N-linked oligosaccharides are required to produce and stabilize the active form of chondroitin 4-sulphotransferase-1

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C4ST-1 (chondroitin 4-sulphotransferase-1) transfers sulphate to position 4 of *N*-acetylgalactosamine in chondroitin. We showed previously that purified C4ST-1 from the culture medium of rat chondrosarcoma cells was a glycoprotein containing approx. 35 % N-linked oligosaccharides. In the present paper, we investigated the functional role of the N-linked oligosaccharides attached to C4ST-1. We found that (i) treatment of recombinant C4ST-1 with peptide *N*-glycosidase F caused a marked decrease in activity, (ii) production of the active form of C4ST-1 by COS-7 cells transfected with cDNA of C4ST-1 was inhibited by tunicamycin, (iii) deletion of the *N*-glycosylation site located at the C-terminal region of C4ST-1 abolished activity, (iv) attachment of a single *N*-glycan at the C-terminal region supported production of the active form of C4ST-1, but the resulting recombinant enzyme was much more unstable at 37 °C than the control recombinant protein,

and (v) truncation of C-terminal region up to the *N*-glycosylation site at the C-terminal region resulted in total loss of activity. These observations strongly suggest that N-linked oligosaccharides attached to C4ST-1 contribute to the production and stability of the active form of C4ST-1. In addition, the N-linked oligosaccharide at the C-terminal region appears to affect the glycosylation pattern of recombinant C4ST; a broad protein band of the wild-type protein resulting from microheterogeneity of N-linked oligosaccharides disappeared and four discrete protein bands with different numbers of N-linked oligosaccharides appeared when the N-linked oligosaccharide at the C-terminal region was deleted.

Key words: chondroitin sulphate, chondroitin 4-sulphotransferase (C4ST), N-linked oligosaccharide, site-directed mutagenesis, tunicamycin.

INTRODUCTION

Chondroitin sulphate chains are composed of repeating disaccharide units, GlcA β 1-3GalNAc β 1-4, with sulphate groups at various positions of the component sugars. CS-A (chondroitin sulphate A) is an isomer containing a GlcA-GalNAc(4-SO₄) repeating unit. CS-A is a component of various extracellular proteoglycans such as aggrecan, and is a receptor for *Plasmodium falciparum*-infected erythrocytes [1–3]. Cytotoxic T-cell lines were found to secrete chondroitin-sulphate-type serglycin contained in cytotoxic granules [4–7], and most chondroitin sulphate contained in serglycin secreted from the cytotoxic T-cells was CS-A [4]. Sulphate groups attached to chondroitin sulphate chains are transferred from PAPS (adenosine 3'-phosphate 5'-phosphosulphate) by sulphotransferases with strict acceptor specificities. Among these sulphotransferases, C4ST-1 (chondroitin 4-sulphotransferase-1) [8,9] and C6ST-1 (chondroitin 6-sulphotransferase-1) [10,11] and GalNAc4S-6ST (*N*-acetylgalactosamine 4-sulphate 6-*O*-sulphotransferase) [12,13] have been purified to homogeneity, and the cDNAs were cloned. The purified C4ST-1, C6ST-1 and GalNAc4S-6ST were all glycoproteins with N-linked oligosaccharides. The contents of N-linked oligosaccharides of these sulphotransferases amount to as much as 30 % of the total molecular mass. At present, however, the role of the N-linked oligosaccharides attached to these sulphotransferases is largely unclear.

Most glycosyltransferases localized to the Golgi membrane contain N-linked oligosaccharides. Deletion of N-linked oligosac-

charides of fucosyltransferases IV [14], fucosyltransferases III, V, VI [15], GD3 synthase [16] and polysialyl transferases [17] was shown to result in a decrease in the enzyme activities or in the alteration of subcellular localization. N-linked oligosaccharides attached to glycosyltransferases were reported to be involved in the stability of GM2 synthase [18]. Mutation of the N-linked glycosylation sites of a lysosomal protein, murine ASM (acid sphingomyelinase), caused a decrease in the enzyme activity [19]. N-linked oligosaccharides on ASM were shown to stabilize the protein via protection from intralysosomal degradation [20]. Cerebroside sulphotransferase contains two N-linked glycosylation sites. The C-terminal site was reported to be essential for enzyme activity [21].

In the present paper, we investigated the functional role of N-linked oligosaccharides attached to C4ST-1, and found that those located to the C-terminal region of C4ST-1 played important roles in the production of the active form of C4ST-1.

