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Functional domains of bovine β-1,4 galactosyltransferase

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A number of N- and C-terminal deletion and point mutants of bovine β-1,4 galactosyltransferase (β-1,4GT) were expressed in *E. coli* to determine the binding regions of the enzyme that interact with *N*-acetylglucosamine (NAG) and UDP-galactose. The N-terminal truncated forms of the enzyme between residues 1–129, do not show any significant difference in the apparent K_ms towards NAG or linear oligosaccharide acceptors e.g. for chitobiose and chitotriose, or for the nucleotide donor UDP-galactose. Deletion or mutation of Cys 134 results in the loss of enzymatic activity, but does not affect the binding properties of the protein either to NAG- or UDP-agarose. From these columns the protein can be eluted with 15 mM NAG and 50 mM EDTA, like the enzymatically active protein, TL-GT129, that contains residues 130–402 of bovine β-1,4GT. Also the N-terminus fragment, TL-GT129NAG, that contains residues 130–257 of the β-1,4GT, binds to, and elutes with 15 mM NAG and 50 mM EDTA from the NAG-agarose column as efficiently as the enzymatically active TL-GT129. Unlike TL-GT129, the TL-GT129NAG binds to UDP-columns less efficiently and can be eluted from the column with only 15 mM NAG. The C-terminus fragment GT-257UDP, containing residues 258–402 of β-1,4GT, binds tightly to both NAG- and UDP-agarose columns. A small fraction, 5-10% of the bound protein, can be eluted from the UDP-agarose column with 50 mM EDTA alone. The results show that the binding behaviour of N- and C-terminal fragments of β-1,4GT towards the NAG- and UDP-agarose columns differ, the former binds preferentially to NAG-columns, while the latter binds to UDP-agarose columns via Mn^{2+}.

Keywords: β-1,4 galactosyltransferase, catalytic domains, site directed mutagenesis, binding studies, deletion mutans

Introduction

Cell surface complex carbohydrates have been implicated in cellular recognition events, such as bacterial and viral adhesions, cell-cell interactions, and tumour progression. The assembly of these complex carbohydrates requires the concerted action of a large number of Golgi resident glycosyltransferases which catalyze the transfer of a single sugar residue to a specific oligosaccharide acceptor. Our understanding of their molecular regulation, genomic organization, cellular targeting, and functional domains has increased by the cloning of several of these enzymes (reviewed in [1]). Although some amino acid sequence homologies have been identified within the catalytic domains of glycosyltransferase families, e.g. within sialyltransferases, fucosyltransferases, the histo-blood group A and B gene products and the galactosyltransferases, no similarities have been found between the families. Despite these differences in the primary sequences of these enzymes, all the glycosyltransferases share a common topology similar to type II membrane proteins. They have a short 6–24 residue long N-terminal cytoplasmic tail, a 16–20 residue long signal anchor transmembrane domain, a stem region of variable length and a C-terminal catalytic domain facing the Golgi lumen. The functions of each of these domains are being studied in several laboratories by expressing cDNA clones in both mammalian and bacterial cells [1]. By transfecting mammalian cells with a variety of glycosyltransferase cDNAs, the role of the transmembrane

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domain in the targeting of these enzymes to Golgi is being investigated [2–6]. It has been shown that the length of the hydrophobic region of the transmembrane domain is an important parameter for its Golgi retention [3]. For the functional analysis of the catalytic domain and large scale production, the secretory forms of these enzymes, which are devoid of the transmembrane domain and part of the stem region, are being expressed in *E. coli* and yeast cells [7–12].

Our laboratory has been studying the functional role of various domains of β-1,4 galactosyltransferase (β-1,4GT), a 402 residue long Golgi enzyme that transfers galactose from the UDP-galactose donor to the non-reducing terminal *N*-acetylglucosamine (NAG) acceptor of glycoproteins and glycolipids, generating a β-1,4 linkage [1]. To determine the sugar nucleotide donor and the sugar acceptor binding regions of β-1,4GT, we have expressed N-terminal deleted forms of the enzyme in *E. coli*. The recombinant proteins mostly accumulated as insoluble inclusion bodies [9]. After solubilization of the inclusion bodies, the enzymatic activity could be regenerated only in the presence of an oxido-shuffling reagent. The amino acid residues 1–129 which include the cytoplasmic tail, the transmembrane domain and the stem region, were shown to be dispensable for enzymatic activity, but the activity was lost when residues 130–142 which includes Cys134 are deleted [9]. Mutation of Cys134 to serine or alanine also resulted in the complete loss of enzymatic activity suggesting that the disulfide bond between Cys134 and Cys247 [13] is essential for its enzymatic activity. One out of a total of three exposed sulphydryl groups near the UDP-galactose binding site has also been implicated in the catalytic activity [14]. Site directed mutagenesis studies of human β-1,4GT [12], in addition to corroborating our conclusions about the importance of the disulfide bond, also showed that mutating Cys340 to serine (Cys342 in bovine β-1,4GT) leads to about a 33-fold increase in the apparent K_m for UDP-galactose.

Comparison of the amino acid sequences of bovine β-1,4- and α-1,3-GTs showed two stretches of similar sequences in the C-terminal region; both the enzymes use UDP-galactose as the sugar nucleotide donor [15–17]. From the site directed mutagenesis studies of human β-1,4GT, several aromatic amino acid residues located in the C-terminal region were shown to be involved in UDP-galactose binding [7, 18]. These results suggest that the UDP-galactose binding site is located in the C-terminal half of the enzyme. To delineate the UDP-galactose and NAG binding regions of β-1,4GT, we have expressed deletion and site-specific mutants of the enzyme as fusion and non-fusion proteins in *E. coli*, and investigated their enzymatic activities and binding to UDP- and NAG-agarose columns. The results of these ongoing studies are reported here.

Materials and methods

MATERIALS

Restriction enzymes and T4 DNA ligase were from New England Biolabs and the polymerase chain reaction (PCR) kit was from Perkin Elmer Cetus Corporation. Plasmid minipreparation kits were from Qiagen and 5′ → 3′ Inc. Oligonucleotides for PCR were synthesized by Genosys. Ampicillin, UDP agarose, NAG agarose were from Sigma Chemical Co. Isopropyl-β-D-thiogalactopyranoside (IPTG) and pGEX-2T were from Pharmacia. pET17b and pET23a vectors and the BL21 (DE-3)/pLysS competent cells were from Novagen. *E. coli* strain JM109 was from Stratagene. AG 1-X8 resin, chloride form, 200–400 mesh was from Bio-Rad. Tris-glycine gels were from Novex.

METHODS

Site directed mutagenesis of β-1,4GT cDNA

General DNA recombinant techniques were carried out as described in Current Protocols in Molecular Biology [19]. pLsGT-1 was used as the DNA template which contained the bovine β-1,4GT sequence to be PCR amplified (Fig. 1). *Bam* HI and *Eco* RI restriction sites were chosen in pGEX-2T, pET17b and pET23a for cloning purposes. The construction of clones pGT-d75, pGT-d129 and pGT-d142 are described in earlier studies [9]. The clones pGT-d119, pGT-d129C134A, pGT-d129NAG and pGT-d257UDP were also generated in pGEX-2T vector by the method described earlier [9]. Clones pEGT-d129 and pEGT-d129NAG were generated in pET-23a vector and clone p17GT-d257UDP in pET17bNB, a modified pET17b vector (Table 1). The PCR primers used for generating these clones are shown in Table 2. The 3′-end PCR primer pc1971-1951 corresponds to the unique *Eco* RI site present in the non-coding region of β-1,4GT (Fig. 1). The 3′-end PCR primer pc_stop_930-907 used for the construction of pGT-d129NAG and pEGT-d129NAG (Table 1) had an *Eco* RI site and a stop codon added. For cloning purposes all 5′-end oligonucleotide primers had a *Bam* HI site and two additional nucleotides. The conditions used for PCR amplification were 1 min at 96 °C, 2 min at 50 °C and 5 min at 72 °C with segment extension of 20 s per cycle for a total of 25 cycles. The PCR products containing the deletion mutants were purified using 5′ → 3′ Insta-mini-prep™ kit, treated with *Bam* HI and *Eco* RI and ligated to the expression vector.

