Vol. 201, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1994 Pages 160-167

EXPRESSION OF SOLUBLE ACTIVE HUMAN ß1,4 GALACTOSYLTRANSFERASE IN *SACCHAROMYCES CEREVISIAE*

Ralf Kleene[1], Christian H. Krezdorn[1], Gabriele Watzele[1], Bernd Meyhack[2], Guido F. Herrmann[3], Christian Wandrey[3] and Eric G. Berger[1,*]

[1]Physiologisches Institut, Universität Zürich, Winterthurerstr.190, CH-8057 Zürich, Switzerland

[2]Ciba-Geigy AG, Biotechnology, K681-1.46, CH-4002 Basel, Switzerland

[3]Institute of Biotechnology, Research Center Jülich, D-52425 Jülich, Germany

Received March 23, 1994

SUMMARY: Sequences coding for the cytoplasmic and transmembrane domains were removed from the cDNA of the human Golgi resident membrane protein ß1,4 galactosyltransferase (gal-T). The remaining sequences coding for the stem and catalytical domains of this glycosyltransferase were fused to sequences coding for the yeast invertase signal sequence. The hybrid was inserted together with a constitutive yeast promoter and a terminator into a *E. coli*/yeast shuttle vector. *Saccharomyces cerevisiae* strain BT150 transformed with this new expression vector expressed enzymically active soluble enzyme, whereas no activity was detectable in mock-transformed yeasts. The enzyme product was identified by HPLC analysis and shown to correspond to the expected product *N*-acetyllactosamine.

The glycosyltransferase *N*-acetylglucosaminide ß1,4 galactosyltransferase (ß1,4 gal-T) (E.C. 2.4.1.38) transfers Gal from UDP-Gal to free GlcNAc or unsubstituted GlcNAc residues in glycoproteins, e.g. ovalbumin [1]. Like many other glycosyltransferases this enzyme is a resident protein of the Golgi apparatus of higher eucaryotes [2]. Like all Golgi-located glycosyltransferases cloned so far (for review see [3]), gal-T is a single peptide with the following domain structure: A N-terminal cytoplasmic domain is followed by a single transmembrane domain and at the luminal site by a stem region and a C-terminal catalytical domain. Enzymically active soluble forms have been described to occur in many body fluids [1, 4, 5]. Indeed, N-terminal analysis [4] or sequencing [6] revealed heterogeneity of the soluble natural enzyme forms that appeared to be truncated by proteolysis [7]. In analogy, soluble forms of glycosyltransferases could be obtained by expressing truncated forms without the transmembrane domain by using recombinant DNA techniques [8].

*Corresponding author. FAX: +1/3640564.

0006-291X/94 $5.00

Complex carbohydrates play an important role in numerous biological functions (for review see [9]). They are attracting great commercial interest as promising pharmaceuticals [10]. Whereas the chemical synthesis of complex carbohydrates is cumbersome because of low yields, need for toxic chemicals and side products, enzymes promise to be powerful and "clean" tools as catalysts for the regio- and stereospecific synthesis of oligosaccharides [11]. Therefore, glycosyltransferases and especially their large scale expression has become one of the prime targets in the pharmaceutical industry.

Beside gal-T several other glycosyltransferases have been heterologously expressed in good yield in higher eucaryotic cells like COS, CHO or SF9 cells (for review see [3]). However, due to the ease and low costs of fermentation yeast is a commercially more attractive expression system than higher eucaryotic cells. In procaryotic cells, such as *E. coli*, the expression level of active gal-T appears very low [12, 13]. We recently reported the successful expression of active membrane-bound human glycosyltransferases in yeast [14, 15]. Since the handling of a membrane protein in purification steps and in its application for organic synthesis is difficult we decided to express a soluble form of gal-T in yeast. For that purpose N-terminal sequences including the transmembrane domain were replaced by the yeast invertase signal sequence [16] which allows translocation into the secretory pathway. Indeed, using a constitutive yeast promoter for expression the soluble form was produced in a biologically active form in *Saccharomyces cerevisiae*. These results demonstrate that yeast is a promising host for the large-scale production of glycosyltransferases.