EXPERIMENTAL

Materials

The following commercial materials were used: H₂³⁵SO₄ was from PerkinElmer; chondroitinase ACII, Δ Di-6S [2-acetamide-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose; Δ Di-6S, 2-acetamide-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulpho-D-galactose; DMEM, Dulbecco's modified Eagle's medium; GalNAc4S-6ST, *N*-acetylgalactosamine 4-sulphate 6-*O*-sulphotransferase; mAb, monoclonal antibody; PAPS, adenosine 3'-phosphate 5'-phosphosulphate; PBS, PAPS-binding domain; PNGase F, peptide *N*-glycosidase F; Tos-Lys-CH₂Cl ('TLCK'), tosyl-lysylchloromethane; Tos-Phe-CH₂Cl ('TPCK'), tosylphenylalanylchloromethane.

Abbreviations used: ASM, acid sphingomyelinase; CS-A, chondroitin sulphate A; C4ST-1, chondroitin 4-sulphotransferase-1; C6ST-1, chondroitin 6-sulphotransferase-1; Δ Di-4S, 2-acetamide-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose; Δ Di-6S, 2-acetamide-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulpho-D-galactose; DMEM, Dulbecco's modified Eagle's medium; GalNAc4S-6ST, *N*-acetylgalactosamine 4-sulphate 6-*O*-sulphotransferase; mAb, monoclonal antibody; PAPS, adenosine 3'-phosphate 5'-phosphosulphate; PBS, PAPS-binding domain; PNGase F, peptide *N*-glycosidase F; Tos-Lys-CH₂Cl ('TLCK'), tosyl-lysylchloromethane; Tos-Phe-CH₂Cl ('TPCK'), tosylphenylalanylchloromethane.

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Table 1 Nucleotide sequences of oligonucleotide primers used for introduction of mutation by site-directed mutagenesis

Amino acid mutation	Codon change	Nucleotide sequences of primers (5' → 3')
N205S	AAC → AGC	CACGCAGAAGTACAGCACCTCCTCCACAAGC GCTTGTGGAAGGAGGTGCTGACTTCTGCGTG
N223S	AAC → AGC	CCGACGCCAGCGGAAGAGCGCCACGAGGAGGCC GGGCTCTCGTGGCGCTCTCCGCTGGCGTCGG
N321S	AAC → AGC	CCACGGAGTCTTCCAGAGCATCAGCGCCG CGGCGCTGATGCTCTGGAAGAACTCCGTGG
N342S	AAC → AGC	GACTTTTAAATGTTCAAGTACTAGTGCCAACTACC GGTAGTTTGGCACTGAGTAGTGAACATTAAGT
K125Q	AAG → CAG	CTACTGCTAGTGCCCAAGTAGCGTGCACTGAG CCAGTTGGTGCACGCTACCTGGGACATAGCAGTAG
P346Stop	CCA → TGA	GTTCAACTACTCAGTGTGAACTACCTGAAGTTGG CCAATTCAGGTAGTTTCACTAGTAGTGTGAAC
N347Stop	AAC → TAG	CAACTACTCAGTGCCATAGTACCTGAAGTTGG CCAATTCAGGTACTATGGCACTGAGTAGTTG
Y348Stop	TAC → TAG	CAACTACTCAGTGCCAACTAGCTGAAGTTGG CCAATTCAGCTAGTTTGGCACTGAGTAGTTG
L349Stop	CTG → TAG	CTCAGTGCCAACTACTAGAAGTTGGATTAG CTAATCCAATCTAGTAGTTTGGCACTGAG

mAb (monoclonal antibody), FLAG peptide, pFLAG-CMV2, DMEM (Dulbecco's modified Eagle's medium), foetal bovine serum, Tos-Lys-CH₂Cl ('TLCK', tosyl-lysylchloromethane), Tos-Phe-CH₂Cl ('TPCK', tosylphenylalanylchloromethane), PMSF and pepstatin were from Sigma. PNGase F (peptide N-glycosidase F) was from Roche Applied Science. [³⁵S]PAPS was prepared as described in [22]. Chondroitin (squid skin) was prepared as described in [23].