Construction of pET17NB

In order to obtain clones that would express non fusion proteins, Novagen's pET17b vector was modified to generate the pET17bNB vector. pET17b was digested with *Nde* I and *Spe* I and ligated to a pair of annealed oligonucleotides 5′-TATGGGATCCGCTAGCA-3′ and

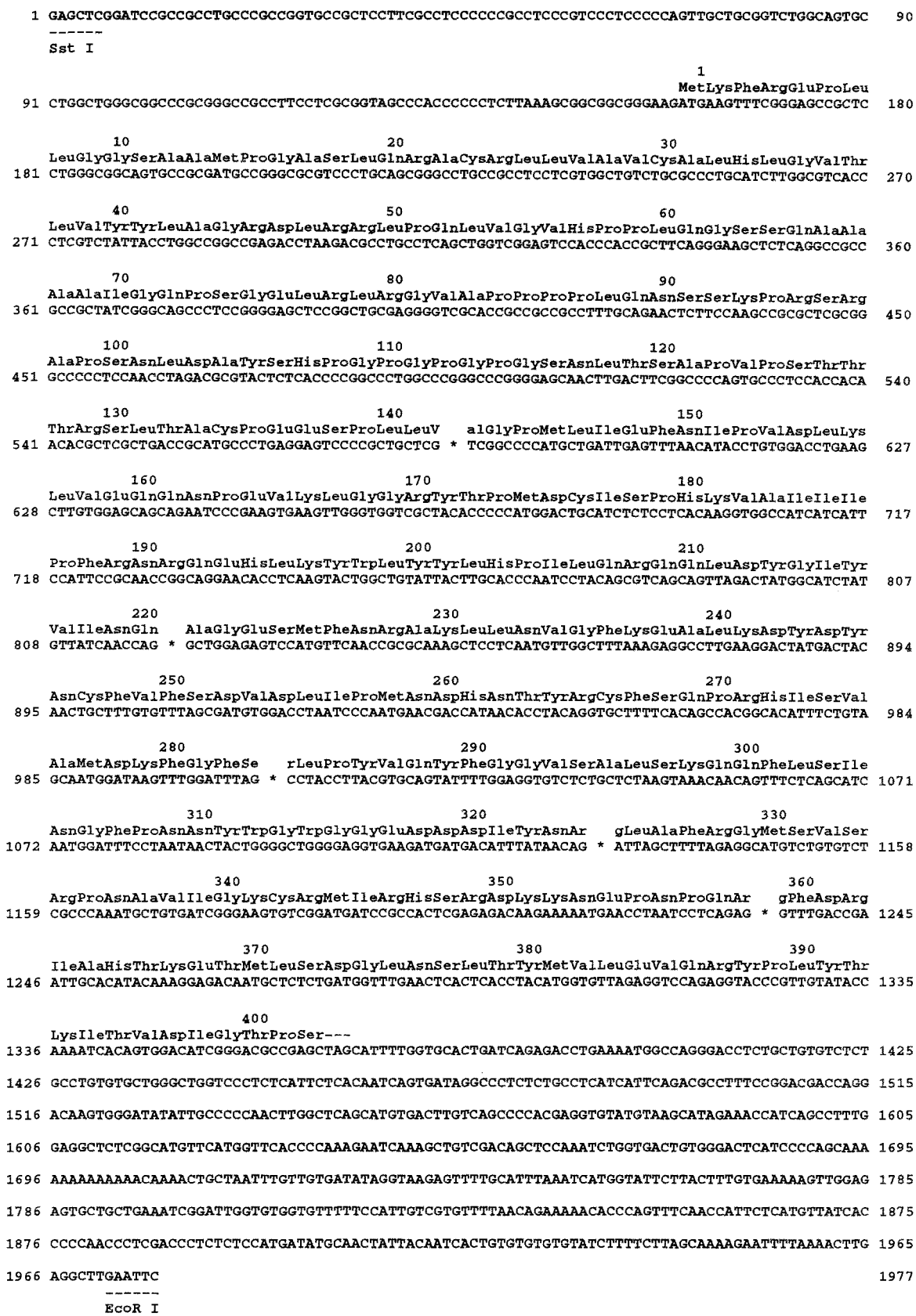

Figure 1. Sequence from *Sst* I to *Eco* RI site of the clone pLsGT [2] that was used for PCR amplification and subcloning into the expression vectors pGEX-2T, pET17b and pET23a. The predicted amino acid sequence of β-1,4GT is shown above the nucleotide sequence. Asterisk (*) represents the positions of the introns in the gene.

Table 1. Summary of the β-1,4GT mutant constructs and the recombinant proteins

Name of the clone	*Primers used (5′ end/3′-end)*	*Vector*	*Name of the protein*	*The first and last residues of β1,4GT and additional sequences fused at the* N*-terminus*	*Enzyme activity*	*Salt requirement for solubility*
pGT-d75	None[a]	pGEX-2T	GST-GT75	GST - GlySerProGlyThr - Glu^{76} . . . . . Ser^{402}	+	Low salt
pGT-d119	p517-540/pc1971-1951	pGEX-2T	GST-GT119	GST - GlySer - Ser^{120} . . . . . Ser^{402}	+	Low salt
pGT-d129	p547-570/pc1971-1951	pGEX-2T	GST-GT129	GST - GlySer - Ser^{130} . . . . . Ser^{402}	+	Low salt
pGT-d142	p586-609/pc1971-1951	pGEX-2T	GST-GT142	GST - GlySer - Gly^{143} . . . . . Ser^{402}	−	Low salt
pGT-d129C134A	p547-570__mut/pc1971-1951	pGEX-2T	GST-GTC134A	GST - GlySer - Ser^{130} . . Ala^{134b} . . . Ser^{402}	−	Low salt
pGT-d129NAG	p547-579/pc__stop__930-907	pGEX-2T	GST-GT129NAG	GST - GlySer - Ser^{130} . . . . . Pro^{257}	−	Low salt
pGT-d257UDP	p931-966/pc1971-1951	pGEX-2T	GST-GT257UDP	GST - GlySer - Met^{258} . . . . . Ser^{402}	−	Low salt
pEGT-d129	p547-570/pc1971-1951	pET-23a	TL-GT129	T7 Tag - GlySer - Ser^{130} . . . . . Ser^{402}	+	High salt
pEGT-d129NAG	p547-579/pc__stop__930-907	pET-23a	TL-GT129NAG	T7 tag - GlySer - Ser^{130} . . . . . Pro^{257}	−	High salt
p17GT-d257UDP	p931-966/pc1971-1951	pET17NB	GT-257UDP	MetGlySer - Met^{258} . . . . . Ser^{402}	−	High salt

[a]The construction of the clone pGT-d75 is as described in [24].
[b]Residue 134 is Cys in all the constructs except in this mutant where it has been changed to Ala.

