Characterization of a Novel Polypeptide *N*-Acetylgalactosaminyltransferase (dGalNAc-T3) from *Drosophila*

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Polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases) catalyze the initial reaction of mucin-type *O*-glycosylation. Here, we report the first biochemical characterization of one of the *Drosophila* GalNAc-transferases, dGalNAc-T3. This enzyme retains conserved motifs essential for the catalytic activity, but is a novel isozyme in that it has several inserted sequences in its lectin-like domain. Northern hybridization analysis of this isozyme identified a 2.5-kb mRNA in *Drosophila* larva. Biochemical characterization was carried out using the recombinant soluble dGalNAc-T3 expressed in COS7 cells. dGalNAc-T3, which required Mn²⁺ for the activity, had a pH optimum ranging from pH 7.5 to 8.5, and glycosylated most effectively at 29–33 °C. Its *K_m* for UDP-GalNAc was 10.7 μM, which is as low as that of mammalian isozymes. dGalNAc-T3 glycosylated the peptides containing a sequence of XTPXP or TTAAP most efficiently. The enzyme was irreversibly inhibited by *p*-chloromercuriphenylsulphonic acid, indicating the presence of essential Cys residues for the activity.

Key words *N*-acetylgalactosaminyltransferase; mucin; *Drosophila*; *O*-glycosylation; insect; peptide

Glycosylation of proteins is one of the most important post-translational modifications in the cell. Mucin-type *O*-glycosylation, which is characterized by the sugar-peptide linkage GalNAcα1→Ser (or Thr), is widely distributed in many secretory and membrane glycoproteins, and is involved in important biological functions such as cell–cell recognition, host–pathogen interaction, and protection of proteins from proteolytic degradation.^{1,2)} The initial step of mucin-type *O*-glycosylation is catalyzed by UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases) (EC 2.4.1.41), by transferring GalNAc from UDP-GalNAc to Ser and Thr residues of proteins.³⁾ GalNAc-transferases are regarded as essential enzymes for *O*-glycan biosynthesis because they define the number and the positions of mucin-type carbohydrates on the polypeptides. Recent progress in the molecular cloning of GalNAc-transferases has revealed a large gene family in mammals, nematodes, and insects.^{4–23)} The evolutionary conservation of GalNAc-transferases stresses the biological importance of the mucin carbohydrates.

Searches of the *Drosophila* genome database indicated the presence of at least 14 GalNAc-transferases, and nine of these isozymes cloned have been shown to encode functional enzymes.^{17,24)} Moreover, the developmental importance of the GalNAc-transferases in *Drosophila* is demonstrated by the lethal mutations in the l(2)35Aa allele coding for dGalNAc-T1.^{17,23)} However, *Drosophila* GalNAc-transferases have not been biochemically characterized so far. Thus, their activity has been determined under the same conditions as the mammalian enzymes. Here, we report the detailed biochemical properties of one of the *Drosophila* GalNAc-transferases (dGalNAc-T3). We also demonstrate an extensive analysis of the substrate specificity of dGalNAc-T3, in which synthetic peptides from insect mucins as well as mammalian ones were used as acceptors to assess the possibility of dGalNAc-T3 glycosylating mucin polypeptides in insects.

MATERIALS AND METHODS

Materials An EST clone GH09147 (GadFly annotation CG4445), encoding for dGalNAc-T3, was purchased from Research Genetics, Inc. A panel of peptides was synthesized using a Multipin Peptide Synthesis Kit (Chiron Mimotopes).

