UDP-N-acetyl-\(\alpha\)-D-galactosamine:polypeptide N-Acetylgalactosaminytransferase

IDENTIFICATION AND SEPARATION OF TWO DISTINCT TRANSFERASE ACTIVITIES*

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Using a defined acceptor substrate peptide as an affinity chromatography ligand we have developed a purification scheme for a unique human polypeptide, UDP-GalNAc-polypeptide N-acetylgalactosaminytransferase (GalNAc-transferase) (White, T., Bennett, E. P., Takio, K., Sørensen, T., Bonding, N., and Clausen, H. (1995) J. Biol. Chem. 270, 24156–24165). Here we report detailed studies of the acceptor substrate specificity of GalNAc-transferase purified by this scheme as well as the GalNAc-transferase activity, which, upon repeated affinity chromatography, evaded purification by this affinity ligand. Using a panel of acceptor peptides, a qualitative difference in specificity between these separated transferase preparations was identified. Analysis of GalNAc-transferase activities in four rat organs and two human organs also revealed qualitative differences in specificity. The results support the existence of multiple GalNAc-transferase activities and suggest that these are differentially expressed in different organs. As the number of GalNAc-transferases existing is unknown, as is the specificity of the until now cloned and expressed GalNAc-transferases (T1 and T2), it is as yet impossible to relate the results obtained to specific enzyme proteins. The identification of acceptor peptides that can be used to discriminate GalNAc-transferase activities is an important step toward understanding the molecular basis of GalNAc O-linked glycosylation in cells and organs and in pathological conditions.

Glycosylation of proteins in eukaryotes is fundamental for the integrity of the individual cell and the organism as a whole (Varki, 1993). A number of different types of protein glycosylations have been identified (for a recent review see Lis and Sharon, 1993). Biosynthesis of the initial glycosylation of the protein backbone has been established in most cases and the involved glycosyltransferases partly characterized. In several cases characterization of glycosylation sites has identified peptide motifs that suggest the nature of the acceptor substrate specificities of transferases initiating protein glycosylation.

Thus N-linked asparagine glycosylation is restricted to the sequence -Asn-Xaa-Ser/Thr- (where Xaa may be any amino acid except proline). Proteoglycan-type glycosylation of serine is restricted to -Ser-Gly-Xaa-Gly- (Bourdon et al., 1987). The GlcNAc-type glycosylation of serine or threonine appears to be adjacent to an acidic amino acid and within two residues of a proline (Haltiwanger et al., 1992). The fucose-type glycosylation of serine/threonine seems to be restricted to the peptide sequence -Gly-Gly-Thr/Ser-Cys-, although the enzyme has yet to be characterized (Harris and Spellman, 1993).

In contrast, a defined peptide motif for GalNAc O-glycosylation (mucin type) and the equivalent yeast Man-type glycosylation of serine/threonine has not emerged. A number of studies have attempted to identify a consensus sequence for mammalian GalNAc O-glycosylation by studying sequences around identified glycosylation sites (Gooley et al., 1991; O'Connell et al., 1991; Wilson et al., 1991; Elhammer et al., 1993) as well as by testing the peptide substrate specificity of the GalNAc-transferase activity in crude and pure form (O'Connell et al., 1992; Wang et al., 1992, 1993; Elhammer and Kornfeld, 1986; Hagen et al., 1993; O'Connell and Tabak, 1993; Gooley and Williams, 1994; Nishimori et al., 1994a, 1994b). It is clear from these studies that the GalNAc-transferase must have broad acceptor substrate specificity, but it is likely that our understanding of this broad motif is shadowed by the involvement of several GalNAc-transferases. As described in the accompanying paper (White et al., 1995) a novel GalNAc-transferase has been isolated and cDNA cloned, which, together with the previously cloned bovine GalNAc-transferase (Homa et al., 1993), clearly establishes the existence of at least two distinct enzymes. By analogy, Strahl-Bolsinger et al. (1993) provided evidence that more than one polypeptide O-mannosyltransferase exists in yeast.

The total number of existing GalNAc-transferases is unknown, but it is very likely that our knowledge of this family of transferases will expand rapidly. Assigning detailed acceptor substrate specificity toward the cloned and expressed GalNAc-T1 and -T2 awaits comparative studies of recombinant transferases, and data obtained so far using purified enzyme preparations are likely to be biased by copurified mixtures of enzymes (Homa et al., 1993; White et al., 1995; Wang et al., 1993). To begin to understand the potential differential specificity of multiple GalNAc-transferases we have begun search-

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1 The abbreviations used are: GalNAc-transferase, UDP-GalNAc-polypeptide N-acetylgalactosaminytransferase; GalNAc-T1, polypeptide GalNAc-transferase originally cloned by Homa et al. (1993); GalNAc-T2, polypeptide GalNAc-transferase as cloned by White et al. (1995); HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; hCG-β, human chorionic gonadotropin-β.
ing for acceptor substrate peptides that would show differences as acceptors for different GalNAc-transferase preparations.