Table 2. Primers used for the construction of β-1,4GT mutants

5′-end primers (with *Bam* HI site)

p517-540	517	5′-CGGGATCCTCGGCCCCAGTGCCCTCCACCACA-3′ 540
p547-570	547	5′-CGGGATCCTCGCTGACCGCATGCCCTGAGGAG-3′ 570
p586-609	586	5′-CGGGATCCGGCCCCATGCTGATTGAGTTTAAC-3′ 609
p547-570_mut	547	5′-CGGGATCCTCGCTGACCGCA[GCG]CCTGAGGAG-3′ 570
p547-570	547	5′-CGGGATCCTCGCTGACCGCATGCCCTGAGGAGTCCCCGCTG-3′ 579
p931-966	931	5′-CGGGATCCATGAACGACCATAACACCTACAGGTGCTTTTCACAG-3′ 966
		Bam HI

3′-end primers (with *Eco* RI site)

pc1971/1951	1971	5′-TGGAATTCAAGCCTCAAGTTTTAAAATTC-3′ 1951
pc_stop_930-907	930	5′-TGGAATTCCTATGGGATTAGGTCCACATCGCTAAA-3′ 907
		Eco RI

The restriction enzyme sites at the 5′-end are underlined. The nucleotide positions in the template GT sequence (pLsGT; Fig. 1) where the primer hybridizes are indicated in the primer names. 'pc' in the 3′-end primer names indicate that the primers are complementary to the sequence in pLsGT. The Cys134Ala mutation is enclosed in a box in the primer p547–570_mut.

5′-CTAGTGCTAGCGGATCCCA-3′ to reintroduce *Bam* HI and *Nhe* I sites. This modified vector has an ATG codon in the *Nde* I site next to the *Bam* HI site. Thus, cloning in the *Bam* HI site will produce a protein having Met-Gly-Ser tripeptide preceding the recombinant protein.

Expression of deletion and site-specific mutants of β-1,4GT in E. coli *and isolation and solubilization of inclusion bodies*

JM109 and BL21 (DE3)/pLysS competent cells were transformed with the pGEX-2T and pET vector derivatives, respectively as per manufacturer's protocols. Transformed cells were grown overnight at 37 °C in LB broth containing 50 μg ml^{-1} ampicillin. For BL21-(DE3)/pLysS cells, in addition to ampicillin, 34 μg ml^{-1} chloramphenicol was also added. Overnight cultures were diluted 1:10 in LB broth containing appropriate antibiotics and grown at 37 °C until OD_{600} was 0.4. The cultures were induced with 0.4 mM IPTG for 3 h at 37 °C. The inclusion bodies were isolated, denatured with 5 M guanidine HCl, renatured and dialysed as previously described [9]. To increase the solubility of the recombinant proteins TL-GT129, TL-GT129NAG and GT-257UDP, varying concentrations of NaCl (from 150 mM to 1 M) were added to both the renaturation and the dialysis buffers. Bio-Rad protein dye reagent was used for the estimation of protein concentration and pre-cast NOVEX gels for SDS-PAGE analysis. Coomassie Blue stained gels were scanned with an Eagle Eye scanner from Stratagene. The scanned gels were analysed with the Image analysis program (version 1.55f) from NIH.

^{35}S or ^{3}H Methionine labelling of GST-GT129, GST-GT142, and GST-GTC134A fusion proteins

The bacterial clones were grown overnight at 37 °C in M9 medium containing 20% sucrose, 1 M $CaCl_2$, 1 mg ml^{-1} vitamin B1, and a mixture of 20 amino acids at 2 mg ml^{-1}. The overnight cultures were diluted 1:10 in fresh M9 medium devoid of cysteine and methionine, grown for 1 h at 37 °C followed by the addition of IPTG to a final concentration of 0.3 mM and 5 mCi of L-^{35}S-cysteine and L-^{35}S-methionine or 2 mCi of ^{3}H-methionine. *E. coli* cultures were incubated for an additional 6 h.

Bacterial cells were harvested as previously described [9]. The bacterial pellets were processed and the radiolabelled proteins extracted from the inclusion bodies. Aliquot of the renatured and dialysed recombinant GST fusion proteins were bound to and eluted from glutathione-4B sepharose, UDP- and NAG-agarose columns. Incorporation of radioactivity was calculated in the eluted fractions.

Binding of recombinant proteins to affinity columns

The binding to the affinity columns was carried out at 4 °C with pre-incubated buffers. Renatured fusion and non-fusion protein solutions were applied to a 0.25 ml bed volume of either NAG- or UDP-agarose column which were pre-equilibrated with a 25 mM cacodylic buffer, pH 7.6, containing 25 mM $MnCl_2$, 10 mM β-mercaptoethanol, 0.1% Triton X-100. For NAG-agarose columns, 0.5 mM UMP was also included in the equilibration buffer. In some cases competing sugars were dissolved in this buffer. After loading of the samples, the columns were washed with 2 ml of equilibration buffer. The elution buffer for both the UDP- and NAG-agarose columns consisted of 25 mM cacodylate pH 7.6, 10 mM β-mercaptoethanol, 0.1% Triton X-100, 50 mM EDTA and 15 mM NAG. The concentration of the elution buffer components varied in some experiments as discussed in Results. GST fusion proteins were bound to and eluted from glutathione-4B sepharose column as described previously [9]. Fractions of 0.25 ml were collected from each column and aliquots were analysed by SDS-PAGE.

β-1,4GT assays

Activity of the recombinant β-1,4GTs was measured as described earlier using 1–2 ng of protein [9]. Kinetic studies were carried out with NAG, chitobiose, and chitotriose as acceptors at various UDP-[^{3}H]galactose concentrations (50–400 μM). The data from triplicate experiments was plotted and the apparent K_m values were determined by Lineweaver-Burk analysis.

Results

CONSTRUCTION OF β-1,4GT CLONES AND THE EXPRESSION OF RECOMBINANT PROTEINS

β-1,4GT Deletion mutants fused to GST domain

As reported previously clones pGT-d75, pGT-d129 and pGT-d142, constructed in the pGEX-2T vector and coded for residues 76–402, 130–402 and 143–402 of β-1,4GT, respectively, were fused to the C-terminal end of the GST domain [9]. The proteins accumulated as inclusion bodies in *E. coli* which could be solubilized in 5 M guanidine HCl. To regenerate the enzymatic activity, the solubilized proteins had to be renatured in the presence of oxido-shuffling reagents. For this study, one more deletion construct, pGT-d119, was included which was constructed as described in Methods. To delineate the binding regions for NAG- and UDP-galactose, two clones corresponding to the N- and C-terminal portions of the catalytic domain were constructed. The clones pGT-d129NAG and pGT-d257UDP coded for residues 130–257 and 258–402 of β-1,4GT, respectively, were fused to the C-terminus of the GST domain (Table 1). All the constructs coded for the protein which had successive deletions from the N-terminus except for pGT-d129NAG which also had a deletion from the C-terminus. The corresponding GT sequences coded by these clones which are fused to the GST domain are shown in Table 1.