Northern Blot Analysis *Drosophila* larva poly A⁺ RNA (Clontech) was electrophoresed in the denaturing condition in a 1.0% agarose-formaldehyde gel with 20 mM 4-morpholinepropanesulfonic acid/5 mM sodium acetate buffer, pH 7.0. Separated RNA was then transferred from a gel to a positively charged nylon membrane (Roche) with 0.05 M NaOH. The filter membrane was briefly washed with 2× stand saline citrate (SSC), and then baked for 20 min at 50 °C to immobilize RNA on the membrane. Following the prehybridization in the hybridization buffer (0.2 M sodium phosphate buffer, pH 7.5, containing 0.1% sodium dihydrogen pyrophosphate, 2 mM ethylenediamine tetraacetic acid, and 7% sodium dodecylsulfate (SDS)) for 8 h at 68 °C, the membrane was hybridized overnight at 68 °C with the ³²P-labeled dGalNAc-T3-specific probe, which was prepared by polymerase chain reaction (PCR) with primer pairs, DmG-PS and DmG-PA, designed for amplification of nucleic acid residue 1–600 bp, in the presence of [α-³²P]TTP, 3000 Ci/mmol. The membrane was then washed in 2×SSC, containing 0.1% SDS, and subsequently in 0.1×SSC, containing 0.1% SDS, 3 times for 10 min at 68 °C, respectively.

Expression of Recombinant Soluble dGalNAc-T3 in COS7 Cells For the construction of soluble dGalNAc-T3, a cDNA fragment, deleted with the sequences for putative cytoplasmic tail, the transmembrane domain and a portion of the stem region, was amplified by PCR using dGalNAc-T3 cDNA as a template and a primer set of 5'-GAGATGC GATATCCGAAACCG-3' and 5'-AATGTCCGGGCCCCG-GCATGA-3'. The nucleotides in the primers represented in boldfaces are mutated to introduce EcoRV and ApaI restriction sites (underlined) in the amplified cDNA, respectively. Following the amplification, the PCR product was digested

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with EcoRV and ApaI, and purified by a QIAquick Gel Extraction Kit (Qiagen). The digest was ligated into the BamHI (blunt-ended) and the XhoI sites of a mammalian expression vector, pInsProA, using Ligation High (Toyobo). pInsProA contains a cDNA fragment encoding for the insulin signal sequence and the Protein A-IgG binding domain inserted into pcDNA3.1 (Invitrogen).²⁵⁾ The generated construct coding for the truncated dGalNAc-T3 (Fig. 1) fused with the signal sequence and the IgG-binding domain of Protein A was introduced into *E. coli* strain DH5 α (Toyobo). Colonies were screened by PCR, and the plasmid (pIP- Δ dGNT3) containing the truncated dGalNAc-T3 was obtained using the QIAprep Miniprep (Qiagen). pIP- Δ dGNT3 was then transfected into COS7 cells using FuGENE6 (Roche). Three days after transfection, the secreted recombinant enzyme in the conditioned medium was purified as described below.

Purification and Western Blot Analysis of Recombinant dGalNAc-T3 The secreted enzyme in the culture medium was purified on IgG-Sepharose (Amersham Pharmacia Biotech) as follows: Five hundred microliters of the conditioned medium were mixed with 10 μ l of IgG-Sepharose and incubated under constant rocking at 4 °C for 2 h. The IgG-Sepharose adsorbed with the recombinant dGalNAc-T3 was washed three times with 25 mM imidazole buffer (pH 7.2) containing 300 mM NaCl and 1% Triton X-100, and three times with 25 mM imidazole buffer (pH 7.2) containing 100 mM NaCl. The adsorbed dGalNAc-T3 on the resins was used for the enzyme assay as described below. For quantitation of the recombinant enzyme, the washed resins were suspended in the SDS-polyacrylamide gel electrophoresis loading buffer, boiled at 100 °C for 3 min, and centrifuged at 12000 rpm for 10 min. The resulting supernatant was loaded on the gel. The electrophoresed proteins in the gel were transferred to a polyvinylidene difluoride membrane (Bio-Rad), and visualized by incubating the blot with an alkaline phosphatase-conjugated rabbit antibody to mouse IgG (Cappel), followed by staining with nitrobluetetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Intensity of the bands on the immunoblots was determined using the LAS-1000PLUS (Fuji) to evaluate the concentration of recombinant dGalNAc-T3 in the medium.