Materials and Methods

Strains and plasmids

The *S. cerevisiae* strain BT150 (*MATα, his4, leu3, ura3, pra1, prb1, prc1, cps1*) deficient in protease A and B and carboxypeptidase Y and S was a gift from Dr. Hinnen (Ciba-Geigy AG).

Construction of pDPsGT

The cDNA coding for gal-T was isolated from HeLa cells using the PCR method [17]. The primers used for amplification corresponded to published sequence information [18]. The PCR product was inserted into pUC18 resulting in the plasmid p4AD113.

For the expression of a soluble gal-T a short constitutive yeast *PHO5* promoter variant and sequences coding for the signal sequence of yeast invertase were linked in a first cloning step to truncated cDNA encoding a soluble form of gal-T. In order to obtain this construct the constituents were prepared as follows:

(a) The gal-T cDNA was first excised from the plasmid p4AD113 by digestion with EcoRI. The fragment was isolated and then partially digested with MvnI. A 1.05 kbp MvnI-EcoRI fragment showing the gal-T cDNA sequence from nucleotide position 134 to 1197 was isolated.

(b) From the vector p31/*PHO5*(-173)RIT a BamHI-XhoI fragment was excised. This 0.24 kbp fragment comprises the sequences of the promoter region from nucleotide position -9 to -173 of the yeast *PHO5* gene [19, 20] and the sequences coding for the invertase signal sequence [16]. The promoter *PHO5*(-173) has no upstream regulatory sequences and, therefore, behaves like a constitutive promoter. The 0.24 kbp fragment was cut with HgaI (Biolabs) and isolated. The recognition site of HgaI is positioned on the antisense strand downstream of the invertase signal sequence. The cut creates a 5` DNA overhang which coincides with the end of the coding sequences of the invertase signal sequence.

(c) The following pair of synthetic oligonucleotides was annealed by slowly cooling down after heating to 68° C:

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(1) 5'CTGCACTGGCTGGCCG 3'
(2)       3'GACCGACCGGC 5'
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The resulting linker was designed to link the sequences coding for the invertase signal sequence and the truncated gal-T cDNA in frame.
(d) pUC18 was digested with EcoRI and BamHI and treated with calf intestinal alkaline phosphatase.
The fragments obtained from (a) and (b), the adaptor from (c) and the vector from (d) were ligated and transformed into *E. coli* strain DH5α (Gibco-BRL) according to [21]. Transformants were characterized by restriction analysis and DNA sequencing.
In a second cloning step the expression vector was constructed as follows:
(e) The recombinant plasmids having the constitutents in the proper array were first cut with SalI and then partially digested with EcoRI. The 1.35 kbp cassette comprises the promoter and sequences coding for the invertase signal sequence linked in the correct reading frame to sequences encoding the soluble gal-T starting at amino acid position 42.
(f) The plasmid p31R10 was cut with HindIII. The ends were filled by Klenow polymerase. The plasmid was then digested with EcoRI and the 0.39 kbp EcoRI-blunt end fragment containing the *PHO5* terminator sequences was isolated.
(g) The yeast/*E. coli* shuttle vector pDP34 [22] was linearized by BamHI digestion. After polishing the ends by treatment with Klenow polymerase the plasmid was cut with SalI.
The fragments obtained from (e) and (f) and the vector (g) were ligated and transformed into DH5α. Transformants were characterized by restriction analysis. The recombinant plasmid pDPsGT containing the expression cassette comprising the promoter, the sequences coding for invertase signal sequence, the linker, the truncated cDNA coding for soluble gal-T and the terminator sequences were transformed into yeast strain BT150 using the lithium acetate transformation method [23]. Ura^+ transformants were selected and analysed for enzymic activity.