Constructs in pET vectors

In an attempt to produce β-1,4GT sequences as non-fusion proteins, we constructed clones in pET17b vector. The vector was modified to have the *Bam* HI site next to the *Nde* I site which carries the ATG start codon. The PCR amplified DNA sequences corresponding to the regions coding for 130–402, 130–257 and 258–402 residues of the β-1,4GT were cloned in the *Bam* HI/*Eco* RI site of the vector. After insertion of a DNA fragment in the *Bam* HI site, the clone produces a protein that has three additional residues at the N-terminus. Low levels of proteins were produced from the constructs which coded for residues 130–402 and 130–257, even after varying the conditions of bacterial growth and induction. Only the clone p17GT-d257UDP, coding for residues 258–402 of β-1,4GT, gave high yields of the protein.

To increase the expression levels of fragments coding for residues 130–402 and 130–257 of β-1,4GT, we constructed the clones in the pET23a vector which produces a protein with an additional 12 amino acids (T7 Tag) at the N-terminus. The clones pEGT-d129 and pEGT-d129NAG (Table 1) produced high levels of proteins in BL21(DE3)pLysS cells when cultures were induced with 0.4 mM IPTG for a period of 3 h. The high levels of proteins produced using this vector may be due to the sequences in T7-tag corresponding to nucleotides with high codon usage in *E. coli*.

SOLUBILITY PROPERTIES OF THE RECOMBINANT GT PROTEINS

All the constructs described in Table 1 produced recombinant proteins as insoluble inclusion bodies and required 5 M guanidine HCl for solubilization. In order to regenerate enzymatic activity from the proteins GST-GT75, GST-GT119, GST-GT129 and TL-GT129 oxido-shuffling reagent had to be present during renaturation conditions, while proteins containing any residues between 143 and 402 of β-1,4GT did not require any oxido-shuffling reagent during renaturation conditions.

The recombinant proteins fused at the C-terminus of

the GST domain remain soluble in the presence of low salt (150 mM NaCl) during renaturation and dialysis [9]. In contrast, under the same conditions, proteins fused to the T7-tag or the protein GT-257UDP precipitated out during renaturation. In order to avoid precipitation of these proteins higher salt concentrations (1 M NaCl) were required both during washing of inclusion bodies, and during renaturation and dialysis of the proteins. The SDS-PAGE analysis after renaturation of the recombinant proteins and before passing through the affinity columns is shown in Fig. 2.

REQUIREMENT FOR OXIDO-SHUFFLING REAGENT FOR REGENERATING THE ENZYMATIC ACTIVITY OF GST-GT129 OR TL-GT129

To regenerate the activity from the 5 M guanidine HCl solubilized and denatured inclusion bodies of GST-GT129 or TL-GT129, an oxido-shuffling reagent is required during the dilution and renaturation step (Table 3). If the renaturation is carried out in the absence of the oxido-shuffling reagent protein shows only about 10% of the activity compared with the treated sample. However, the activity of the untreated sample can be fully restored if the sample is pretreated with the oxido-shuffling reagent for 1 h before the activity is measured. The residual 10% activity observed with the samples which were renatured in the absence of the oxido-shuffling reagent is possibly due to air oxidation since this activity can be reduced if the renaturation step is carried out in the presence of 10 or 50 mM dithiothreitol (DTT). Furthermore, the soluble form of GST-GT129 or TL-GT129 in *E. coli*, even though it represents a small fraction of the total amount of inclusion bodies produced in *E. coli* upon IPTG induction, also shows a three-fold increase in its activity if the protein is treated with the oxido-shuffling reagent (Table 3). Unlike TL-GT129 (or GST-GT129), which contains Cys 134, the mutant GST-GTC134A, where Cys 134 has been mutated to alanine, cannot be activated with the oxido-shuffling reagent. These results show that the enzymatic activity of the protein requires Cys 134 that has been shown to be disulfide bonded to Cys 247, and thus explains the need for the oxido-shuffling reagent to form this disulfide bond.

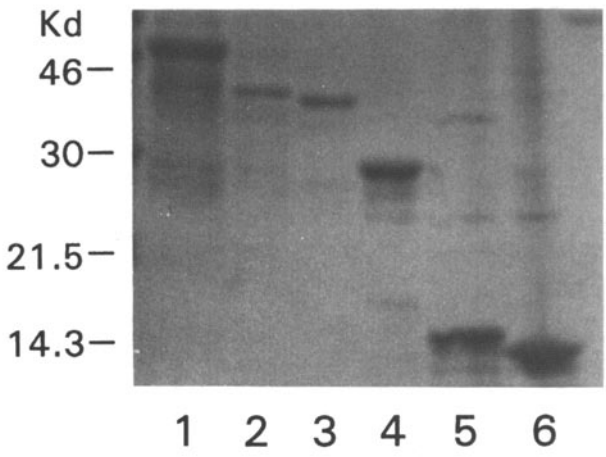

Figure 2. SDS-PAGE analysis of renatured and dialysed recombinant proteins before binding to affinity columns. Insoluble bacterial inclusion bodies were solubilized in denaturing buffer as indicated in Materials and methods. GST fusion proteins: GST-GT129 (lane 1), GST-GT257UDP (lane 2) and GST-GT129NAG (lane 3), and proteins devoid of the GST domain: TL-GT129 (lane 4), GT-257UDP (lane 5) and TL-GT129NAG (lane 6). An aliquot of the recombinant proteins was subjected to electrophoresis under reducing conditions. Major protein bands correspond to the recombinant proteins. Protein standard markers (K_d).

Table 3. Enzymatic activation of the recombinant TL-GT129[a]

Sample	*Overnight treatment*	*Treatment before assay*[b]	*Activity (%)*
Inclusion bodies	–	–	10
Inclusion bodies	ox-sh	–	100
Inclusion bodies	–	ox-sh	100
Inclusion bodies	50 mM DTT	–	1
Inclusion bodies	10 mM DTT	–	7
Inclusion bodies	10 mM DTT	ox-sh	22
Soluble fraction	–	–	30
Soluble fraction	ox-sh	–	100

[a]Protein samples in 5 M guanidine HCl, were slowly diluted 1:50-fold in a solution with or without oxido-shuffling reagent (ox-sh) or with DTT, shaken overnight at 4 °C and assayed.
[b]After overnight treatment, some samples were treated with ox-sh for 1 h prior to assay.

KINETIC CONSTANTS OF RECOMBINANT AND BOVINE MILK β-1,4GT

The kinetic constants of enzymatically active recombinant proteins were measured to determine whether the N-terminal deletions would affect the apparent K_ms of the proteins for NAG, chitobiose and chitotriose acceptors and for the sugar nucleotide donor, UDP-galactose. The appparent K_m values for NAG, among the fusion proteins, did not show much variation (Table 4). However, in comparison to bovine milk GT there was approximately a two-fold increase in the apparent K_m. In contrast to the monosaccharide NAG, the apparent K_m for the disaccharide chitobiose and the trisaccharide chitotriose, showed no difference among the deletion proteins and with the milk bovine GT except for a minor difference with GST-GT119. Also the apparent K_m for NAG among the fusion proteins and milk bovine GT was about two- to three-fold higher than the apparent K_m for chitobiose and chitotriose. For UDP-galactose the apparent K_ms for the deletion mutants and bovine milk GT ranged between 0.02 and 0.06 mM (data not shown).

Table 4. Apparent K_m values (mM) of bovine milk and recombinant β-1,4GT[a]

	NAG	*Chitobiose*	*Chitotriose*
Bovine GT	1.75	0.58	0.50
GST-GT75	2.35	0.75	1.00
GST-GT119	3.08	0.75	0.60
GST-GT129	2.83	0.75	1.00

[a]The apparent K_m values for the sugar acceptors were calculated from triplicate experiments. These values varied for NAG and chitobiose by ±0.25 and for chitotriose by ±0.12.