Here we present evidence that affinity chromatography using a defined synthetic acceptor substrate peptide resulted in separation of two different GalNAc-transferase activities and that these appear to be differentially expressed in organs. The results thus provide the first evidence for the involvement of at least two GalNAc-transferase specificities in GalNAc O-linked glycosylation initiation. The study identified acceptor peptides capable of discriminating different GalNAc-transferase activities, which should prove valuable for detailed studies of the specificity of identified and cloned enzymes in this family.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human placentas were collected (6–24 h postdelivery) after informed consent at the Herlev Hospital, Copenhagen; human liver tissue was obtained at routine autopsy. Porcine and ovine submaxillary glands were bought from Pel-Freez. Synthetic peptides were custom synthesized by Carbiotech (Copenhagen, Denmark) or Neosystems (Strassburg, France) with amino acid and mass spectrometry analysis for sequence confirmation. The peptide sequences studied are listed in Table I.

**Polypeptide GalNAc-transferase Assay**

The standard enzyme reaction mixture consisted of 25 mM Tris-HCl (pH 7.4), 0.25% Triton X-100, 5 mM MnCl₂, 5 mM CDP-choline, 5 mM 2-mercaptoethanol, 0.05 mM UDP-[14C]GalNAc (4,000 cpm/nmol), 250 µg peptide, and enzyme in a final volume of 100 µl. Unless otherwise stated, assays were incubated for 10 min at 37°C followed by Dowex 1 ion exchange (formic acid form) chromatography and scintillation counting. Combinations of substrate and enzyme source were evaluated at least once by C-18 chromatography (C2C18 3.2 Smart System, Pharmacia Biotech Inc.) to ensure stability of peptide and that incorporated [14C]GalNAc was associated with the peptide. Furthermore, to exclude the possibility that crude enzyme preparations from organs degraded or by other means blocked the acceptor substrate peptides, the human liver enzyme assay was studied in two ways: 1) time course preincubation of peptides with human liver enzyme 0–24 h at 37°C without sugar nucleotide followed by evaluation of acceptor substrate accessibility using human placenta enzyme preparation with sugar nucleotide; 2) mass spectrometry of the time course preincubation study.

When measuring Kₐ and Vₘₐₓ for different peptide substrates the UDP-[14C]GalNAc concentration was increased to 0.2 mM (4,000 cpm/ nmol), and the enzyme concentration was 0.5 milliunits/ml. Preparative glycosylation of peptides was performed with 0.5 µmol of peptide, 5 milliunits of enzyme, and 5–100 µmol of UDP-[14C]GalNAc in a final volume of 1 ml. The glycopeptide was purified by C-18 reverse phase chromatography, and the glycopeptide-containing fractions were detected by scintillation counting.

**Structure Determination**

Matrix-assisted Laser Desorption/Ionization Mass Spectrometry—Samples were dissolved in 0.1% trifluoroacetic acid to a concentration of approximately 0.05 µg/µl. One µl of sample solution was applied to a stainless steel probe tip precoated with 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in acetone, 15 µg/µl) and washed thoroughly before introduction into the mass spectrometer (Vorm et al., 1994).

All mass spectra were obtained on a Bruker reflex Time of Flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Data were acquired by a LeCroy 9450A 400 megasamples/s digital storage oscilloscope (LeCroy Corporation, Chestnut Ridge, NY) from which single shot spectra were transferred to a MacIntosh Quadra 950 computer (Apple Computer Inc., Cupertino, CA) via a National Instruments NI DAQ GPIB controller board (National Instruments, Austin, TX).

Control of data acquisition parameters, the transfer and subsequent averaging of spectra, as well as further data processing were carried out using the computer program LaserOne, which was written in ThinkC (Symantec Corporation, Cupertino, CA) by M. Mann and P. Mortensen, EMBL, Heidelberg, Germany.

All mass spectra were obtained in the linear mode and calibrated using a singly charged matrix ion, which provided a mass accuracy of approximately 0.1%.

**Amino Acid Sequencing—Peptides and glycopeptides (GalNAc-glycosylated) were sequenced automatically (Applied Biosystems 470), and the phenylthiohydantoin derivatives were analyzed by on-line high performance liquid chromatography. GalNAc-glycosylated sites were identified by the loss of the Ser/Thr-phenylthiohydantoin signal and the appearance of pseudo-peaks.

