phage lysate was amplified by PCR using primers EBHC100 and EBHC204 at 0.5 µm using 40 cycles of 95°C for 45 s, 55°C for 15 s, and 72°C for 15 s. Three sublibraries found to contain TE3 cDNA clones were further assayed by PCR using EBHC202 (5'-GCGCATCCGACGAAAAAAGCTTCAGGTT-3') or EBHC204 (5'-GGGATCTCTTCAGAATCTGAGTCC-3') primers combined with the cDNA vector primers to estimate lengths of cDNA inserts for selection of sublibraries with most sequences. Amplifications were performed for 35 cycles of 95°C for 45 s, 55°C for 1 s, and 72°C for 2 min.

Two sublibraries generated 3' PCR products (EBHC204/cDNA) of approximately 1000 bp, and two sublibraries generated TE3 5' PCR products of approximately 1200 bp. PCR products were subcloned into pT7T3U19 and sequenced. Both sublibraries were subjected to phage amplification (approximately 40-fold) by liquid culture phage amplification (15), giving a sublibrary titer of approximately 10^7 pfu. Lysate was amplified by PCR using primers EBHC100 and EBHC204 at 0.5 µm using 40 cycles of 95°C for 45 s, 55°C for 15 s, and 72°C for 15 s. Three sublibraries found to contain TE3 cDNA clones were further assayed by PCR using EBHC202 (5'-ACCAGATCTCCAACGATG-3') and EBHC204 (5'-TGGGATCTCTTCAGAATCTGAGTCC-3') primers combined with the cDNA vector primers to estimate lengths of cDNA inserts for selection of sublibraries with most sequences. Amplifications were performed for 35 cycles of 95°C for 45 s, 55°C for 1 s, and 72°C for 2 min.

Genomic DNA was isolated from sublibraries with most sequences and used in RT-PCR reactions with mRNA from a variety of human organs and cell lines. A single DNA fragment of approximately 250 bp was amplified from all templates (Fig. 2). Hybridization with oligonucleotide probes specific for GalNAc-T1 and -T2 was amplified from all templates. Twenty µl of culture supernatant after incubation at 37°C for 20 min. Controls included untransformed Sf9 cell culture medium and a control pAcGP67-OE-sol of the enzymatically nonfunctional histo-blood group O2 cDNA and as described previously for the blood group A CDNA (21). Co-transfection of SF9 cells with pAcGP67-constrasts and Baculo-Gold® DNA was performed according to the manufacturer's description. Briefly, 0.5 µg of construct was mixed with 0.05 µg of Baculo-Gold DNA and co-transfected in SF9 cells in 24-well plates. 48h post-transfection recombinant virus was amplified in 6-well plates at dilutions of 1:10 and 1:50. Titer of amplified virus was estimated by titration in 24-well plates with monitoring of enzyme activity. Transfection assays were performed on supernatants of SF9 cells in 6-well plates infected with virus at titer 1:1000 to 1:5000 representing end point dilutions giving optimal enzyme activity.

The 196-bp products from RT-PCR of MKN45 mRNA that were resistant to TCA-3 probes specific for GalNAc-T1 or -T2 (EBHC112, 5'-CTTTGGAATT-GTATCATGCTA-3', and EBHC45, 5'-TGGGTCACGCTTGGAGATC-3', respectively).

Polymerase on a model 480 thermocycler (Perkin Elmer). Products were sequenced. Two sets of sequences differing from GalNAc-T1 and -T2 but exhibiting a high degree of similarity were identified, and sequence information from one set of identical clones designated TE3 was used for the isolation of 5' and 3' sequences outside the GalNAc-transferase motif.