BINDING STUDIES OF N-TERMINAL DELETION AND SITE SPECIFIC MUTANTS OF β-1,4GT PROTEINS

The fusion proteins that contain the coding sequences between residues 143 and 402 of β-1,4GT and the Cys134Ala mutant, GST-GTC134A, are enzymatically inactive (Table 1). To determine if the substrate bindings were affected, the fusion proteins GST-GT142 and GST-GTC134A, were tested for their binding affinity to NAG- and UDP-agarose columns and compared with that of the enzymatically active protein GST-GT129. Since these fusion proteins contain a GST domain that binds to glutathione-sepharose 4B, the affinity binding of the renatured proteins to this matrix provided an internal control of the extent to which the different binding domains of the fusion proteins fold under the renaturation conditions used in this study. Initially we used either ^{35}S or ^{3}H labelled proteins for binding studies. After renaturation of either ^{35}S or ^{3}H labelled GST-GT129 about 80–95% bound to NAG- and UDP-agarose columns, and approximately 60–70% of the bound protein could be eluted with 15 mM NAG and 50 mM EDTA (Table 5). On the other hand, only about 35% of the proteins bound to glutathione-sepharose 4B column (data not shown) indicating that the renaturation and binding conditions are not optimal for the GST domain. The enzymatically inactive deletion mutant, GST-GT142, and also the point mutant GST-GTC134A (data not shown), bound 87–90% to the NAG-column which is comparable to the binding of the active GST-GT129 protein. Again only 35% of these proteins bound to the glutathione-sepharose 4B column. The bound proteins to NAG-agarose column could be eluted with either 15 mM NAG or 50 mM EDTA or with a better efficiency using a combination of both. Almost 100% of the NAG bound mutant proteins could be eluted in the presence of 15 mM NAG and 50 mM EDTA. The proteins could not be eluted with 15 mM glucose thus indicating NAG dependent specific binding to the column. The mutant proteins showed reduced binding to the UDP-agarose column. Only about 65% bound to the column compared with GST-GT129. These results show that the mutant proteins bind to the UDP-column with lesser affinity than the enzymatically active fusion protein (GST-GT129), suggesting that the region in the mutant proteins that binds to the UDP-column may have been structurally altered.

Since the behaviour of the mutant proteins on the UDP-agarose column was different from the behaviour of the enzymatically active protein, we tested the binding of the proteins containing residues 130–257 and 258–402 of β-1,4GT, either fused to or devoid of the GST domain, on NAG and UDP columns. Binding of these proteins was not influenced by either the presence or absence of the fused domain. The proteins TL-GT129NAG and GT-257UDP which correspond to the N- and C-terminus regions of the enzymatically active TL-GT129, were bound to NAG- and UDP-agarose columns. An aliquot of the bound and eluted proteins were analysed by SDS-PAGE (Fig. 3) and the stained gels were scanned. The data showed that 80–90% of the N-terminus fragment, TL-GT129NAG, binds to and is eluted from the NAG-agarose column with 15 mM NAG, or 50 mM EDTA, or a combination of the two as does the enzymatically active protein TL-GT129, with the exception that it elutes earlier from the column than the active protein (Table 6). The bound protein could not, however, be eluted with 15 mM glucose showing that the binding to the NAG-agarose column is NAG specific. The binding of TL-GT129NAG to an UDP-agarose column is low compared with TL-GT129, and only 40% of the bound protein elutes from the UDP-agarose

Table 5. Binding of GST-β-1,4GT fusion proteins to NAG- and UDP-agarose columns[a]

No.	*Labelled protein*	*NAG-agarose*		*UDP-agarose*	
		Bound (% of input)	Eluted (% of bound)	Bound (% of input)	Eluted (% of bound)
1	^{35}S-GST-GT129	95.2	71.1	86.7	54.3
2	^{3}H-GST-GT129	85.9	67.9	81.1	58.9
3[b]	^{35}S-GST-GT142	87.8	99.6	66.4	100.0
	^{3}H-GST-GT129	90.0	72.4	84.1	100.0

[a]Input cpms were counted in 30 μl of each radiolabelled protein. % of input bound was determined after subtracting unbound cpms. The binding and elution conditions are described in Methods.
[b]^{3}H-GST-GT129 and ^{35}S-GST-GT142 were mixed and renatured together. ^{3}H-GST-GT129 was used as an internal control to monitor the efficiency of renaturation of ^{35}S-GST-GT142.

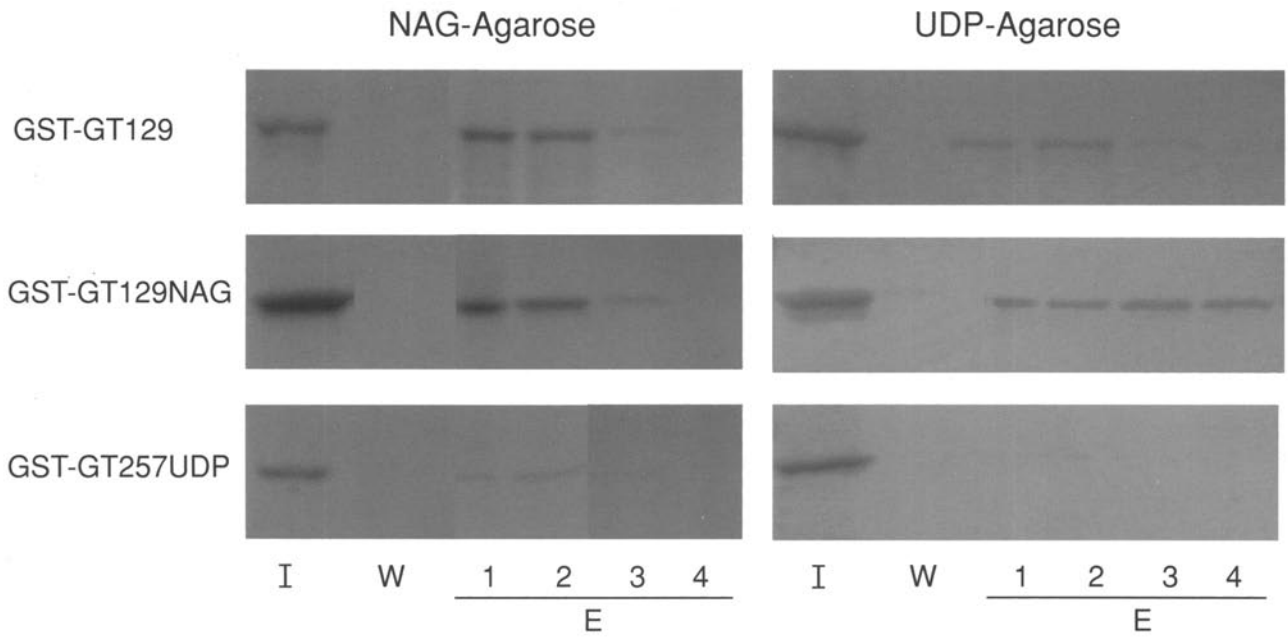

Figure 3. Electrophoretic analysis of β-1,4GT recombinant proteins on 14% Novex gels. Renatured and dialysed GST-GT129, the N-terminus fragment GST-GT129NAG and C-terminus fragment GST-GT257UDP fragment were bound to and eluted from NAG- (left panel) and UDP-agarose (right panel) columns as described under Materials and methods. Aliquots of each fraction were analysed on gels under reducing conditions and proteins visualized by Coomassie Blue staining. Lane I, input sample; Lane W, wash fraction; E, eluted fractions 1–4 (indicate successive order of elution of the proteins from the columns, 1: first eluted, 4: last eluted fraction). The three proteins separated on NAG and UDP columns were analysed by densitometry (Eagle Eye Scanner) using Image analysis program (version 1.55f) from NIH.