**Protein Determination**

Protein concentrations were determined by the method of Bradford (Bio-Rad protein assay) using bovine serum albumin as standard.

**Purification of GalNAc-transferase from Different Tissues**

The human placenta, and ovine and porcine submaxillary GalNAc-transferase was purified as described in the accompanying paper (White et al., 1995). Briefly, Triton X-100 extracts of organs were applied to Cibacron blue 3GAG-agarose and transferase eluted with 1.5 M KCl (step 1, see Table I). The eluted and dialyzed preparation was passed through a DEAE-Sephalocolumn (step 2). The eluted enzyme was applied to an S-Sepharose column after pH adjustment to 6.5 and the transferase activity eluted by a NaCl gradient (step 3). The pooled enzyme fractions were then applied to a Muc2 affinity column (Muc2 acceptor substrate peptide coupled to cyanogen bromide-activated Sepharose) in the presence of UDP and Mn²⁺, and bound enzyme eluted with EDTA in the absence of UDP (step 4). The eluted enzyme was dialyzed and concentrated (dialysis concentrator Spectro) and is hereafter referred to as the purified transferase preparation. Triton X-100 (0.1%) was included throughout the entire purification procedure.

In some experiments Triton X-100 was exchanged at the S-Sepharose step (step 3) by slow overnight washing with n-octyl glucoside and maltoside, and further purification was performed in n-octyl glucoside-containing buffers that were otherwise as described.

**Analysis of GalNAc-transferase Specificity in Rat and Human Organs**

Male rat or human organs were homogenized in water. After centrifugation at 10,000 × g the pellet was resuspended in water and centrifuged. The final pellet was resuspended in extraction buffer containing 1.5% Triton X-100, 2 mM EDTA, 100 mM NaCl, and 25 mM Tris-HCl (pH 6.5) and extracted for 2 h at 4°C. Because of the limited stability of the organ homogenate, detailed substrate analysis was performed on the Cibacron-purified preparations. Cibacron-purified transferase refers to the pooled 1.5 × KCl eluate dialyzed and concentrated by a dialysis concentrator. No significant differences in the substrate specificity between the crude homogenate and the Cibacron-purified transferase preparations could be measured; however, the crude transferase homogenate showed quite variable activities and was difficult to characterize.

**RESULTS**

**Purification of Porcine and Ovine GalNAc-transferase**

Table I summarizes the purification of ovine and porcine submaxillary GalNAc-transferase activity from 500 g of tissue using Muc2 affinity chromatography. Purification of the human placenta GalNAc-transferase was similar, as reported in the accompanying paper (White et al., 1995).

The purification scheme used gave a quantitatively different result for the submaxillary gland activity compared with human placenta. The initial Cibacron chromatography of ovine and porcine gland extracts yielded the same results as for the human placenta transferase. However, the yields of the ovine and porcine transferases were considerably lower at the peptide affinity chromatography step compared with human placenta. Since the human transferase purified by the same procedure using Triton X-100 as detergent in the affinity chromatography was found to be the soluble fragment without the hydrophobic transmembrane segment, this difference in yield could be related to a relatively lower ratio of soluble versus membrane-bound transferase. Human placenta tissue was obtained 6–24 h after delivery (stored at room temperature) and after freezing was subsequently thawed at 4°C for 1–3 days before extraction. In contrast the animal glands were quick frozen by the supplier (Pel-Freez) and thawed at 4°C overnight.
In separate experiments (not shown) the unbound fraction of the Muc2 affinity chromatography of porcine gland enzyme was reapplied to the Muc2 affinity column after detergent exchange from Triton X-100 to n-octyl glucoside and maltoside. Detergent exchange of the nonretained material from the Muc2 peptide column using n-octyl glucoside and maltoside followed by repeated Muc2 peptide affinity chromatography run in these detergents resulted in a considerably higher purification yield, although even repeated application on the column failed to absorb more than 50% of the transferase activity measured with the Muc2 peptide substrate. The human placenta preparation behaved similarly except for the initially higher yield of the first chromatography run in Triton X-100. Ion exchange Mono S chromatography performed without detergent on the porcine and ovine preparations obtained after detergent exchange and Muc2 affinity purification resulted in total loss of enzyme activity from the void volume to a molecular weight of approximately 100,000 (not shown), all suggestive of a very high activity. Gel filtration of the same preparations gave a spread of activities and Muc2 affinity purification resulted in total loss of enzyme activity. Gel filtration of the same preparations gave a spread of activities.