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yielded two sublibraries (number 8 and number 1) containing long 3\' and 5\' sequences outside the TE3 probe area. This strategy facilitated identification of cDNA clones with long 5\' and 3\' inserts and allowed us to compare multiple 5\' and 3\' sequences obtained within the isolated cDNA clones to identify and avoid intron containing sequences. Two PCR products of 1000 bp from sublibrary 8 and two PCR products from sublibrary 1 of 1200 bp were selected, subcloned, and sequenced. The sequences of these PCR products exhibited similarity to the sequences of GalNAc-T1 and -T2. One 5\' cDNA clone 1.5\' possessed a 1300-bp open reading frame but was not considered to contain the complete coding sequence, because it lacked a putative hydrophobic transmembrane region. A second screen using an antisense primer EBHC211 (5\' region of clone 1.5\') yielded another 5\' clone, 12.5\', which included additional 550 bp of 5\' sequence including a potential transmembrane region. As shown in Fig. 4, the combined sequences of the selected cDNA clones contained an 1902-bp open reading frame. Multiple alignment analysis (DNASIS, Hitachi) of human GalNAc-T1, -T2, and -T3 and the \textit{C. elegans} gene (accession number L16621) revealed a variable insert 61-amino acid motif. Sequences given are compared with the human GalNAc-T1 (3) where an asterisk indicates an identical amino acid or base. The location and sequence of primers (EBHC100/EBHC106) used in the RT-PCR cloning strategy are indicated. The sequence of the GalNAc-transferase motif of the isolated TE3 DNA shown to represent GalNAc-T3 is also shown.

Expression of GalNAc-T3—Expression of the pAcGP67-GalNAc-T3-sol construct in Sf9 cells resulted in a 20–100-fold increase in GalNAc-transferase activity in culture medium of infected cells compared with uninfected controls or cells infected with the histo-blood group O2 gene (Table I). GalNAc-transferase activity with the Muc2 acceptor substrate peptide was increased 20-fold, and activity with the HIV-V3 peptide was increased nearly 100-fold. In contrast, expression of GalNAc-T1 and -T2 constructs only increased the GalNAc-transferase activity 3.5-to-4-fold.

3. E. P. Bennett and H. Clausen, unpublished observations.
Background levels of GalNAc-transferase activity in uninfected cell medium was higher than in control infected cell medium, probably as a result of the production and release of endogenous Sf9 GalNAc-transferase due to the larger number of cells in uninfected cultures. Furthermore, background enzyme activity varied significantly among different acceptor substrate peptides. The peptide Muc2 yielded the highest background and HIV-V3 peptide yielded the lowest activity. In an early attempt to express functional pAcGP67-GalNAc-T3, constructs were made that were truncated either at the 5' or 3' end (data not shown). Interestingly, constructs lacking the COOH-terminal or NH2-terminal amino acid were completely inactive, indicating that both the stem region and the COOH-terminal region are important for maintaining a catalytically active protein, a feature also found for the α3-galactosyltransferase (14).

Northern Blot Analysis of Human Organs—Northern blots with mRNA from 16 human adult and 5 fetal organs were probed with GalNAc-T1, -T2, and -T3 (Fig. 6). Similar to previous results using the multiple tissue Northern blot, MTN I, GalNAc-T1 hybridized to two mRNAs of approximately 3.4 and 4.1 kb (1), whereas GalNAc-T2 hybridized to a 4.5-kb mRNA. Variable amounts of a smaller 2–3-kb mRNA were also detected with this probe (3). Hybrization of these probes to multipletissue Northern blots, MTNII and fetal MTN, resulted in slightly different estimated mRNA sizes for all GalNAc-Ts. This discrepancy is probably due to differences in the parameters of gel electrophoresis and the marker positions assigned by the supplier. GalNAc-T3 hybridized to a 3.6-kb mRNA (estimated from MTN I) highly expressed in pancreas and testis, which was weakly expressed in kidney, prostate, ovary, intestine, and colon. A very low level of GalNAc-T3 mRNA was also detected in adult placenta and lung as well as fetal lung and kidney. In adult spleen GalNAc-T3 hybridized to a larger 4.2-kb mRNA (estimated from MTN II).

DISCUSSION

This study presents data on the cloning, sequencing, and expression of a third member of a growing family of polypeptide transferase activity toward Muc2 and MucSC peptide substrates.