Table 6. Binding of β-1,4GT fragments to NAG- and UDP-agarose columns[a]

Protein	*Amino- and carboxy-terminal residues*	*NAG-agarose column*		*UDP-agarose column*	
		Eluted (% of bound)	*Elution condition*	*Eluted (% of bound)*	*Elution condition*
TL-GT129	Ser130, Ser402	80–90	15 mM NAG 50 mM EDTA	80–90	15 mM NAG 50 mM EDTA
TL-GT129NAG	Ser130, Pro257	80–90	15 mM NAG 50 mM EDTA	60–70[b]	30 mM NAG
GT-257UDP	Met258, Ser402	35–40	15 mM NAG 50 mM EDTA	5–10	50 mM EDTA

[a]Binding of the proteins to the NAG- and UDP-agarose columns were done as described in Methods. Results shown represent average of duplicate experiments.
[b]Only 40% of the input protein bound to the UDP-agarose column as compared to other samples which bound 80–90% of the input.

column. In contrast, GT-257UDP, residues 258–402 of β-1,4GT, binds equally well to both NAG- and UDP-agarose columns, but only 40% and 10%, respectively, is eluted from each of these columns. These results show that GT-257UDP binds to UDP-agarose much more tightly than TL-GT129 or TL-GT129NAG.

In order to elute the tightly bound proteins from the UDP-agarose columns, different conditions of elution were tried. The addition of 30 mM NAG or 30 mM D-galactose to the elution buffer without 50 mM EDTA, enhances elution of TL-GT129NAG but does not affect the elution of TL-GT129 or GT-257UDP. On the other hand, the bound TL-GT129 and GT-257UDP can only be eluted from the UDP column in the presence of 50 mM EDTA and 15 mM NAG (Table 6). The results suggest that both the active protein containing residues 130–402

(TL-GT129) and the C-terminal fragment (GT-257UDP) of β-1,4GT may bind to the UDP-column via Mn^{2+}. On the other hand, binding of TL-GT129NAG, residues 130–257 of β-1,4GT, to the UDP-column may not involve Mn^{2+} and thus can be easily eluted with 15 mM NAG.

Discussion

Carbohydrate structures on glycoproteins and glycolipids are synthesized by the coordinated action of glycosyltransferases. Molecular cloning techniques to date have yielded the characterization and grouping of several of these Golgi resident enzymes into at least four gene families [20]. Although little primary sequence similarities have been found between members of distinct glycosyltransferase families, they share a common topology. They all are type II membrane proteins with approximately the same length and domain structure, suggesting that this topology plays an important role in their targeting to the Golgi and for performing their catalytic function.

Initial studies on the structure and function of the cloned transferases have attempted to define the domain boundaries of glycosyltransferases. Results from our laboratory have suggested that truncation of the cytoplasmic tail of β-1,4GT does not affect synthesis or Golgi localization [3, 21]. Moreover, the transmembrane domain of the enzyme (Fig. 4) must be intact for proper processing of the protein in mammalian cells, and can be interchanged among glycosyltransferases without affecting synthesis or localization [3]. Residues Arg24, Leu25, His33, Cys29 and His32, located within the transmembrane domain, are critical for β-1,4GT to be retained in the Golgi [3, 5]. In addition, the hydrophobic length of the transmembrane domains of glycosyltransferases, is an important parameter for their Golgi retention, which on the average is shorter than the corresponding regions in the proteins that are targeted to the plasma membrane (16 versus 20 residues) [3]. It has been proposed that shorter transmembrane domains may interact with the thinner lipid bilayer at their site of synthesis, while longer transmembrane domains may interact with the thicker lipid bilayer, initiating their sorting and movement into the proper cellular compartments [3].

In vivo, the soluble forms of glycosyltransferases are derived from the membrane forms by proteolytic cleavage at the stem region. The soluble forms are enzymatically active and contain the binding sites for its substrates, the sugar nucleotide donor and the acceptor glycoconjugates, and for metal ions. The functional analysis of the catalytic domain of β-1,4GT has been carried out by expressing the protein in *E. coli* [7, 9]. In these cells the recombinant fusion proteins have been shown to accumulate in inclusion bodies as insoluble precipitates that

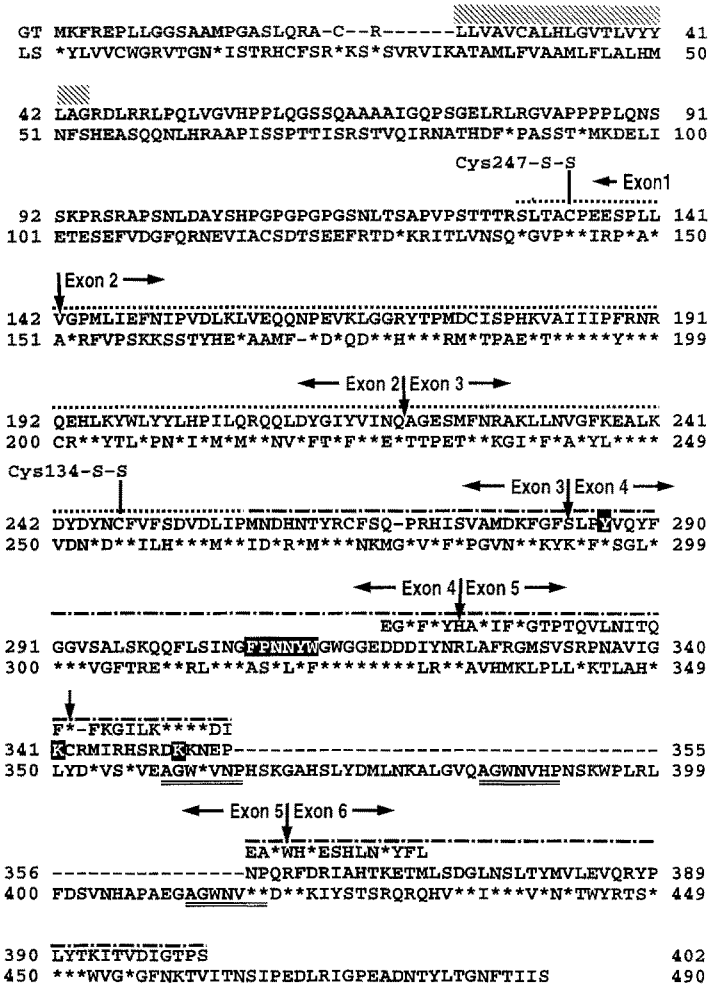

Figure 4. Comparison of the amino acid sequence of bovine β-1,4GT (GT) and *Lymnaea stagnalis* (LS) β-1,4GlcNAcT [28]. A portion of α-1,3GT sequence has been shown above the GT sequence. The conserved amino acids between the sequences are indicated by asterisks (*) and the missing amino acids by dashes. The membrane spanning domain of β-1,4GT has been shown above the nucleotide sequence (hatched box). Repeats in β-1,4GlcNAcT are double underlined. Exon boundaries of GT are indicated ([29, 30] and our unpublished results). Cysteines involved in disulphide bond are at positions 134 and 247 of β-1,4GT. Cys 342 involved in UDP-galactose binding [12] is marked with a down arrow. Dots (. . . . .) and broken lines (– . – . –) above GT sequence correspond to the sequences present in TL-GT129NAG and GT-257UDP, respectively. Residues within the black boxes have been shown to be involved in UDP-galactose [7, 15, 17, 18] and NAG binding sites [7, 16].