Separation of two GalNAc-transferase Activities by Affinity Chromatography on Muc2 Peptide

As shown in Fig. 1 the apparent $K_m$ for the Muc2 peptide of the Muc2 affinity chromatography-purified transferase was significantly lower than the crude transferase before chromatography and the unbound transferase activities. Combined with the finding that the Muc2 affinity chromatography appeared to bind only a fraction of the GalNAc-transferase activity even after repeated chromatography led us to study the substrate specificity of the GalNAc-transferase activities during this purification step. Table II shows the peptides, their sequences, and the apparent $K_m$ with human placenta transferase as a dialyzed S-Sepharose eluate (step 3) before the Muc2 affinity step.

Analysis of the substrate specificity of enzyme preparations before the Muc2 affinity purification, the unbound flow-through fraction, and the bound and eluted enzyme preparations from the column using various synthetic peptide substrates is presented in Table III and Fig. 2. Human placenta and ovine and porcine submaxillary transferases in the Muc2 affinity-purified form all contained both threonine and serine transferase activity as measured by the human chorionic gonadotropin-β peptide, which has only serine acceptor sites, although with a very low activity. Strikingly, all of the Muc2 affinity-purified transferase preparations (step 4) failed to glycosylate the HIV-V3 peptide sequence, whereas the enzyme preparations before affinity chromatography as well as the nonretarded materials from the affinity chromatography column readily utilized this substrate. The product of the HIV-V3 peptide glycosylated by pre-Muc2 affinity-purified enzyme preparations (step 1 or 3) was confirmed as containing a single GalNAc residue attached to the single Thr in an undegraded peptide by amino acid sequencing (Fig. 3) and mass spectrometry (not shown). Although the HIV-V3 peptide is an acceptor of in vitro enzymatic glycosylation it is not known if indeed this site serves as an in vivo O-glycosylation site. HIV gp120 is, however, O-glycosylated, and GalNAc-Ser/Thr epitopes have been identified (Hansen et al., 1992; Merkle et al., 1991).

To exclude that this difference could be ascribed to soluble versus membrane forms of enzymes tested, the apparently membrane-bound form of the GalNAc-transferase was purified and analyzed after detergent exchange. As shown in Table III the difference in substrate specificity was consistent also for the porcine transferase purified after detergent exchange. Several peptides with sequences overlapping the HIV-V3 peptide were analyzed to exclude a specific problem with the peptide design of HIV-V3, and all peptides showed the same reaction pattern with the transferase preparations tested. In Table III only the peptide HXB2 with the same internal sequence as HIV-V3 but with extended sequence in both NH2 and COOH termini is shown.

The HIV-V3 acceptor substrate peptide was identified by

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### Table I

| Protein activity | Yield | Specific activity | Purification |
|------------------|-------|------------------|--------------|
| Volume (ml)      | Protein (mg) | Activity (units) | % | units/mg | -fold |
| Triton X-100 extract | 1,500 | 20,200 | 16.3 | 100 | 0.00081 | 1.0 |
| 1. Cibacron blue eluate | 400 | 1,710 | 4.7 | 29 | 0.0028 | 3.5 |
| 2. DEAE flow-through | 475 | 713 | 3.8 | 23 | 0.0053 | 6.5 |
| 3. S-Sepharose eluate | 60 | 194 | 1.3 | 0.08 | 0.0068 | 8.4 |
| 4. Muc2 column eluate | 200 | 10 | 1.1 | 0.07 | 0.11 | 123.5 |

Ovine submaxillary gland purification steps

| Protein activity | Yield | Specific activity | Purification |
|------------------|-------|------------------|--------------|
| Volume (ml)      | Protein (mg) | Activity (units) | % | units/mg | -fold |
| Triton X-100 extract | 1,500 | 5,748 | 22.2 | 100 | 0.0039 | 1.0 |
| 1. Cibacron blue eluate | 375 | 653 | 6.4 | 29 | 0.0098 | 2.5 |
| 2. DEAE flow-through | 450 | 387 | 4.4 | 23 | 0.011 | 2.8 |
| 3. S-Sepharose eluate | 55 | 70 | 1.1 | 0.04 | 0.014 | 3.6 |
| 4. Muc2 column eluate | 1.5 | 0.75 | 0.07 | 0.003 | 0.09 | 25.4 |

Porcine submaxillary gland purification steps

| Protein activity | Yield | Specific activity | Purification |
|------------------|-------|------------------|--------------|
| Volume (ml)      | Protein (mg) | Activity (units) | % | units/mg | -fold |
| Triton X-100 extract | 1,500 | 20,200 | 16.3 | 100 | 0.00081 | 1.0 |
| 1. Cibacron blue eluate | 400 | 1,710 | 4.7 | 29 | 0.0028 | 3.5 |
| 2. DEAE flow-through | 475 | 713 | 3.8 | 23 | 0.0053 | 6.5 |
| 3. S-Sepharose eluate | 60 | 194 | 1.3 | 0.08 | 0.0068 | 8.4 |
| 4. Muc2 column eluate | 200 | 10 | 1.1 | 0.07 | 0.11 | 123.5 |

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A one unit of enzyme is defined as the amount of enzyme that will transfer 1 μmol of GalNAc from UDP-GalNAc in 1 min using the standard reaction mixture as described under "Experimental Procedures" with 25 μg of Muc2 peptide as acceptor substrate.