Expression plasmids

The plasmids pFLAGmC4ST containing full-length cDNA of mouse C4ST-1 with the N-terminal FLAG tag were constructed as described previously [9]. Site-directed mutagenesis was carried out by PCR using a QuikChange™ site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions. N-glycosylation sites, Asn-Xaa-Ser/Thr motifs, were disrupted by substituting asparagine (Asn²⁰⁵, Asn²²³, Asn³²¹ and Asn³⁴²) with serine. The PAPS-binding domain (5'-PSB) was destroyed by substituting glutamine for Lys¹²⁵. The truncated mutants lacking four to seven amino acid residues located at the C-terminal were generated by substituting a stop codon for Pro³⁴⁶, Asn³⁴⁷, Tyr³⁴⁸ or Leu³⁴⁹. The nucleotide sequences of all the mutated cDNAs were confirmed. The sequences of all the PCR primers used for the site-directed mutagenesis are shown in Table 1.

Cell culture and transfection of COS-7 cells

The plasmids prepared as above were transfected into COS-7 cells by the DEAE-dextran method as described previously [9]. The cells were extracted with 0.15 M NaCl, 10 mM Tris/HCl, pH 7.2, 10 mM MgCl₂, 2 mM CaCl₂, 0.5 % (v/v) Triton X-100, 20 % (v/v) glycerol and protease inhibitors (50 µM Tos-Lys-CH₂Cl, 30 µM Tos-Phe-CH₂Cl, 300 µM PMSF and 3 µM pepstatin) for 30 min on a rotary shaker. The extracts were centrifuged at 10000 *g* for 10 min. The C4ST activity in the supernatant fractions was measured. The cellular extracts were applied to an anti-FLAG mAb-conjugated agarose column (Sigma). The absorbed materials were eluted with a buffer containing FLAG peptide under the conditions recommended by the manufacturer. The purified protein solution (1.5 ml) was used to determine the C4ST activity

and visualized with Western blot as described below before or after PNGase F digestion. The affinity-purified recombinant protein without mutation showed a broad protein band. After PNGase F digestion, a single protein band was detected at the migration position of 42 kDa that agreed well with the molecular mass, 42 111 Da, calculated from the cDNA. To measure the C4ST activity secreted into the culture medium, the transfected cells were cultured for 24 h in a medium containing 10 % (v/v) serum, and then for 24 h in serum-free medium (Cosmedium-001; Cosmo Bio, Tokyo, Japan). The C4ST activity contained in the serum-free medium was determined.

To determine the effects of tunicamycin, COS-7 cells were plated in 6-cm-diameter culture dishes at a density of 5.5×10^5 cells/dish. After 24 h, transfection with the cDNAs was carried out using TransFast (Promega), according to the manufacturer's instructions. After transfection in the serum-free medium, 2 vol. of DMEM containing 10 % (v/v) serum was added. After 4 h, tunicamycin was added to the culture medium at the final concentrations indicated in Figure 1(C), and the culture was continued for a further 45 h. The cellular extracts were prepared as described above.

CHO-K1 (Chinese-hamster ovary) cells were cultured in F-12 medium containing penicillin (100 units/ml), streptomycin (50 µg/ml) and 10 % (v/v) foetal bovine serum. Transfection of cDNAs into CHO-K1 cells was carried out using TransFast as above.

Western blot analysis

SDS/PAGE was carried out according to the method of Laemmli [24]. The affinity-purified proteins were boiled in an SDS sample buffer with 10 % (w/v) 2-mercaptoethanol and then separated by SDS/PAGE (10 % gels). Proteins were electrophoretically transferred on to Hybond ECL® (enhanced chemiluminescence) membranes (Amersham Biosciences) at 60 V for 4 h. For immunoblotting, non-specific binding sites were blocked with 5 % (w/v) dried skimmed milk in TBST (20 mM Tris/HCl, pH 7.6, 130 mM NaCl and 0.1 % Tween 20) at 4 °C for 16 h. An anti-FLAG M2 mAb (Sigma) was used at a 1:10000 dilution. Blots were developed with a polyclonal anti-mouse IgG antibody coupled to horseradish peroxidase diluted to 1:8000, using an ECL® detection kit and HyperfilmECL® (Amersham Biosciences). Molecular masses of polypeptides were calculated from the migration distance of a molecular-mass standard (Sigma) run in every gel. Protein bands in Hybond ECL® membrane were visualized by Amido Black staining. Amounts of FLAG-tagged fusion proteins were estimated by immunoblotting of these proteins together with FLAG-tagged bacterial alkaline phosphatase (FLAG-BAP, Sigma) as a standard as described previously [25].