Background levels of GalNAc-transferase activity in uninfected cell medium was higher than in control infected cell medium, probably as a result of the production and release of endogenous Sf9 GalNAc-transferase due to the larger number of cells in uninfected cultures. Furthermore, background enzyme activity varied significantly among different acceptor substrate peptides. The peptide Muc2 yielded the highest background and HIV-V3 peptide yielded the lowest activity. In an early attempt to express functional pAcGP67-GalNAc-T3, constructs were made that were truncated either at the 5' end or 3' end (data not shown). Interestingly, constructs lacking the COOH-terminal or 55 NH2-terminal amino acid were completely inactive, indicating that both the stem region and the COOH-terminal region are important for maintaining a catalytically active protein, a feature also found for the α3-galactosyltransferase (14).

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cDNA Cloning and Expression of GalNAc-T3

Table I

Expression of GalNAc-T3 in Sf9 cells

| Constructs | Specific activitya | Muc2 | Muc5 | HIV-V3 |
|------------|-------------------|------|------|--------|
| pAcGP67-GalNAc-T3-sol | 2.05 | 0.77 | 0.97 |
| pAcGP67-GalNAc-T2-sol | 1.98 | 1.24 | 0.03 |
| pAcGP67-GalNAc-T2-sol | 1.20 | 0.66 | 0.02 |
| pAcGP67-GalNAc-T3-sol | 0.08 | 0.02 | 0.01 |
| Uninfected cells | 0.12 | 0.04 | 0.01 |

a One unit of enzymes is defined as the amount of enzyme that transfers 1 μmol GalNAc in 1 min using the standard reaction mixture as described under “Experimental Procedures” with 50 μg of peptide as acceptor substrate.

GalNAc-transferases. A putative GalNAc-transferase motif of 61 amino acids that is highly conserved in sequence among GalNAc-T1, GalNAc-T2, and C. elegans homologue was used to search for potential additional members of the polypeptide GalNAc-transferase family. The screening strategy included an RT-PCR strategy similar to that reported for the sialyltransferase family (12, 23) followed by restriction enzyme analysis as a selection procedure. This method allowed us to eliminate or reduce “background” for the two known GalNAc-transferases, GalNAc-T1 and GalNAc-T2, and clearly distinguish novel RT-PCR products of the same size as those for GalNAc-T1 and -T2. Two novel DNA fragments were identified and sequenced. The present study presents data about one of these.

The novel cDNA was shown by expression in insect cells to have polypeptide GalNAc-transferase activity that transfers 1 μmol GalNAc in 1 min using the standard reaction mixture as described under “Experimental Procedures” with 50 μg of peptide as acceptor substrate.

Fig. 5. Multiple sequence alignment (DNASIS, Hitachi) of human GalNAc-T1 (T1), GalNAc-T2 (T2), GalNAc-T3 (T3), and C. elegans (C) predicted protein ZK.688.8. Introduced gaps are shown as hyphens, and aligned residues identical to GalNAc-T1 are indicated by colons. Hydrophobic sequences representing putative transmembrane domains are indicated by underlining. The first step of mucin-type O-glycosylation is mediated by GalNAc-transferase activity in total extracts of various cell lines and organs (4, 27). GalNAc-T3 also catalyzed glycosylation of mucin-type acceptor sequences such as Muc2 and Muc5, which can also be glycosylated by GalNAc-T1 and -T2. In a previous study we found that the enzyme activity that mediated glycosylation of the HIV peptide also utilized the Muc2 substrate by cross-competitive glycosylation (4). This finding is consistent with the substrate specificity reported here for GalNAc-T3, suggesting that GalNAc-T3 may represent this particular enzyme; however, additional enzymes may also show related specificities. Detailed analysis of individual GalNAc-transferases with a large panel of peptides and structural confirmation of the specific acceptor sites utilized for GalNAc-glycosylation will be necessary to fully understand the specificity of the individual members of the enzyme family.