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**FIG. 1.** $K_m$ determination of ovine GalNAc-transferase at different stages of purification. Ovine GalNAc-transferase was purified on the Muc2 peptide affinity column (step 5). $K_m$ values were measured as described under "Experimental Procedures" and calculated from a Lineweaver-Burk double reciprocal plot to 231, 254, and 50 μM for the precolumn preparation (S-Sepharose eluate, step 4), the nonretarded material, and the eluate, respectively.
application of 15-mer overlapping peptides covering the entire
sequence of the HIV-III envelope protein gp120 as acceptor
substrate in the GalNAc-transferase assay in an attempt to
predict O-linked glycosylation sites by in vitro enzyme assay
(Clausen et al., 1994). Subsequent analysis of these peptide
sequences by the prediction model of Elhammer et al. (1993)
has shown that the glycosylation probability of the HIV-V3
peptide sequence is 0 and is therefore not predicted by the
model.

To characterize the different transferase activities further,
competitive glycosylation experiments were performed. Since
the GalNAc-transferase preparation before the Muc2 affinity
chromatography was capable of glycosylating the HIV-V3 pep-
tide as well as the Muc2 peptide, it was pertinent to analyze if
one enzyme utilized both of these substrates. As shown in Fig.
2A the Muc2 peptide was a competitive inhibitor of HIV-V3
peptide glycosylation suggestive of one enzyme utilizing both
substrates. Control experiments using Muc1 and Muc2 peptide
with Muc2 affinity-purified transferase also showed competi-
tive inhibition of Muc2 glycosylation (Fig. 2B). Surprisingly,
the HIV-V3 peptide, which was not glycosylated by the Muc2
affinity-purified transferase, was found to be an inhibitor of
Muc2 glycosylation using this enzyme preparation. Increasing
amounts of HIV-V3 peptide to Muc2 affinity-purified transferase
showed competitive inhibition of Muc2 glycosylation. These
results suggest that Muc2 peptide affinity chromatography
separates two distinct transferase activities with over-
lapping specificity concerning the Muc2 acceptor substrate but
which are distinguishable with respect to the HIV-V3 peptide.

Organ Differences in GalNAc-transferase Activities

The findings that different GalNAc-transferase activities
could be separated and that acceptor peptides that could
differentiate these were identified made it possible to analyze
to potentially differential organ expression of these activities.

As described under “Experimental Procedures” the GalNAc-
transferase preparations used were partially purified either to
step 1 or to step 3 (inclusive). As such transferase preparations
may include interfering proteolytical activity, the following
steps were taken to exclude this. 1) Routine assays included
identification of product by Dowex 1 chromatography, but in all
combinations of peptide and enzyme preparations C-18 chro-
matography of the reaction mixture revealed the same UV
profile. Potentially incorporated [14C]GalNAc was always
found associated with the peptide peak (only when peptides
containing multiple acceptor sites were used, were products
clearly separable from the unglycosylated peak by the
conditions used). 2) Mass spectrometric analysis of substrate
peptides after prolonged incubation with one of the organ en-
zyme preparations (human liver, purification step 1) showed
no evidence of degradation (Fig. 4). 3) Sequential incubation of
acceptor substrate peptides first with an organ enzyme prepa-
ration not capable or only poorly capable of incorporating Gal-
NAc (human liver, purification step 1) and second with an
organ enzyme preparation capable of incorporating GalNAc
(human placenta, purification step 3) showed little or no loss/
decreased accessibility of acceptor substrates (Fig. 5).