Assay of sulphotransferase activity

C4ST activity was assayed by the method described previously [8]. The standard reaction mixture contained 2.5 µmol of imidazole-HCl, pH 6.8, 1.25 µg of protamine chloride, 0.1 µmol of dithiothreitol, 25 nmol (as glucuronic acid) of chondroitin, 50 pmol of [³⁵S]PAPS (approx. 5.0×10^5 c.p.m.) and enzymes in a final volume of 50 µl. To determine the *K_m* for chondroitin, 1.25 µg of protamine chloride was replaced with 0.25 µmol of CaCl₂, because the optimal protamine concentration depended on the concentration of chondroitin [23]. The reaction mixtures were incubated at 37 °C for 20 min, and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, ³⁵S-labelled glycosaminoglycans were isolated by precipitation with ethanol, followed by gel chromatography with a fast desalting column, as described previously [8], and radioactivity was determined. To determine C4ST

and C6ST activity, ^{35}S -labelled chondroitin was digested with chondroitinase ACII, and the radioactivity of unsaturated disaccharides ($\Delta\text{Di-4S}$ and $\Delta\text{Di-6S}$) separated by paper chromatography was counted. To determine the V_{max} for PAPS and chondroitin, the sulphotransferase activity of the recombinant proteins was normalized to the density of the protein bands on the Western blot detected by the anti-FLAG antibody.

Digestion with PNGase F

The affinity-purified recombinant C4ST-1 with or without mutations was precipitated with 10% trichloroacetic acid, and the precipitates were washed with acetone. Alternatively, the affinity-purified recombinant protein was precipitated with 2 vol. of ethanol containing 1.3% (w/v) potassium acetate. The precipitates were dissolved in 4 μl of buffer 1 (0.5% SDS and 0.15 M Tris/HCl, pH 7.8) and heated at 100°C for 2 min. After cooling, 4 μl of buffer 2 (3.2 mM PMSF, 4% Nonidet P40 and 32 mM EDTA, pH 8.0) and 1 unit of PNGase F were added to the mixture and incubated at 37°C for 18 h. After digestion, the samples were subjected to Western blot as described above. Digestion of the recombinant protein with PNGase F under the non-denaturing conditions was carried out at 37°C in the buffer used for the extraction of the cells, as described above.

RESULTS

Effect of PNGase F digestion on the C4ST activity

To investigate whether removal of N-glycans could affect the C4ST activity, we digested wild-type recombinant C4ST-1 with PNGase F [26] under non-denaturing conditions and assayed the enzyme activity. The C4ST activity decreased with PNGase F digestion over time, and after incubation for 60 min, the activity was 17% of the activity before incubation. On the other hand, in the absence of PNGase F, 47% of the activity was retained after incubation for 60 min (Figure 1A). When protein bands were detected with the anti-FLAG antibody after incubation with PNGase F for 60 min, broad bands of 44 kDa, which was larger than the core protein of 42 kDa, were observed (Figure 1B, lane 4), indicating that PNGase F digestion under the non-denaturing conditions did not remove N-linked oligosaccharides completely. These results suggest that N-linked oligosaccharides attached to C4ST-1 may contribute to the enzyme activity of C4ST-1 or to the stability of the protein.

Effects of tunicamycin on the activity of C4ST-1

COS-7 cells were transfected with C4ST-1 cDNA and cultured in the presence or absence of tunicamycin, and the C4ST activity of the cellular extracts was determined. As shown in Figures 1(C) and 1(D), in the presence of 0.01 $\mu\text{g/ml}$ tunicamycin, most of the fully glycosylated form of C4ST-1, as well as most of the C4ST activity, disappeared, suggesting that attachment of N-glycans to C4ST-1 is required to produce the active form of C4ST-1.

Effects of deletion of one or two N-glycosylation sites

There are four potential N-glycosylation sites in mouse C4ST-1: Asn²⁰⁵, Asn²²³, Asn³²¹ and Asn³⁴². These are shown in Figure 2 as N-1, N-2, N-3 and N-4 respectively. To investigate which of these N-glycosylation sites contribute to enzyme activity, we introduced mutations to each site so that the asparagine residues contained in the consensus Asn-Xaa-Ser/Thr were replaced with serine. In Figure 2, the names of these mutants are shown. The constructed plasmids were transfected into COS-7 cells, and cellular extracts

Figure 1 Effects of PNGase F digestion and tunicamycin treatment on C4ST activity