Growth conditions and preparation of yeast cell lysates
The yeast strain BT150 was grown at 30° C in SD medium [24] or on SD plates. Cells (4 OD_{600}/ml) were harvested by centrifugation and resuspended in 50 mM Tris/HCl, pH 7.4. For disruption glass beads (0.5 mm x 0.45 mm, Braun Melsungen) were added and cells were broken by mechanical lysis using a Kaiser disruptor (B.Braun Melsungen AG, Germany). Ultracentrifugation of lysates was carried out at 100,000g for 60 min at 4° C.

Assay of ß1,4 gal-T
Assay conditions were described in detail previously [14], and carried out using ovalbumin or GlcNAc as acceptor substrates.

HPLC analysis of the product
The samples were separated on a Aminex HPX-87H (Bio-Rad) at 65° C using 0.2 N H_2SO_4 as eluant. The flow rate was 0.8 ml/min. The products were detected by UV spectroscopy using a wavelength of 205 nm.

Results and Discussion

We intended to express a soluble form of gal-T in *Saccharomyces cerevisiae* (Fig. 1). For that purpose the cDNA sequences encoding the cytoplasmic and most of the transmembrane domain was cut off leaving the sequence information of the catalytic domain and the entire stem region. Since all current available evidence indicated that Golgi retention of ß1,4 gal-T is solely due to the transmembrane domain [25], the stem region was left intact. This possibility was not predicted on the basis of data reported for α2,6 sialyltransferase suggesting that part of the stem region appears to specify for Golgi retention [8]. The truncated cDNA was fused in frame to DNA sequences coding for the yeast invertase signal sequence [16]. The invertase signal sequence provides the signal for translocation which should allow the import of the soluble form into the secretory pathway. The invertase signal sequence is expected to be cleaved off during translocation into the ER [26]. For expression in yeast a constitutive *PHO5* promoter variant and the PHO5 terminator were used. A cassette consisting of promoter,