Table I

| Table II | Acceptor substrate peptides used and the apparent Km * of human placenta GalNAc-transferase |
|----------|-------------------------------------------------------------------------------------------------|
| Peptide  | Peptide sequence | Protein (ref.) | Km * (nmol/min/mg) |
|----------|------------------|----------------|-----------------|
| hCG-β    | PRQDSSSSKAPPSLPSRPG | Human chorionic gonadotropin β-chain (1) | ND |
| HIV-V3   | AC-CIRIQPGPGRFTIGKLMNM | HIV IIIB gp120 (2) | 0.50 |
| HXB2     | CNTRKRIRIQPGPGRFTIGK | HIV IIIB gp120 (2) | ND |
| SIV3     | VLCPTMRSSFLHISQSPR | SIVMu225 gp120 (3) | ND |
| Muc1     | APPHGVTSAPDRPAGSTAPC | Human mucin Muc1 tandem repeat (4) | 0.36 |
| Muc1a    | APPHGVTSAPDRPAGGC | Human mucin Muc1 tandem repeat (4) | 0.37 |
| Muc1b    | PDTRPAGSTAPAC | Human mucin Muc1 tandem repeat (4) | 0.50 |
| Muc2     | PTTPITTSTITMTPTPTPTC | Human mucin Muc2 tandem repeat (5) | 0.23 |

* Km values determined with S-Sepharose-purified (step 4) human placenta GalNAc-transferase. References: (1) Birken et al., 1981; (2) Myers et al., 1989; (3) Overbaugh et al., 1992; (4) Gendler et al., 1990; (5) Gum et al., 1989.

ND, not determined.

Table III

| Table III | Substrate specificity of GalNAc-transferase activities before and after Muc2 affinity chromatography |
|-----------|------------------------------------------------------------------------------------------------|
| Purification step | Human placenta | Porcine submaxillary glands | Ovine submaxillary glands |
|              | Pre-Muc2b | Muc2-Pst | Muc2-El | Pre-Muc2 | Muc2-El | Pre-Muc2 | Muc2-El |
|              | nmol/min/mg | | | nmol/min/mg | | nmol/min/mg | |
| Muc2       | 2.6 | 2.7 | 70.8 | 3.6 | 149.1 | 201.2 | 5.9 | 74.6 |
| hCG-β      | 0 | 0 | 0 | 0 | 14.0 | 14.8 | 0.2 | 9.2 |
| HIV-V3     | 1.0 | 0 | 0 | 1.0 | 0 | 0 | 1.0 | 0 |
| HXB2       | 0.5 | ND | 0 | 0.7 | 0 | 0 | 1.1 | 0 |
| SIV3       | 0.2 | ND | ND | 1.3 | 106.7 | ND | 0.4 | ND |

* Specific activities of transferase preparations: Pre-Muc2: 5'-Sepharose eluate (step 3); Muc2-Pst: flow-through of Muc2 affinity column (step 4); Muc2-El: Eluate of Muc2 affinity column (step 4); n-Octyl Muc2-El: eluate of Muc2 affinity column run in n-octyl glucoside detergent rather than Triton X-100 (step 4, modified).

ND, not determined.
the specificity of the Muc2 affinity-purified transferase.

Human Organs—Human placenta and liver were chosen as human counterparts for organ substrate analysis because human liver was found to express significantly more GalNAc-T2 than GalNAc-T1 by Northern analysis (Homa et al., 1993; White et al., 1995) (Table IV). The two organs clearly differed in GalNAc-transferase activity, as liver was unable to glycosylate the HIV-V3 peptide and preferred Muc1b peptide over the Muc1a peptide, as seen for rat kidney, whereas the placenta preparation was able to glycosylate the HIV-V3 peptide and preferred Muc1a to Muc1b.

Species Differences in GalNAc-transferase Activity

Comparison of porcine and ovine submaxillary glands shows no significant variation, since they both contain HIV-V3 activity, but the relative activities with the HIV-V3 and Muc1b substrates were significantly lower.

**DISCUSSION**

In the accompanying paper (White et al., 1995) we describe the purification of a human GalNAc-transferase using a defined acceptor substrate peptide as a major affinity ligand.
During the purification work we found that only a small fraction of enzyme activity was bound to the column even upon repeated chromatography, suggesting that possibly different transferase activities were present. Further support for this hypothesis was found by an observed lower $K_m$ value toward the peptide used (Muc2) for the affinity chromatography in the Muc2 affinity-purified preparation compared with the enzyme preparation immediately before this step and the enzyme preparation that passed through the column. Clearer evidence for the existence of two GalNAc-transferase activities was found by analyzing the acceptor substrates specificity of these enzyme preparations at different stages of the purification. This resulted in the identification of a qualitative difference in glycosylation of a HIV gp120 peptide. This peptide was an excellent substrate during the early steps of purification of the human placenta enzyme as well as the enzyme preparation that passed through the Muc2 affinity column (step 4), whereas the Muc2 affinity-purified enzyme lacked such activity. This phenomenon was found to be species-independent as both ovine and porcine submaxillary gland Muc2 affinity-purified transferase showed the same pattern of activity.