Enzyme Assay The activity of dGalNAc-T3 was determined in a reaction mixture composed of 50 mM imidazole buffer (pH 7.2), 10 mM MnCl₂, 0.5 nmol UDP-[1-³H]GalNAc (approximately 40000 dpm), 5 nmol synthetic peptides, and an appropriate amount of enzyme, in a total volume of 40 μ l. The mixture was incubated at 25 °C for 16 h under constant stirring to maintain a uniform suspension. It was confirmed that the progress of the reaction was linear with respect to the incubation time. Following the incubation, the reaction was stopped by adding 10 μ l of 0.25 M EDTA. The reaction mixture was then applied to an AG1 X-8 anion exchange column (0.5 ml, Cl⁻ form). The pass-through fraction was recovered and the radioactivity was determined. K_m for UDP-GalNAc was obtained by increasing concentrations of UDP-GalNAc from 5 to 400 μ M using MUC2-1 as acceptor. Kinetic parameters were obtained by the double reciprocal plot (1/*v* versus 1/[S]), using standard procedures.

Modification of dGalNAc-T3 with *p*-Chloromercuri phenylsulphonic Acid (PCMPS) Recombinant dGalNAc-

T3 expressed in COS7 cells and adsorbed to the IgG-Sepharose was treated with increasing concentrations (0–0.5 mM) of PCMPS (Sigma) in 0.1 M imidazole buffer (pH 7.2), for 90 min at 25 °C. After incubation with PCMPS, the enzyme adsorbed to the IgG-Sepharose was washed and its activity was determined as described above.

RESULTS AND DISCUSSION

dGalNAc-T3 is a putative type II membrane protein with seven potential *N*-glycosylation sites, and consists of a 10-amino acid *N*-terminal cytoplasmic region, a 23-amino acid transmembrane region, an 84-amino acid stem region, a 345-amino acid putative catalytic region, and a 154-amino acid lectin domain (Fig. 1). It contains a glycosyltransferase 1 motif, which is a conserved sequence commonly found in a wide variety of glycosyltransferases including the GalNAc-transferases, and a DXH sequence, which is a putative binding site for a sugar donor and/or a metal ion.^{26–29)} A Gal/GalNAc-transferase motif is also found in the putative catalytic domain of this isozyme. It is a homologous sequence found in Gal- and GalNAc-transferases, which may be a binding site for both a UDP-sugar and an acceptor substrate.³⁰⁾ At the C-terminus, there is a lectin-like domain called the (QXW)₃ repeats, which consists of three tandemly repeated sequences (α , β , and γ repeats), and occurs only in the GalNAc-transferases among the glycosyltransferase family.³¹⁾ Furthermore, this isozyme contains several amino acid residues essential for catalytic activity as demonstrated by mutational studies of mammalian GalNAc-transferases. It has seven conserved acidic amino acids that are involved in the enzyme activity of human GalNAc-T1.³⁰⁾ It also contains four essential His residues.³²⁾ In addition, it has several Cys residues conserved in the family, most of which are likely involved in forming disulfide bonds necessary for the proper folding of the enzymes. Two Cys residues among them, which are located at the C-terminus of the DXH motif, are also present in this isozyme. It is reported that these are reduced Cys residues, and are involved in binding with UDP-GalNAc.²⁵⁾ Interestingly, dGalNAc-T3 contains a 39 amino acid residue insertion at the N-terminus of the (QXW)₃ α repeat, which is not found in the other GalNAc-transferases. Moreover, it also has a short insertion (approximately 5–10 amino acid residues) in each repeat of the lectin-like domain. This raises the possibility that this isozyme has altered lectin activity. It is reported that the GalNAc-transferases have distinct manners of substrate recognition^{33–35)}; one is based on protein–protein interaction, so-called the initial activity, and is involved in the glycosylation of non-glycosylated peptides, and the other requires the prior addition of GalNAc to peptide substrates to transfer more GalNAc residues to the substrates. The latter activity, called follow-up activity, is based on the recognition of GalNAc residues on the acceptor substrate by the lectin domain of the enzyme. Mutational studies on the rat GalNAc-T1 lectin domain identified an asparagine residue (Asp444) in the α repeat predominantly involved in the lectin activity.³⁴⁾ In dGalNAc-T3, this residue is replaced with arginine. Thus, together with the aberrant insertions, the lectin domain of this isozyme may not be fully functional. In fact, the glycopeptide glycosylation activity of this isozyme is reported to be very low.²⁴⁾