The affinity-purified wild-type recombinant protein was incubated in the presence (○) or absence (●) of PNGase F for various periods as described in the Experimental section, and the C4ST activity of the pre-incubated proteins was determined (A). The C4ST activity before incubation was 56 pmol/min per ml. After incubation in the presence of PNGase F, the recombinant protein was analysed by Western blot using the anti-FLAG mAb after incubation with PNGase F for 0 min (lane 1), 5 min (lane 2), 30 min (lane 3) and 60 min (lane 4) (B). COS-7 cells were transfected with the wild-type C4ST cDNA as described in the Experimental section, and cultured in the presence or absence of tunicamycin for 45 h. The C4ST activity of the cellular extracts was determined (C). After incubation in the absence (lane 1) or presence of 0.001 $\mu\text{g/ml}$ (lane 2) and 0.01 $\mu\text{g/ml}$ (lane 3) tunicamycin, the recombinant protein was analysed by Western blot using the anti-FLAG mAb (D). Results are means $\pm$ S.D. for triplicate data. The molecular mass (in kDa) of the standard protein band (egg albumin) is indicated at the right of the blots, and the glycoform and the core protein of FLAG-C4ST-1 are indicated by arrowhead 1 and arrowhead 2 respectively in (B) and (D).

Figure 2 Schematic diagram of the C4ST-1 mutants generated by site-directed mutagenesis

The recombinant C4ST-1 proteins are depicted by horizontal bars, in which the transmembrane domain (TMD, black box), 5'-PSB (hatched box), 3'-phosphate-binding domain (3'-PB, grey box) and N-glycans ('lollipop') are shown. The FLAG peptide (ellipse) attached at the N-terminal is shown. Four N-glycosylation sites, dubbed N-1, N-2, N-3 and N-4, are indicated above the horizontal bar. Crosses indicate the sites at which mutations were introduced. The name of each mutant is indicated at the left of each bar.

of COS-7 cells were used to determine the C4ST activity after affinity-purification. The C4ST activities of the mutant proteins are shown in Figure 3(A). Introduction of the mutation at N-3 or

Figure 3 Effects of deletion of one or two N-glycosylation sites on the C4ST activity

COS-7 cells were transfected with wild-type or mutant C4ST-1 cDNA, and the C4ST activity of the affinity-purified recombinant protein fractions was determined (A). Results are means $\pm$ S.D. for triplicate cultures. The affinity-purified recombinant proteins obtained from wild-type (lane 1), M-1 mutant (lane 2), M-2 mutant (lane 3), M-3 mutant (lane 4), M-4 mutant (lane 5), M-34 mutant (lane 6), M-1234 mutant (lane 7) and M-PSB mutant (lane 8) were analysed by Western blot before (B) or after (C) digestion with PNGase F. The band density of wild-type recombinant protein in (C) corresponded to 33.5 ng of FLAG-BAP (BAP is bacterial alkaline phosphatase) protein. The molecular mass (in kDa) of the standard protein band (egg albumin) is indicated at the right of the blots. Molecular masses of four distinct protein bands observed in lane 5 were 42, 44, 46 and 48 kDa respectively. The glycoform and core protein of FLAG-C4ST-1 are indicated by arrowhead 1 and arrowhead 2 respectively in (B).

N-4 resulted in a marked decrease in C4ST activity; the highest effect was observed in the M-4 mutant. The M-34 mutant, in which both N-3 and N-4 were mutated, showed little C4ST activity. The observed decrease in the C4ST activity contained in the cellular extracts might have been due to the accelerated secretion of mutant recombinant proteins. To examine this possibility, we determined the C4ST activity contained in the cellular extracts and culture medium. Introduction of the mutation to any N-glycosylation site was found to have no significant effect on the proportion of activity contained in the culture medium (Figure 4A), indicating that the removal of N-linked C4ST-1 oligosaccharides does not alter the rate of secretion.

When the recombinant protein was detected with the anti-FLAG antibody after PNGase F digestion, only a protein band of 42 kDa was detected (Figure 3C). The intensity of the bands of M-1 and M-2 mutants was almost the same as that of the wild-type protein; however, the protein bands of the M-3, M-4, M-34 and M-1234 mutants were weaker than the wild-type recombinant protein. When the C4ST activities of the recombinant proteins were expressed per density of the protein bands obtained after PNGase F digestion, the activities of M-4 and M-3 mutants were 0.26 and 1.2 of the activity of wild-type protein respectively, suggesting that deletion of the N-4 glycan decreased C4ST activity of the recombinant protein. On the other hand, the N-3 glycan did not appear to be required for the production of the active form, but it did appear to reduce the rate of synthesis of the recombinant C4ST. Before PNGase F digestion, the wild-type recombinant protein showed a broad protein band because of the microheterogeneity of N-glycans (Figure 3B, lane 1). Such microheterogeneity has also been observed in purified C4ST-1 [8]. When the N-4 site was