In the course of this work two independent groups have reported the isolation and cloning of a bovine GalNAc-transferase (GalNAc-T1) (Homa et al., 1993; Hagen et al., 1993) that is different from the human GalNAc-transferase reported in the accompanying paper (GalNAc-T2) (White et al., 1995), thus establishing that at least two members of this transferase family exist. The fine specificity of these two GalNAc-transferases has yet to be established in a comparative study of recombinant expressed enzymes. The present results show independently that two distinct transferase activities may be recognized, and substrates capable of distinguishing these have now been identified. Preliminary data suggest that neither human GalNAc-T1 nor GalNAc-T2 utilizes the HIV-V3 peptide (soluble constructs expressed in a baculovirus system). The presented data clearly establish that at least two distinct GalNAc-transferase activities can be identified and separated. The specificity of these activities appears to be overlapping to a large extent as evidenced by competitive substrate analysis (Fig. 2). The competitive inhibitor effect of the HIV-V3 peptide on the Muc2 affinity-purified GalNAc-transferase indicates that the purified transferase recognizes and binds the HIV-V3 peptide but cannot transfer GalNAc to it. Inhibition of glycosylation by non-glycosylating peptides has been noted previously by O’Connell et al. (1992). In addition, an observed de novo appearance of GalNAc-transferase activity during the purification of UDP-Gal:GalNAc:polypeptide N-acetylgalactosaminy transferase may be relevant to this (Brockhausen et al., 1992).

2 E. P. Bennett and H. Clausen, unpublished observation.
Analysis of the substrate specificity of impure GalNAc-transferase preparations may thus be biased by inhibiting factors. The observed difference in specificity of the separated GalNAc-transferase activity may be associated with a cofactor/modulator as found for UDP-Gal GlcNAc 1→4-galactosyltransferase (McGuire et al., 1965); however, several lines of evidence indicate this is not the case. First is the finding that the GalNAc-transferase activities can be physically separated yielding two activities with the purified activity having apparent lower K_m and otherwise broad specificity. Second, transferase activity with a specificity similar to that of the purified transferase is found in crude extracts of certain organs (Table IV). Finally, to date two distinct GalNAc-transferase proteins have been isolated and cDNA cloned, and these show differential organ distribution by Northern analysis (White et al., 1995; Homa et al., 1993).

Detailed understanding of the acceptor-substrate specificity of different GalNAc-transferases clearly has to await cloning and expression of all members of this family of enzymes. It was previously expected that a difference in substrate specificity of multiple enzymes could be related to Ser and Thr acceptor sites (Wang et al., 1992; O’Connell et al., 1992; Harada et al., 1985); however, purified GalNAc-transferase from bovine colostrum has been shown to exhibit specificity for both (White et al., 1995; Homa et al., 1993). The present data corroborate this for GalNAc-T2, showing a near proportional purification of both Thr and Ser activities (Table III), and recombinant expressed GalNAc-T2 showing Ser activity (White et al., 1995). Recently, Wang et al. (1992) showed that purified porcine submaxillary gland GalNAc-transferase, which is reported to be identical to GalNAc-T1 (Roth et al., 1994), exhibits very high substrate specificity for the human erythropoietin sequence -Ala-Ala-Ser-Ala-Ala-. Our preliminary data indicate that recombinant GalNAc-T1 is devoid of such activity, and recombinant GalNAc-T2 is very poor in utilizing this substrate. The reason for these discrepancies is presently unknown but are under study.

In the present study we have worked primarily with acceptor peptides derived from proteins with unknown in vivo O-glycosylation patterns. The peptides derived from human mucin tandem repeats are likely to be glycosylated in vivo at least partly, but for the HIV and SIV peptides it is only known that a few O-glycosylation sites are utilized in vivo on these large glycoproteins (Hansen et al., 1992; Merkle et al., 1991). Recently, Elhammer et al. (1993) proposed a prediction model for O-glycosylation based on the occurrence of amino acid residues positioned ±4 to identified O-glycosylation sites. The prediction model does not identify the HIV-V3 sequence. However, a comparison of the prediction model with an in vitro GalNAc-transferase assay using 32 15-mer peptides covering the entire HIV gp120 protein allowed identification of three out of four sites by both methods; additionally, three sites were identified only by the in vitro enzyme assay (Clausen et al., 1994). Thus, some correlation was found between the statistically predicted sites and in vitro glycosylation, but the in vitro glycosylation assay using crude GalNAc-transferase (Cibacron eluates corresponding to step 1 in Table I) identified additional sites. Whether these sites indeed are glycosylated in vivo is under study, but difficulties in obtaining pure viral envelope proteins in sufficient quantity for structural analysis have hampered this effort. Importantly, the in vitro GalNAc-transferase assay identified sites in the hypervariable V3 loop of different HIV and SIV isolates, and O-glycosylation could therefore mask this principal neutralizing epitope. In fact, the HIV-V3 sequence has been shown to contain a T-cell class I epitope, and the predicted glycosylation sites are positioned in the middle, thus presumably being able to mask the site (Ishikawa et al., 1992; Mouritsen et al., 1994).