Fig. 1. Amino Acid Sequence of dGalNac-T3

Potential *N*-glycosylation sites are indicated by arrows. The DXH sequence in the glycosyltransferase 1 motif and the insert sequences are outlined with solid and dashed lines, respectively. Conserved acidic, histidine, and cysteine residues are indicated by ●, ◆, and △, respectively. ▲ represents the N-terminus of the truncated dGalNac-T3 generated for the enzyme assay.

We then investigated the expression of dGalNac-T3 in *Drosophila* by Northern blot hybridization. A blot containing 1 µg of *Drosophila* larva poly (A)⁺ RNA was probed with a ³²P-labeled 600-bp cDNA fragment under stringent conditions. A single 2.5-kb mRNA was observed, confirming the expression of the dGalNac-T3 transcripts in the larva (Fig. 2).

For the characterization of dGalNac-T3, soluble recombinant dGalNac-T3 was expressed in COS7 cells. dGalNac-T3 cDNA was deleted with the coding region for the cytoplasmic tail and the transmembrane region, and fused with cDNA for an insulin signal sequence and a Protein A-IgG binding domain to the resulting 5'-end of the truncated dGalNac-T3 cDNA. Following the subcloning of the resulting cDNA into the mammalian expression vector, the truncated dGalNac-T3 cDNA was transfected into COS7 cells, and the recombinant enzyme was expressed as the secreted fusion protein, which consisted of Protein A-IgG binding domain fused to N-terminus of the truncated dGalNac-T3. The recombinant secreted protein was purified from culture medium with IgG-Sepharose. Western blot analysis of the purified protein demonstrated the expression of a polypeptide with an expected molecular weight of *ca.* 90 kDa. This recombinant soluble dGalNac-T3 was used for the following experiments.

We first investigated the optimal temperature of dGalNac-T3. Reaction was performed at pH 7.2 at various temperature ranging from 9 °C to 45 °C with synthetic undecapeptide MUC2-1, which contains *O*-glycosylation sites of human MUC2 and is one of the most efficient acceptors of dGalNac-T3 as shown below. dGalNac-T3 exhibited a relatively broad temperature specificity, although it worked most efficiently at temperatures between 29 and 33 °C (Fig. 3A). The optimal temperature of the insect isozyme was significantly lower than that of mammalian GalNac-trans-

Fig. 2. Northern Hybridization Analysis

One microgram of larva poly(A)⁺ RNA was separated by agarose gel electrophoresis and blotted onto a nylon membrane. The blot was probed with ³²P-labeled dGalNac-T3 cDNA.

ferases (40–45 °C).²¹ Interestingly, the *C. elegans* GalNac-transferases have an even lower optimal temperature (20–25 °C).²¹ This indicates that the optimal temperature of enzymes is closely related to the environmental temperature where they inhabit. For the following experiments, the assay was performed at 25 °C. It is a suboptimal temperature, but employed because the flies were kept at this temperature. We then investigated the optimal pH. Reaction was performed in the pH range from 5.4 to 9.0 (Fig. 3B). The enzyme preferred the mild alkaline conditions for the efficient catalysis, with an optimal pH of 7.5–8.5. Bovine GalNac-T1 is similarly reported to have a mild alkaline optimal pH of 7.2–8.6.³⁶ The following enzyme assay was, however, carried out at pH 7.2, since the GalNac-transferases are supposed to reside in the ER and/or the *cis*-Golgi. dGalNac-T3 requires divalent cations for the activity, since its activity was lost upon the addition of EDTA in the reaction mixture. Among the divalent cations tested, Mn²⁺ most effectively enhanced the enzyme activity. The activity in the presence of either Co²⁺

Fig. 3. (A) Optimal Temperature

Reaction was carried out at the temperature between 9 and 45 °C.

