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Construction of recombinant BHK cell lines expressing wild-type and mutants of human α1,3/4-fucosyltransferase

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Abstract

Stable BHK-21 cell lines were constructed expressing: (1) the wild-type form of human α1,3/4-fucosyltransferase (FT3T2); and (2) the secretory form of the enzyme, where amino acid residues 46–361 (S2FT3T2) were coupled at their amino terminus to the signal sequence of interleukin-2. Preliminary results of immunofluorescence microscopy indicated that S2FT3T2 was present in the endoplasmic reticulum and Golgi apparatus of the cells, whereas FT3T2 was localized in the Golgi apparatus. The S2FT3T2 was purified by affinity on a GDP-Fractogel resin and its specificity towards oligosaccharides and glycoproteins was studied by HPAEC-PAD, methylation analysis, MALDI/TOF-MS and ESI/MS-MS. The soluble form of α1,3/4-fucosyltransferase may be used for the in vitro synthesis of the Lewis[a] determinant on carbohydrates and glycoproteins.

Keywords: α1,3/4-Fucosyltransferase; Lewis[a]; BHK cells; Targeting; Golgi

1. Introduction

In inflammatory processes blood cells interact with endothelial cells via selectins. The recognition mechanism has its molecular basis on the Lewis structures and their sialylated and sulfated derivatives. Adhesion mechanisms occurring during metastasis formation are also mediated by the sialyl-Lewis[x] (sLe[x]) and by the sialyl-Lewis[a] (sLe[a]) determinants (reviewed by Lowe, 1994). Molecules containing the sLe[x] and sLe[a] structures can be efficiently used for the inhibition of cellular interactions and, consequently, for the treatment of inflammatory diseases and metastasis.

Fucosyltransferases are key enzymes for the synthesis of these structures both in vitro and in vivo. Fucosyltransferase III has been reported to catalyze the formation of the Le[a], Le[x], and their sialylated derivatives (Lowe et al., 1990). In the present work, we describe the enzymatic synthesis of molecules containing the Le[a] and sLe[a] by using a soluble recombinant form of Fuc-TIII purified from animal, baby hamster kidney (BHK), cells. The use of recombinant glycosyltransferaes with well-known activities and specificities is extremely valuable for the in vitro synthesis of oligosaccharides. This alternative to the traditional synthetic methods of organic chemistry has several advantages such as the increase of yields and decrease of lateral reactions.

2. Materials and methods

2.1. Construction of Fuc-TIII variants

Mutants FT3T2 and S2FT3T2 of the human Fuc-TIII (Fig. 1) were generated by PCR-based site-directed-mutagenesis of a cDNA corresponding to the full length form of the enzyme (Fig. 2). BHK-21 cells were transfected with pCR3 vectors containing the S2FT3T2 and FT3T2 coding sequences by the calcium phosphate precipitation method (Costa et al., 1997).

2.2. Fucosyltransferase activity

The fucosyltransferase activity was determined at 37°C using 20–60 μunits of the enzyme in 50 mM Mops–NaOH buffer pH 7.5 containing 20 mM $MnCl_2$, 0.1 M NaCl, 4 mM ATP, 0.1 mM GDP-[^{14}C]-fucose. 3′-Sialyl-*N*-acetylactosamine (SLN), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), lacto-*N*-fucopentaose (LNFP) and LS-tetrasacchar-

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^{1}MDPLGAAKP^{10}QWPWRRCLAA^{20}LLFQLLVAVC^{30}FFSYLRVSRD
^{40}DATGSPRAPS^{50}GSSRQDTTPT^{60}RPTLLILLWT^{70}WPFHIPVALS
^{80}RCSEMVPGTA^{90}DCHITADRKV^{100}YPQADTVIVH^{110}HWDIMSNPKS
^{120}RLPPSPRPQG^{130}QRWIWFNLEP^{140}PPNCQHLEAL^{150}DRYFNLTMSY
^{160}RSDSDIFTPY^{170}GWLEPWSGQP^{180}AHPPLNLSAK^{190}TELVAWAVSN
^{200}WKPDSARVRY^{210}YQSLQAHLKV^{220}DVYGRSHKPL^{230}PKGTMMETLS
^{240}RYKFYLAFEN^{250}SLHPDYITEK^{260}LWRNALEAWA^{270}VPVVLGPSRS
^{280}NYERFLPPDA^{290}FIHVDDFQSP^{300}KDLARYLQEL^{310}DKDHARYLSY
^{320}FRWRETLRPR^{330}SFSWALDFCK^{340}ACWKLQQESR^{350}YQTVRSIAAW
^{360}FT