Our analysis of the acceptor-substrate specificity of GalNAc-transferase preparations from different organs demonstrated both quantitative and qualitative differences (Table IV). A number of control experiments ruled out that these differences in specificity were a result of degradation and/or unknown blocking of acceptor substrate peptides. The most striking finding was that the HIV-V3 peptide indicated a qualitative distinction between enzyme extracts, thus agreeing with our interpretation that the Muc2 affinity chromatography results in the separation of two distinct GalNAc-transferase activities.

The differential organ expression of GalNAc-transferase activities using HIV-V3 peptide was further corroborated by analysis of two partial sequences of the Muc1 20-mer tandem repeat (Table IV). Interestingly, the ability to glycosylate HIV-V3 correlated with Muc1a glycosylation and lack of HIV-V3 enzyme activity with Muc1b glycosylation. Northern analysis of GalNAc-T1 (Homa et al., 1993) and GalNAc-T2 (White et al., 1995) expression indicated that human kidney and liver preferentially express GalNAc-T2, and these organs (rat kidney, human liver) appear to express a substrate specificity in agreement with that found for GalNAc-T2. The finding of apparent differential organ glycosylation of the in vitro identified glycosylation sites in the Muc1 tandem repeat (Muc1a: -Thr-Ser-; Muc1b: -Ser-Thr-) may be important for understanding the molecular basis of cancer-associated epitopes mapped to the Muc1 tandem repeat (Gendler et al., 1990). A number of antibodies to Muc1 have been generated, and most of these map to the knob-like structure defined by -Ala-Pro-Asp-Thr-Arg-Pro- (Taylor-Papadimitriou et al., 1993). Flanking these

### Table IV

| Organs              | Muc2     | HIV-V3   | Muc1a    | Muc1b    |
|---------------------|----------|----------|----------|----------|
| Rat ventricle       | 1.02 ± 0.41 | 260 ± 11 | 667 ± 8  | 625 ± 38 |
| Rat kidney          | 837 ± 52  | 12 ± 0.5 | 193 ± 12 | 1,142 ± 50 |
| Rat testes          | 857 ± 43  | 393 ± 8  | 1,896 ± 57 | 40 ± 5  |
| Rat salivary glands | 2,146 ± 148 | 303 ± 18 | 1,384 ± 194 | 198 ± 18 |
| Porcine salivary glands | 7,423    | 1,649    | 4,376 | 2,981 |
| Ovine salivary glands | 6,391    | 1,889    | 3,293 | 2,041 |
| Human placenta      | 918 ± 18  | 440 ± 22 | 841 ± 67 | 390 ± 12 |
| Human liver         | 331 ± 30  | 2 ± 0.02 | 75 ± 5  | 509 ± 49 |

*GalNAc-transferase preparations were dialyzed, concentrated Cibacron eluates (step 1) were used for rat organs and S-Sepharose eluates (step 3) for porcine and ovine salivary glands and human placenta. Peptide substrate concentrations were 50 μg in a standard reaction assay. S.D. is given for triplicate assays.
repeated knobs are the -Thr-Ser- and -Ser-Thr- motifs, which the present study indicates are differentially glycosylated by independent GalNAc-transferases. Structural analysis of in vitro glycosylated Muc1 tandem repeats using breast and pancreatic cell line extracts as well as semipurified human placenta GalNAc-transferase (purification steps 1 and 3) indicates that only the flanking sites are glycosylated and that the single Thr in the knob tip (-Pro-Asp-Thr-Arg-) is left unglycosylated (Nishimori et al., 1994a, 1994b). If the observed difference in vitro glycosylation reflects the in vivo processing this finding may have implications for the structure of Muc1 expressed in different organs and in cancer cells.

In conclusion, the present study provides evidence that different GalNAc-transferase activities are involved in the initiation of GalNAc O-glycosylation and that these are differentially expressed in cells and organs. Identification of suitable acceptor substrates capable of distinguishing such transferase activities is believed to be a significant step forward in the understanding of GalNAc O-glycosylation processing and will be valuable for characterization of the substrate specificity of different GalNAc-transferase genes as these are cloned and expressed.

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