(B) Optimal pH

Reaction was performed in 20 mM of the three different buffers. pH 5.4–7.2, cacodylate buffer; pH 6.9–8.1, imidazole buffer; pH 7.5–9.0, Tris buffer.

Fig. 4. Apparent K_m Determination for UDP-GalNAc

Reaction conditions are described under Materials and Methods.

Assay with these peptides indicated that dGalNAc-T3 has broad substrate specificity capable of glycosylating almost all of the peptides from both insect and mammalian sequences, but with a considerably distinct reaction rate depending on the peptide sequences (Table 1). Concerning the peptides derived from insects, mucin like-2 (MPTAGTPPP^{IG}), -4 (LP-PVT^{TT}PAPG), -6 (PADV^{TTAAP}GG) -8 (AWIPT^{TT}PIPG), and hemomucin-2 (TTTT^{TT}PKPTG) were excellent acceptors. Comparison of their sequence revealed that all the peptides except mucin like-6 contain XTPXP (underlined above), a sequence that is defined as “a mucin box” and is reported to be an efficient *O*-glycosylation site by Takeuchi *et al.*⁴⁰) A sequence TTAAP contained in mucin like-6 also seemed to be a good acceptor site, since mammalian MUC7-1 (TTAAP^{TT}PSAG) and MUC7-6 (PPE^{TTAAP}PTG), which similarly contain TTAAP, were glycosylated as efficiently as mucin like-6. It should, however, be noted that some peptides containing XTPXP were poor acceptors. For example, mucin like-11 (RCESD^{TT}PPPG), -12 (EEEI^{TT}PRPG), and hemomucin-3 (TTTP^{TT}KPTTKTG) were not glycosylated efficiently. These peptides, however, are characterized by the presence of several charged amino acids (represented in the boldface in the above sequences), which are unfavorable residues for *O*-glycosylation,^{38,41}) in and around the XTPXP sequences. As to the mammalian synthetic peptides, eight peptides were glycosylated at rate higher than 200 μmol GalNAc/h/mmol peptide. Seven of these contain XTPXP or TTAAP (MUC2-1, 2-3, 2-7, 5AC-4, 7-1, 7-3, and 7-6), indicating again that dGalNAc-T3 preferentially glycosylates the peptides containing these acceptor sites. Among the poor acceptors, there were peptides, such as MUC2-8 and MUC5AC-1, which contain XTPXP. This clearly demonstrates that the presence of a favorable primary sequence (*e.g.* XTPXP and TTAAP) is necessary but not a prerequisite for the efficient *O*-glycosylation by dGalNAc-T3. Efficiency of the *O*-glycosylation would be influenced by several other factors than the primary sequence of the acceptor site as well, such as the amino acid sequence flanking the acceptor site and the secondary structure of the peptide.