Figure 4 Effects of deletion of one N-glycosylation site of C4ST-1 on the secretion of C4ST-1 from COS-7 cells (A) and C4ST activity expressed by CHO cells (B)

(A) COS-7 cells were transfected with wild-type or mutant C4ST-1 cDNA as described in the Experimental section, and the C4ST activity of the cellular extracts (open bars) and the culture medium (closed bars) were determined. (B) CHO cells were transfected with wild-type or mutant C4ST-1 cDNA, and the C4ST activity of the cellular extracts was determined. Results in both panels are means $\pm$ S.D. for triplicate cultures.

Table 2 Comparison of the K_m between the wild-type recombinant protein and M-4 mutant protein

Recombinant protein	K_m		V_{max} (relative values)	
	PAPS (μ M)	Chondroitin (mM)	PAPS	Chondroitin
Wild-type	0.5	2.1	1.0	1.0
M-4 mutant	1.1	2.7	0.85	0.35

disrupted (M-4 mutant), the broad protein band observed in the wild-type recombinant protein disappeared and four distinct protein bands with molecular masses of 48, 46, 44 and 42 kDa were detected (Figure 3B, lane 5). These protein bands apparently correspond to different glycoforms with different numbers of N-glycans; 48, 46, 44 and 42 kDa bands probably bear three, two, one and zero N-glycans respectively. The M-PSB mutant, in which Lys¹²⁵ located in the putative 5'-PSB was replaced with glutamine, lost C4ST activity completely, but showed the broad protein band (Figure 3B, lane 8), suggesting that even fully glycosylated protein can be inactive for other reasons.

The effects of the deletion of the N-glycosylation sites were determined using CHO cells instead of COS-7 cells. Although the specific activity was lower in CHO cells, essentially the same relative activities as seen in COS-7 cells for the different mutants were obtained (Figure 4B), showing that the effects of N-link addition on activity is observed in multiple cell types.

The K_m and V_{max} for PAPS and chondroitin were determined using wild-type recombinant protein and M-4 mutant recombinant protein purified with an anti-FLAG-antibody-conjugated affinity column (Table 2). The K_m for PAPS of the M-4 mutant was about twice that of the wild-type protein, and the V_{max} for chondroitin of the M-4 mutant recombinant protein was about one-third that of the wild-type protein. These data with purified enzymes are consistent with those obtained with impure samples, and show that the differences reside within the enzyme protein itself.

Activity of C4ST-1 mutants containing a single N-glycosylation site

As observed above, deletion of the N-4 site resulted in marked loss of activity. To determine if attachment of the N-glycan at the N-4

Figure 5 Heat stability of the mutant C4ST-1 recombinant proteins

The recombinant proteins from wild-type (●), M-123 mutant (■) and M-4 mutant (▲) were incubated at 37°C for the indicated period, and then the C4ST activity was determined at 25°C. Results are means $\pm$ S.D. for three determinations.

site is sufficient for C4ST activity, we prepared mutants containing only one N-glycosylation site. In Figure 2, these mutants and their names are shown. When the C4ST activity of these mutant proteins was determined at 37°C, the activity was hardly detected in all the mutants (results not shown). We assumed that the low activity may be due to denaturation of the mutant proteins during the incubation at 37°C. To investigate such a possibility, we determined the activity at 25°C after pre-incubation at 37°C for various periods (Figure 5). After pre-incubation at 37°C for 20 min, approx. 75% of the activity of wild-type protein was retained, whereas the activity of the M-123 mutant was decreased to less than 25% of the activity before pre-incubation. We also determined the heat stability of M-4 mutant (Figure 5); M-4 mutant protein showed a similar denaturation curve to that of wild-type protein. These results indicate that the mutant protein containing a single N-glycan is extremely unstable at 37°C, whereas deletion of N-4 glycan resulted in the decrease in the enzyme activity, but not in the stability. Based on these observations, we determined the C4ST activity of these mutant proteins at 25°C (Figure 6). The M-123 mutant containing only N-glycan at N-4 showed approx. 30% of the wild-type protein, while the M-1234 mutant devoid of all N-glycans exhibited little activity. The importance of N-4 glycan was also shown by the fact that, when the C4ST activities of the recombinant proteins were expressed per density of the protein bands, the activities of M-123 mutant were 0.42 of the activity of wild-type protein, and the activities of the other mutants were less than half of the activity of M-123. It is evident that the M-123 mutant was actually glycosylated, because a 44 kDa protein band was observed in addition to the band of the 42 kDa core protein (Figure 6B, lane 5). Although the M-134 and M-124 mutants were also glycosylated (Figure 6B, lanes 3 and 4), the C4ST activity of these mutants was much lower than that of M-123, suggesting that N-glycans attached to N-2 and N-3 may contribute mainly to the stability of the protein rather than to the production of the active enzyme. On the other hand, the M-234 mutant showed no glycoform, and its C4ST activity was the same level as that of M-1234. These results clearly indicate that attachment of the N-glycan at N-4 is sufficient for the production of the active enzyme.