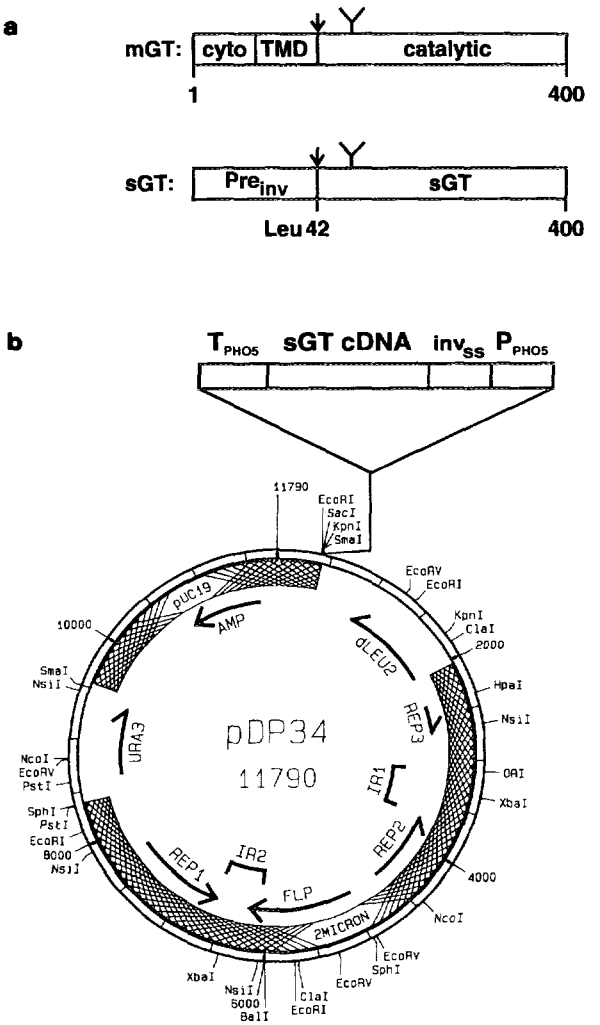

Fig. 1. Construction of gal-T expression vector pDPsGT. a) Schematic representation of the domain structure of the membrane-bound gal-T (mGT) and the construction of the soluble gal-T (sGT). Cytoplasmic (cyto), transmembrane (TMD) and catalytic domains and invertase presequence (Pre_{inv}) are shown. b) A cassette consisting of a constitutive *PHO5* promoter variant (P_{PHO5}), sequences coding for the yeast invertase signal sequence (inv_{SS}), an adaptor and a truncated cDNA of gal-T (sGT cDNA) and the *PHO5* terminator (T_{PHO5}) was inserted into the yeast expression vector pDP34 (for details see Materials and Methods).

sequences coding for invertase signal sequence fused in frame to the truncated cDNA coding for gal-T and terminator was constructed and inserted into the *E. coli*/yeast shuttle vector pDP34. The new expression vector was designated as pDPsGT and is expected to express soluble gal-T with leu_{42} at the N-terminus. The expression vector was transformed into the yeast strain BT150. For control the strain was mock-transformed with pDP34 without any inserted cassette.

Lysates were prepared from the different transformants and analysed for enzymic activity. For activity measurements two different acceptor substrates of gal-T were used: GlcNAc and

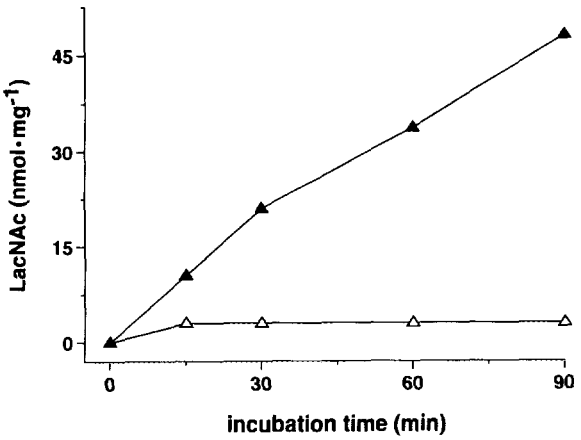

Fig. 2. Determination of enzymic activity. 50 µl lysate (corresponding to 50 µl culture) at a concentration of 2.7 mg protein · ml^{-1} was added to the reaction mixture containing 1 µmol $MnCl_2$, 10 µmol Tris/HCl (pH 7.4), 1 µmol GlcNAc, 0.2 µmol UDP-Gal and 20 nmol UDP[^{14}C]Gal (final specific activity: 2500 cpm · $nmol^{-1}$). After the indicated time periods the reaction mixture was applied to a AG1-X8 column and the flow-through was counted in a ß-scintillation counter.
BT150/pDP34 (open triangles); BT150/pDPsGT (filled triangles).

ovalbumin. In lysates derived from mock-transformed yeasts no gal-T activity was detectable at all, whereas in lysates of yeasts transformed with pDPsGT enzymic activity was readily detectable. Fig. 2 shows the corresponding linear time curve of transfer of radioactive Gal from UDP-Gal to GlcNAc. Under the conditions specified in the legend of Fig. 2, the increase was linear up to 90 min of incubation. The yield of activity was app. 200 mU/ l culture by assuming a similar specific activity of recombinant gal-T and the human milk enzyme. The enzyme also catalysed the transfer of Gal to ovalbumin (not shown). The K_m values were determined for UDP-Gal and GlcNAc and for glucose in the lactose synthetase reaction [1] in the presence of α-lactalbumin. The kinetic parameters are summarized in Tab. 1 in comparison to human milk gal-T and recombinant full-length gal-T [14].

Tab. 1: Michaelis constants. One substrate was kept under saturating condition while the other was varied and the incubation time was 1h. The parameters were assessed using the Enzfitter software package.