Finally the effect of Cys modification of dGalNAc-T3 was investigated. Tenno *et al.* reported that the modification of mammalian GalNAc-T1 with a Cys-specific reagent, PCMPS, irreversibly inhibited the enzyme with a K_i of 0.03 mM and that Cys212 and Cys214, which are C-terminal to the DXH motif, are the sites of the modification and are involved in the binding with UDP-GalNAc.²⁵) dGalNAc-T3,

or Ni^{2+} was 60 and 20% of that of Mn^{2+} , respectively. Neither Ca^{2+} , Cu^{2+} , Mg^{2+} , nor Zn^{2+} was effective. dGalNAc-T3 exhibited a similar divalent cation requirement as that of the mammalian GalNAc-transferases.³⁶) Taking these observations together, the activity of dGalNAc-T3 was determined in the following experiments at 25 °C with the pH 7.2 buffer containing 10 mM MnCl_2 . We then investigated the affinity of UDP-GalNAc with the enzyme (Fig. 4). The K_m value (10.7 μM) of dGalNAc-T3 for UDP-GalNAc is as low as those of the mammalian GalNAc-transferases.³⁷) In order to investigate the acceptor substrate specificity of dGalNAc-T3, a number of peptides were synthesized and used for the assay (Table 1). The peptide sequences were obtained from typical mucin-like molecules of *Drosophila* and human origin that are characteristically rich in Ser, Thr, and Pro residues. As for the putative insect mucins, we searched for genes coding for a putative mucin-like molecule in the *Drosophila* database. We then examined the *O*-glycosylation probability of the candidate sequences according to the method described by Elhammer *et al.*,³⁸) and the peptide sequences with a high probability were chosen for the peptide synthesis (mucin like-1 to -14, Table 1). We also prepared the peptides containing putative *O*-glycosylation sites found in insect hemomucin, which is a putative self-defense protein found in hemocytes, in the gut.³⁹) In addition to insect peptide, peptides with sequences from mammalian mucins and glycophorin were included. All the peptides were synthesized onto the Gly residue covalently bound to the multipins. The synthesized peptides, therefore, have a C-terminal Gly residue.

Table 1. Substrate specificity of dGalNAc-T3

Peptide	Sequence	Activity ^{a)}	Peptide	Sequence	Activity ^{a)}
<i>Drosophila</i>			Mammals		
Mucin like-1	PPVVPFTPLPG	107.4	MUC2-9	TPTGTQTPTTG	25.0
Mucin like-2	MPTAGTPPIPG	299.3	MUC2-10	TQTPTTTTPTG	38.6
Mucin like-3	PGGDSSSYQPG	20.0	MUC3-1	HSTPSFTSSIG	20.3
Mucin like-4	LPPVTTTPAPG	904.3	MUC3-2	SFTSSITTTEG	19.2
Mucin like-5	SEVTTTLVPAG	25.6	MUC3-3	SITTTETTSYG	48.9
Mucin like-6	PADVTTAAPGG	543.9	MUC3-4	TETTSHTSPSG	18.3
Mucin like-7	SDAESSGPVPG	20.4	MUC4-1	TSSVSTGHATG	27.1
Mucin like-8	AWIPTPPPIPG	1171.5	MUC4-2	STGHATSLPVG	20.7
Mucin like-9	QWDTPISTPG	19.7	MUC4-3	ATSLPVTDTSG	22.5
Mucin like-10	PTPISTPACEG	13.8	MUC4-4	PVTDTSSVSTG	17.0
Mucin like-11	RCESDTPPPPG	79.5	MUC5AC-1	GTPSPVPTTG	147.6
Mucin like-12	EEEIPATPRPG	52.9	MUC5AC-2	SPVPTTSTTSG	18.8
Mucin like-13	GSPCTTDNPSG	18.9	MUC5AC-3	TTSTTSAPGTG	191.5
Mucin like-14	EPPITTPHCSG	5.8	MUC5AC-4	TSAPGTTSPSG	519.5
Hemomucin-1	PTTTTTTTTPG	89.8	MUC5B-1	SSTPGTAHTLG	19.0
Hemomucin-2	TTTTTTPKPTG	604.6	MUC5B-2	GTAHTLTVLVTG	18.3
Hemomucin-3	TTTPKPTTKTG	31.9	MUC5B-3	TLTVLTATG	19.7
Hemomucin-4	PKPTTKTTTG	20.1	MUC5B-4	LTTATTPTAG	36.6
Hemomucin-5	PEPSKPKVKRG	16.5	MUC5B-5	ATTPTATGSTG	34.3
Hemomucin-6	TTPTTPTPTG	19.9	MUC5B-6	TATGSTATPSG	31.7
Mammals			MUC5B-7	STATPSSTPGG	39.8
MUC1-1	PDTRPDAPGSG	18.8	MUC7-1	TTAAPPTPSAG	516.3
MUC1-2	PDAPGSTAPPG	87.9	MUC7-2	PPTPSATTPAG	44.1
MUC1-3	GSTAPPAHGVG	33.9	MUC7-3	SATTPAPPSSG	2310.6
MUC1-4	PPAHGVTSAPG	266.7	MUC7-4	PAPPSSSAPPG	29.3
MUC1-5	GVTSAPDTRPG	35.2	MUC7-5	SSSAPPETTAG	24.8
MUC2-1	PTTTPSPITG	532.5	MUC7-6	PETTAAPPTG	483.6
MUC2-2	PSPPTTTTTG	108.9	Glycophorin-1	VLLLSAIVSIG	21.3
MUC2-3	ITTTTTPLPTG	267.5	Glycophorin-2	SAIVSISASSG	20.0
MUC2-4	TTPLPTTPSG	127.9	Glycophorin-3	SISASSTGTVG	25.3
MUC2-5	PTTTPITTTG	45.8	Glycophorin-4	SSTTGVMHTG	26.9
MUC2-6	PITTTTTVTPG	139.1	Glycophorin-5	GVAMHTSTSSG	23.0
MUC2-7	TTVTPTPTPG	429.4	Glycophorin-6	HTSTSSSVTKG	32.7
MUC2-8	TPTPTPTGTQG	57.5			