The first step of mucin-type O-glycosylation is mediated by at least three and probably more GalNAc-transferases. The data presented here clearly show that GalNAc-T3 exhibits a different acceptor substrate specificity than GalNAc-T1 and -T2. Among a panel of acceptor substrate peptides (Table I), GalNAc-T3 was found to glycosylate a peptide derived from the HIV envelope glycoprotein gp120, which did not serve as substrate for GalNAc-T1 or -T2 (4, Table I). This peptide was identified as an acceptor substrate during analysis of enzyme activity in total extracts of various cell lines and organs (4, 27). GalNAc-T3 also catalyzed glycosylation of mucin-type acceptor sequences such as Muc2 and Muc5, which can also be glycosylated by GalNAc-T1 and -T2. In a previous study we found that the enzyme activity that mediated glycosylation of the HIV peptide also utilized the Muc2 substrate by cross-competitive glycosylation (4). This finding is consistent with the substrate specificity reported here for GalNAc-T3, suggesting that GalNAc-T3 may represent this particular enzyme; however, additional enzymes may also show related specificities. Detailed analysis of individual GalNAc-transferases with a large panel of peptides and structural confirmation of the specific acceptor sites utilized for GalNAc-glycosylation will be necessary to fully understand the specificity of the individual members of the enzyme family.

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In contrast to GalNAc-T1 and -T2, expression of GalNAc-T3 appears to be highly regulated and mainly found in pancreas and testis; weak expression is found in a few other organs including placenta. Interestingly, approximately 200 bp covering part of the GalNAc-transferase motif of GalNAc-T3 were recently sequenced from a pancreatic expressed sequence library (EMBL accession number T11328). The lack of GalNAc-T3 expression in human liver correlates with the finding that organ extracts from human liver lacked GalNAc-transferase activity utilizing the HIV peptide, whereas expression of GalNAc-T3 mRNA in human placenta is in agreement with GalNAc-transferase activity using the HIV peptide in extracts of placenta (4). One interpretation of these data is that differential expression of different GalNAc-transferases can result in O-glycosylation of distinct sites on a given protein. The biological significance of this is unclear. There are a few studies on O-glycosylation sites, and these are limited to analysis of the functional activity or stability of a protein with or without a single O-glycan singly carrying O-glycosylation. On one hand, there may be a large number of acceptor sites (28), because assignments of O-glycosylation sites are difficult to perform (29).

The results presented here suggest that cell-, organ-, and species-specific differences in the position of O-glycosylation may occur as a result of differential expression of polypeptide GalNAc-transferases. In searching for potential motifs of O-glycosylation by analyzing serine and threonine residues carrying O-glycans in glycoproteins, one may need to consider the GalNAc-transferase repertoire of the cell of origin (30, 31). The existence of a transferase family of unknown size possibly exceeding three members displaying differential acceptor substrate specificity and cell/organ distribution suggests that mucin-type O-glycosylation is a much more defined and controlled process than previously recognized. In this respect, previous studies aimed at identifying consensus sequence motifs for O-glycosylation may not have identified such because of the unknown level of complexity. The data reported here suggest that O-glycosylation in terms of sites is less random than previously suggested and that more defined acceptor substrate peptide sequences may be recognized for each of the individual GalNAc-transferases. With the individual GalNAc-transferases expressed as recombinant proteins, it may be possible to determine primary peptide sequence motifs for the individual enzymes, which could be useful for predicting O-glycosylation in vivo by a given cell type.

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9. Schacht, H. (1994) in Molecular Glycobiology (Fukuda, M., and Hindsgaul, FIG. 6. Northern blot analysis of human tissues. Multiple human Northern blots (MTN I, MTN II, and fetal MTN) from Clontech as labeled was probed with 32P-labeled GalNAc-T1 probe, TEB1 (T1), GalNAc-T2 probe, TEB2 (T2), and GalNAc-T3 probe, TEB3 (T3), respectively.
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