	K_m		
	UDP-Gal	GlcNAc	Glc/ α-lactalbumin [1]
	µM	mM	mM
recombinant soluble gal-T	25	2.3	7.0
recombinant full-length gal-T [2]	28	3.6	12.0
soluble human milk gal-T [2]	82	2.8	4.9

1) lactose synthetase activity
2) see [15]

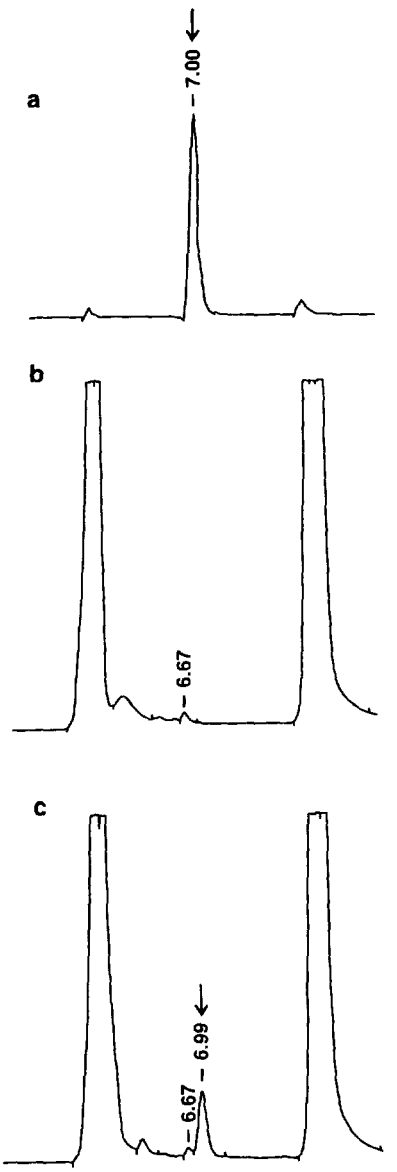

Fig. 3. HPLC analysis of the product. Authentic LacNAc was separated and the retention time was determined to be 7 min (a). The reaction mixtures using either lysate of BT/pDP34 (b) or BT/pDPsGT (c) was separated on HPLC. The position of LacNAc is indicated by the arrow. Note: the peaks showing a retention time of 6.67 do not correspond to the authentic LacNAc profile.

The reaction product was characterized by HPLC analysis using LacNAc as a reference (retention time = 7 min)(Fig. 3a). When lysates of mock-transformed cells (BT150/pDP34) were used in the reaction the HPLC separation profile showed no peak corresponding to LacNAc (Fig. 3b). However, when lysate of BT150/pDPsGT was applied for the reaction, a peak showing the retention time of LacNAc was detected (Fig. 3c). By integration of the

LacNAc peak areas of the reference and the reaction product formed by BT/pDPsGT, respectively, the product could be quantified and the corresponding enzyme activity determined to be approx. 200 mU/ l culture. This value was identical to that determined by using the radioactive enzyme assay.

Ultracentrifugation of lysates was carried out in order to analyse whether the enzyme was soluble or not. Up to 95 % of total activity was found in the 100.000g supernatant, whereas less than 5 % was present in the pellet indicating that the enzyme is a soluble protein (data not shown). This result was confirmed by the finding that addition of detergent during or following cell disruption did not lead to an increase in total enzyme activity suggesting that the gal-T was not membrane associated like the membrane-bound forms which were activated by Triton-X100 [14].

Taken together, the results clearly demonstrate that enzymically active gal-T can be expressed as soluble form in *S. cerevisiae*. This expression system will hopefully prove useful for large scale production of other glycosyltransferases.

ACKNOWLEDGMENTS

This work was supported by grant 2305.1 of the KWF to EGB. We thank C. Gasser for the preparation of the artwork.

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