Effect of C-terminal truncation of the recombinant C4ST-1

The observation that deletion of the N-glycosylation site at the C-terminal region caused a marked decrease in C4ST activity suggests important roles of the C-terminal N-glycan in producing the active form of C4ST-1. The C-terminal N-glycan is attached to

Figure 6 C4ST activity of the recombinant proteins containing a single N-glycosylation site

COS-7 cells were transfected with wild-type or mutant C4ST-1 cDNA, and the C4ST activity of the affinity-purified recombinant protein fractions were determined (A). Results are means $\pm$ S.D. for triplicate cultures. The affinity-purified recombinant proteins obtained from wild-type (lane 1), M-234 mutant (lane 2), M-134 mutant (lane 3), M-124 mutant (lane 4), M-123 mutant (lane 5) and M-1234 mutant (lane 6) were analysed by Western blot (B). The band density of wild-type recombinant protein corresponded to 19.2 ng of FLAG-BAP (BAP is bacterial alkaline phosphatase) protein. The molecular mass (in kDa) of the standard protein band (egg albumin) is indicated at the right of the blot. Molecular masses of two protein bands observed in lanes 3–5 were 42 and 44 kDa respectively. The glycoform and the core protein of wild-type recombinant protein are indicated by arrowhead 1 and arrowhead 2 respectively.

Asn³⁴², which is the eleventh amino acid residue from the C-terminal. We investigated the role of the C-terminal N-glycan by another approach, in which the polypeptide of the recombinant C4ST-1 was truncated from the C-terminal. The C4ST activity of the truncated proteins decreased as the number of amino acid residues removed increased (Figure 7A). When seven amino acids located at the C-terminal were removed, the C4ST activity was completely abolished (Figure 7A, Δ 7aa). In a Western blot of these C-terminally truncated proteins using the anti-FLAG antibody, the protein of the Δ 4aa mutant showed a broad protein band, as observed in the wild-type recombinant protein (Figure 7B, lane 2), while the protein of the Δ 7aa mutant showed four distinct sharp protein bands of 48, 46, 44 and 42 kDa respectively, as observed in M-4 mutant recombinant protein (Figure 7B, lane 5). From the similar separation pattern, it seems highly unlikely that the N-4 site of the Δ 7aa mutant is glycosylated. These observations also support the important roles of the N-glycan attached to the N-4 site.

DISCUSSION

The purified C4ST-1, C6ST-1 and GalNAc4S-6ST were shown to be glycoproteins containing as much as 30% of N-glycans. The functional roles of such abundant N-glycans have not been clarified. In the present paper, we examined the functional roles of N-glycans attached to C4ST-1. Digestion of the recombinant C4ST-1 with PNGase F resulted in marked loss of C4ST activity, suggesting that N-glycans may be involved in maintaining the active form *in vitro*; however, in this experiment it was not clear whether N-glycans are required for the enzyme activity or for the

Figure 7 Effects of C-terminal truncation on C4ST activity

COS-7 cells were transfected with wild-type or mutant C4ST-1 cDNA, and the C4ST activity of the affinity-purified recombinant protein fractions were determined (**A**). Results are means $\pm$ S.D. for triplicate cultures. The affinity-purified recombinant proteins obtained from wild-type (lane 1), Δ 4aa mutant (lane 2), Δ 5aa mutant (lane 3), Δ 6aa mutant (lane 4) and Δ 7aa mutant (lane 5) were analysed by Western blot (**B**). The molecular mass (in kDa) of the standard protein band (egg albumin) is indicated at the right of the blot. Molecular masses of four distinct protein bands observed in lanes 4 and 5 were 42, 44, 46 and 48 kDa respectively. The glycoform and core protein of the wild-type recombinant protein are indicated by arrowhead 1 and arrowhead 2 respectively. Amino acid sequences of the C-terminal regions of the C-terminal truncation mutants are shown in (**C**).