can be solubilized in 5 M guanidine HCl [9]. The solubilized β-1,4GT expressed as a GST fusion protein, was renatured in a mixture of oxido-shuffling reagent and 150 mM NaCl. After renaturation, the enzymatic activity was recovered and the fusion proteins remained in solution. In the present study the deleted forms of β-1,4GT were expressed as non-fusion proteins in *E. coli*

using the pET17b vector which was modified so that the translational initiation codon AUG preceded the β-1,4GT sequence. The protein GT-257UDP, that started from Met258 of β-1,4GT (Table 1), was expressed to high levels. However, expression of the proteins that started from Ser130 of β-1,4GT (GT129 and GT129NAG) was inefficient and only very low levels of the proteins were produced. There is ample evidence to show that the expression of foreign genes in bacterial cells may be complicated by protein instability or inefficient mRNA translation [22], and this is thought to be due to the presence of codons at the N-terminal end of the foreign genes that are rarely used in *E. coli*. To overcome this we used another vector that coded for a 12 amino acid long peptide fused to the N-terminal end of β-1,4GT sequence. The 12 amino acid long peptide is comprised of codons which are commonly used in *E. coli*. This system has a significant advantage over the GST fusion protein which gave yields of only 2 mg GST-fusion protein per l of culture [9]. Cleavage of the fusion protein with thrombin resulted in the release of the GST domain with approximately an eight-fold decrease in the final yield of β-1,4GT. In contrast, the yield of the recombinant protein TL-GT129, devoid of the GST domain, was about 2 mg l^{-1} of culture with 80% of the activity of bovine milk β-1,4GT. TL-GT129 and TL-GT129NAG also accumulated within inclusion bodies which required solubilization in 5 M guanidine HCL, and TL-GT129 required an oxido-shuffling reagent for regeneration of the enzymatic activity. We observed that the deleted forms of β-1,4GT, devoid of the GST domain do not remain in solution even after the addition of 150 mM NaCl, but moderate increase in NaCl concentration during renaturation and dialysis inhibited precipitation of these proteins. It was observed that 1 M NaCl was just enough to prevent the precipitation of the recombinant proteins. The high salt concentration seems to have the same effect as the extra sequence present at the N-terminal region of the fusion proteins, in that it prevents the aggregation and subsequent precipitation of β-1,4GT. The GST domain fused to β-1,4GT helps the fusion protein to remain in solution at low salt concentration.

In vivo β-1,4GT transfers galactose to the NAG residues of a variety of bi-, tri-, or tetraanntenary N-linked oligosaccharides of glycoproteins that exit from Golgi. To test the hypothesis that the residues 75–129 of β-1,4GT, are not involved in catalysis, but may influence the apparent K_m values towards sugar acceptors that are the *in vivo* substrates, we have initially analysed the transfer of galactose to mono-, bi- and trisaccharide structures. Kinetic study revealed that the N-terminal deletion mutants have nearly the same apparent K_m values for NAG, chitobiose and chitotriose as those of bovine milk GT (Table 4). From the present data it seems that the presence of N-terminal 75–129 residues does not affect transfer of galactose to mono-, bi- and trisaccharide structures. Also the soluble form of human β-1,4GT, that was produced in *E. coli*, does not show any change in the apparent K_m values between the linear oligosaccharide acceptors, agalactopoly-*N*-acetyllactosamine and lacto-*N*-triose II [23]. The secretory form of bovine β-1,4GT, which starts either from residue 76 or 96 [24], has been shown to transfer galactose 10 times faster to the NAG residue attached to the α-1,3 arm of the biantennary N-linked oligosaccharide than to the NAG attached to α-1,6 arm [25]. The N-terminal deletion mutants generated in the present study should be of help to answer whether the N-terminal sequences of β-1,4GT, which include the stem region, provide an extended binding surface for the branched oligosaccharide that may result in the preferences towards various acceptors. If the 1.7-fold difference in the apparent K_m of GST-GT119 for chitotriose compared with GST-GT75 or GST-GT129, observed in the present studies, reflects that any such structural preferences remain to be determined.

Although a number of studies show that the catalytic domain of β-1,4GT contains binding sites for sugar donor and acceptor substrates [7, 9, 12, 18], it is still not clear which regions of the protein are involved in their binding. The present study shows that the protein GST-GT142 which starts from residue 143 of β-1,4GT, in spite of being catalytically inactive binds to an NAG-agarose column with the same affinity as the catalytically active GST-GT129. Similarly GST-GT129C134A, which starts from residue 130 of β-1,4GT and has Cys134 mutated to Ala134, shows no enzymatic activity, but still binds to an NAG-agarose column. Both GST-GT142 and GST-GTC134A also bind to a UDP-agarose column but with lesser affinity than the enzymatically active GST-GT129. These results indicate that the structural integrity of the two substrate binding regions is to some extent still maintained in the renatured protein. Our results suggest that the folding of the protein seems not to be dependent on the disulfide bond formation. The protein that has been renatured in the absence of an oxido-shuffling reagent can be activated if treated subsequently with this reagent indicating that the disulfide bond formation is required for eliciting the activity of the protein. There are other proteins, for example RNAse T1 and dihydrofolate reductase, which also fold in the absence of disulfide bond formation [26, 27]. The absence of a disulfide bond between Cys134 and Cys247 may alter the UDP binding region to a higher degree than the NAG binding region thus affecting its binding to a UDP-agarose column.

Since enzymatically inactive β-1,4GT proteins, containing residues 143–402 of β-1,4GT, can still bind to NAG and UDP agarose columns, we produced N- and

C-terminal halves, either as GST fusion proteins or devoid of the GST domain, to test if one or both of these fragments show binding preferences towards NAG- or UDP-agarose columns. Our selection of the two halves was based on the following observations. β-1,4GT and α-1,3GT, both use UDP-galactose as the sugar nucleotide donor and also show some sequence homologies at the C-terminal half of the catalytic domain (Fig. 4 and [15–17]). The cross linking and mutational data have identified some of the residues involved in the UDP-galactose binding of β-1,4GT which are also localized in the C-terminal half of the enzyme [16, 18]. This data, together with our observation that the catalytically inactive GST-GT142 can bind both columns, prompted us to generate the protein fragments TL-GT129NAG and GT-257UDP, containing residues 130–257 and 258–402 of β-1,4GT, respectively, to test their binding properties towards NAG- and UDP-agarose columns. The fusion domain had no influence on the binding behaviour of the proteins since the proteins which were either fused to or devoid of the GST domain at the N-terminal end of β-1,4GT sequences behaved in the same way in their binding towards NAG- or UDP-agarose columns. The N-terminal half, TL-GT129NAG (residues 130–257), just as the enzymatically active TL-GT129 (residues 130–402), bound to and eluted with either 15 mM NAG or 50 mM EDTA or a combination of both from the NAG-agarose column. This binding is specific since 15 mM glucose cannot elute the protein from the column. The fragment TL-GT129NAG, as opposed to TL-GT129, bound to the UDP-agarose column with lesser affinity, and could also be eluted from this column with only 30 mM NAG. This result suggests that the region between residues 130–257 of β-1,4GT may be involved in the binding to NAG but may also contain some residues that bind to UDP. On the other hand, the C-terminal fragment, GT-257UDP, that bound to both agarose columns was partially eluted from the NAG-agarose but could hardly be eluted from the UDP-agarose column. To elute the bound protein from the UDP-column we varied the conditions of the elution buffer. However, only a small fraction of GT-257UDP was eluted from the column with buffer containing only 50 mM EDTA.