a) The activity is indicated as $\mu\text{mol GalNAc/h/mmol peptide}$. Origin of *Drosophila* mucin like peptides is as follows, mucin like-1 and -2; CG11570, mucin like-3; CG12480, mucin like-4; CG13722, mucin like-5; CG13817, mucin like-6 and -7; CG2779, mucin like-8; CG8697 (larval cuticle protein 2), mucin like-9 and -10; CG17058 (peritrophin A), mucin like-11 and -12; CG17014, mucin like-13; CG6004, mucin like-14; CG10287 (Gasp).

containing both Cys residues corresponding to mammalian Cys212 and Cys214, was also incubated with increasing concentrations of PCMPS. PCMPS effectively inhibited dGalNAc-T3 with an apparent K_i of 0.05 mM (Fig. 5). This raised the possibility that two Cys residues C-terminal to DXH in dGalNAc-T3 are also involved in the recognition of UDP-GalNAc.

In summary, we have characterized one of the *Drosophila* GalNAc-transferases (dGalNAc-T3). Recombinant dGalNAc-T3 expressed in COS7 cells was catalytically active and characterized by the low optimal temperature, compared with the mammalian enzymes. dGalNAc-T3 glycosylated most of the mucin-like peptides, with strong preference to the sequences containing XTPXP and TTAAP, and did not require prior *O*-glycosylation of the peptides. This suggests that this isozyme may be responsible for the initiation of insect mucin *O*-glycosylation. dGalNAc-T3 is expected to have low follow-up activity, as deduced by the primary structure of the enzyme, and, in fact, Ten Hagen *et al.* detected very low follow-up activity.²⁴⁾ It would be reasonable to predict the presence of the so-called follow-up isozymes in insects, and a recent study reports the presence of insect isozymes with glycopeptide glycosylation activity.²⁴⁾ Cooperation of dGalNAc-T3 with these isozymes would lead to the

Fig. 5. Inhibition of dGalNAc-T3 with PCMPS

Purified dGalNAc-T3 was incubated with increasing concentrations of PCMPS, and assayed for activity as described under Materials and Methods.

more efficient glycosylation of mucins.

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