stability of the active protein. We previously found that the activity of purified C4ST from the culture medium of chondrosarcoma cells decreased to 20% after 4 months, even when the enzyme was stored at -20°C , and that the purified C4ST required dithiothreitol for the activity [8]. The observed decrease in C4ST activity of the recombinant C4ST-1 during incubation at 37°C may represent an unstable property of C4ST, as observed in the purified enzyme. Tunicamycin has been used to investigate the role of N-glycans attached to glycosyltransferases [15,27] and sulphotransferases [21]. Treatment with tunicamycin of the cells that transiently express C4ST-1 also caused a decrease in the C4ST activity. Treatment with tunicamycin appears to affect not only the production of the active enzyme, but also the rate of metabolic decay of C4ST, because the protein bands detected by the anti-FLAG antibody became markedly weak.

The N-glycan attached to the C-terminal N-glycosylation site of C4ST-1 (N-4) was found to be most important for the production of active C4ST-1. The importance of the N-glycan attached to the C-terminal was reported in cerebroside sulphotransferase [21].

Figure 8 ClustalW alignment of the C-terminal conserved region of C4ST family proteins

The predicted amino acid sequences were aligned. White letters in black boxes and boxed letters indicate identical and similar amino acid residues respectively among these proteins. N-glycosylation sites contained in C4ST-1 are indicated as N-3 and N-4.

The reason the N-glycan attached to the N-4 site stimulates C4ST activity is not clear. The N-glycan may interact directly with substrates, and thereby increase the affinity for the substrates. Alternatively, the N-glycan may interact with chaperones which reside in the lumen of the endoplasmic reticulum, and contribute to the formation of active conformation. For the production of active C4ST, the N-glycan attached to the N-4 site appears to be sufficient, because the M-123 mutant protein showed C4ST activity; however, the mutant protein was much more unstable than the wild-type protein. N-glycans attached to N-2 and N-3 may contribute mainly to the stability of C4ST-1.

Removal of the C-terminal N-glycosylation site of C4ST-1 affected not only the C4ST activity, but also the band pattern of the protein detected by the anti-FLAG antibody. A broad protein band was mainly detected in the wild-type recombinant protein, whereas four distinct protein bands were observed for the M-4 mutant protein. As judged from the difference in the molecular mass between each protein band, these protein bands are thought to correspond to glycoforms with zero, one, two and three N-glycans. These observations suggest that blockade of attachment of the N-glycan to the N-4 site generates a variability of substitution at the other sites which is not seen in the wild-type protein.

When seven C-terminal amino acids residues were removed, the C4ST activity was totally lost. As a result of the deletion of seven amino acids, the N-4 site was exposed at the C-terminal. Efficiency of N-glycosylation was reported to be affected by C-terminal truncation of rabies virus glycoprotein [28]; however, the mechanism of the effects of C-terminal truncation is likely to be different from that of our observation, because the effects for rabies virus glycoprotein were seen when changes were made up to 68 residues away. The absence of N-glycan at the N-4 site of the Δ 7aa mutant appeared to be supported by the band pattern on SDS/PAGE; four discrete protein bands were also observed as in the M-4 mutant. The results obtained from the C-terminal truncation also showed the important role of the N-glycan attached to the N-4 site.

To date, several genes belonging to the C4ST family have been reported: mouse and human C4ST-1 [8,29,30], human C4ST-2 [29], human C4ST-3 [31], human and mouse GalNAc4ST-1 [32–35], human and mouse GalNAc4ST-2 [34–36], human D4ST [37], and human and rat HNK-1 [38,39]. ClustalW alignment of these genes showed five conserved sequences. Two of these sequences correspond to the putative PAPS-binding domains (5'-PBS and 3'-phosphate-binding domain). The C-terminal region was highly conserved among these genes (Figure 8). In the C-terminal conserved region, the N-glycosylation site corresponding to the N-4 site of C4ST-1 was also present in C4ST-3, GalNAc4ST-1 and GalNAc4ST-2. These observations suggest that the N-glycan attached to the C-terminal of the C4ST family proteins may play an important role, as observed in C4ST-1.

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