Mutational analysis of human β-1,4GT has identified a stretch of residues between 305 and 309 (corresponding to bovine residues 307–311) that may be involved in UDP-galactose binding (Fig. 4 and [7, 18]). Furthermore, by chemical modifications with periodate cleaved UDP and trace acetylation analysis, residues Lys341 and Lys351 of bovine GT have also been identified in UDP-galactose binding [16]. These residues are within the GT-257UDP fragment, as well as in the enzymatically active GST-GT129 or TL-GT129. However, only the latter binds to and also elutes from NAG and UDP-columns. This observation may suggest that residues 130–257 which form a disulfide bond between Cys134 and Cys247, and are absent in GT-257UDP, help to fold the UDP binding region correctly. The observation that GST-GT129NAG (or TL-GT129NAG) containing residues 130–257, binds to and elutes from the NAG column in a similar manner to the active protein GST-GT129 (or TL-GT129), further supports this hypothesis. Furthermore, Yadav and Brew [13] have also made observations that suggest that the sugar acceptor site of β-1,4GT is at the N-terminal 150 residue region containing a Cys134-Cys247 disulfide bond.

Our results clearly show binding behaviour differences in the N- and C-terminus halves of β-1,4-GT towards the NAG- and UDP-agarose columns. These results are consistent with other data suggesting that the region between residues 130 and 257, which contains a single disulfide bond in β-1,4-GT is involved mainly in acceptor binding, while the UDP-galactose binding region lies in the C-terminus half. A cDNA clone that codes for β-1,4-*N*-acetylglucosaminyltransferase (β4-GlcNAcT) was recently isolated from the pond snail *Lymnaea stagnalis* [28]. It transfers NAG from the sugar nucleotide donor, UDP-NAG, to an oligosaccharide acceptor, NAG, that is also used by β-1,4GT. Sequence comparison between β4-GlcNAcT and β-1,4GT shows homologies within their N-terminal region. The N-terminal homologous region of β-1,4GT is coded by exons 2,3 and 4 of the gene in bovine, murine and human species (Fig. 4 and [28–30]). The NAG-agarose binding data of the fragment TL-GT129 or TL-GT129NAG, residues 130–257 of β-1,4GT coded by exons 2 and 3, would support the homology observation that the two enzymes which transfer sugar to the same sugar acceptor, NAG, show maximum sequence homology in the same region. In the C-terminus of β-GlcNAcT there are, however, additional sequences in the region that correspond to the protein sequence coded by exon 5 of β-1,4GT (Fig. 4 and [29, 30]). These additional sequences in the exon 5 of β-1,4GT would disrupt the putative UDP-galactose binding site but may, on the other hand, introduce a binding site for UDP-NAG. Furthermore, the sequence of β-1,4GT coded by exon 5 and the boundaries of its neighbouring exons 4 and 6 show some degree of homology with α-1,3GT, the enzyme that also uses the same nucleotide sugar donor, UDP-galactose. Chemical cross-linking and mutational analysis have identified UDP-galactose binding residues that are also in the region coded by exons 4 and 5 (Fig. 4). Our observation that the fragment 258–402 does not bind well to NAG-agarose, but binds tightly to the UDP-agarose column, supports the hypothesis that in this region of β-1,4GT lies the sugar nucleotide binding site.

The three-dimensional structures of several plant and mammalian lectins reveal that the sugar binding specificity of lectins in some cases may not be determined by

the linear amino acid sequence (primary sequence) but may depend on the secondary or tertiary structure of the protein [31, 32]. There are, however, some examples where in some cases a contiguous polypeptide chain has been shown to be involved in sugar binding [33, 34]. It remains, however, to be determined if the binding regions for the sugar acceptor and sugar donor substrates in glycosyltransferases, and β-1,4-GT in particular, are generated by contiguous polypeptide chains. Nevertheless, the results presented here and from various other laboratories can be summarized in a schematic diagram depicting the essential components involved in the catalytic action of β-1,4-GT (Fig. 5). It is reasonable to assume that to generate the glycosidic linkage the two substrates have to be in close proximity. The major binding surface for the sugar acceptor and the nucleotide sugar donor, which may lie in the N- and C-terminal halves of the catalytic portion of the protein, respectively, have to overlap at the catalytic surface. For generating β-glycosidic linkage the O4-H of NAG has to be in the proximity of the C1 of galactose (UDP-α-galactose) at the catalytic site, and inversion of configuration at C1 has to occur. The disulfide bond between Cys134 and Cys247, which is required during catalysis, but not for folding or binding to the substrates (data presented here and [9, 12]), may be involved at the time of the catalytic act either to constrain the molecular flexibility of the activated complex, or by being in close proximity to the catalytic site, to share the electronic orbitals of the bonded disulfides with the activated complex. This may further involve the thiol of Cys342 which is also required for catalysis [12, 14], thus forming a catalytic triad involving thiol-disulfide exchange as has been observed in other systems (reviewed in [35]). In addition, Mn^{2+} (or Ca^{2+}) which has been implicated to be involved in catalysis [14] may be bonded to the phosphate of the nucleotide sugar donor, and to the carboxyl groups of the aspartic acid residues 318–320 (DDDI) of the protein, which are near the UDP-galactose binding region. His347 is also present in this region and may participate during the catalytic event. A second Mn^{2+} ion which is about 18 ± 3 Å away from the catalytic Mn^{2+} ion, has been implicated in the structural integrity of the protein [14], and may be involved in the binding of the sugar acceptor site as happens with metal ion dependent (Ca^{2+}) sugar binding lectins. Further studies involving specific mutations, mutations in the fragments generated in this study and the three-dimensional structure determination of galactosyltransferase should facilitate in describing the structural details of the binding of substrates and the catalytic mechanism involved.

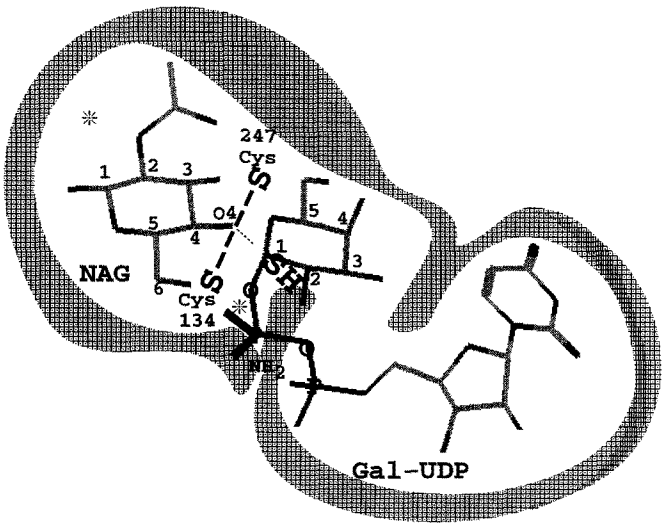

Figure 5. Schematic representation of the substrates of β-1,4GT at the active site and the binding surface. Star (*) represents Mn^{2+}.

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