Fig. 1. Amino acid sequence of Fuc-TIII. Cytoplasmic domain, Met-1 to Arg-15; transmembrane domain, Cys-16 to Leu-34 (underlined); stem region, Arg-35 to Pro-61; catalytic domain, Thr-62 to Thr-361. Asn-154 and Asn-185 are occupied N-glycosylation sites. (Adapted from Kukowska-Latallo et al., 1990)

ide[a] (LST-a) were used at a 0.33 mM concentration in the presence of 0.42 mM GDP-Fuc.

2.3. Methylation analysis of carbohydrates

For methylation analysis, oligosaccharides were permethylated according to Hakomori (1964), purified on a Sephadex LH20 column, hydrolyzed, reduced, and peracetylated as described (Nimtz et al., 1990). Separation and identification of partially methylated alditol acetates were performed on a Finnigan gas chromatograph, equipped with a 30 m DB5 capillary column, connected to a Finnigan GCQ ion trap mass spectrometer.

2.4. Analytical HPAE-PAD of native and desialylated oligosaccharides

A Dionex BioLC System (Dionex, Sunnyvale, CA, USA) equipped with a CarboPac Pal column (4 × 250 mm) was used in combination with a pulsed amperometric detector following the methodology described by Nimtz et al. (1993).

2.5. Matrix assisted laser desorption–ionization time of flight mass spectrometry (MALDI/TOF-MS)

2,5-Dihydroxybensoic acid was used as UV-absorbing matrix 10 mg ml^{-1} 2,5-dihydroxybenzoic acid were dissolved in 10% ethanol in water. For analysis by MALDI/TOF-MS the solutions of the native or reduced and permethylated oligosaccharides were mixed with the same volume of matrix. One microlitre of the sample was spotted onto a stainless steel tip and dried at room temperature. The concentration of the analyte mixtures were approximately 10 pmol μl^{-1}. Measurements were performed on a Bruker REFLEX™ MLDI/TOF mass spectrometer using a N_2 laser (337 nm) with 3 ns pulse width and 107 to 108 W cm^{-2} irradiance at the surface (0.2 mm^2 spot) Spectra were recorded at an acceleration voltage of 20 kV using the electron for enhanced resolution.

2.6. Indirect Immunofluorescence

S2FT3T2 and FT3T2 were detected essentially according to Munro (1991).

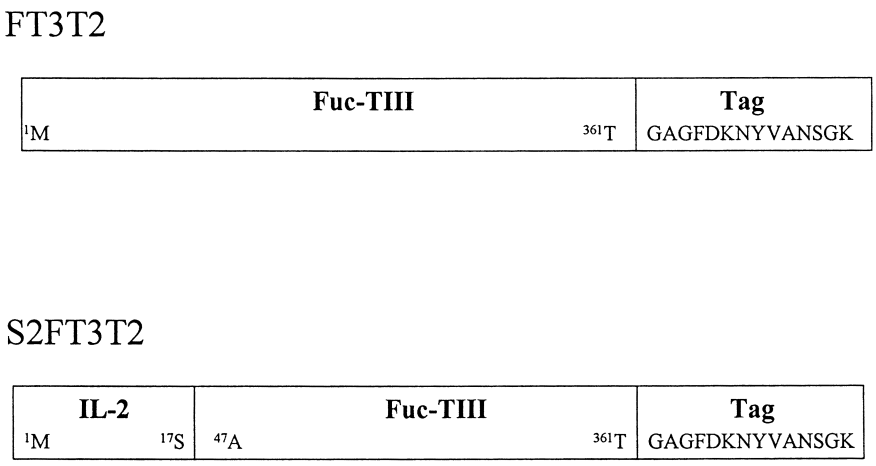

Fig. 2. Solubilization of Fuc-TIII. FT3T2 is derived from Fuc-TIII by addition of a tag at the C-terminus of the enzyme. S2FT3T2 was solubilized from Fuc-TIII by the addition of the signal sequence of interleukin-2 to the N-terminal Ala-47 from Fuc-TIII. The same tag was added at the C-terminus of the enzyme. The site directed metagenesis was performed by PCR and the mutants obtained were cloned into the pCR3 vector.

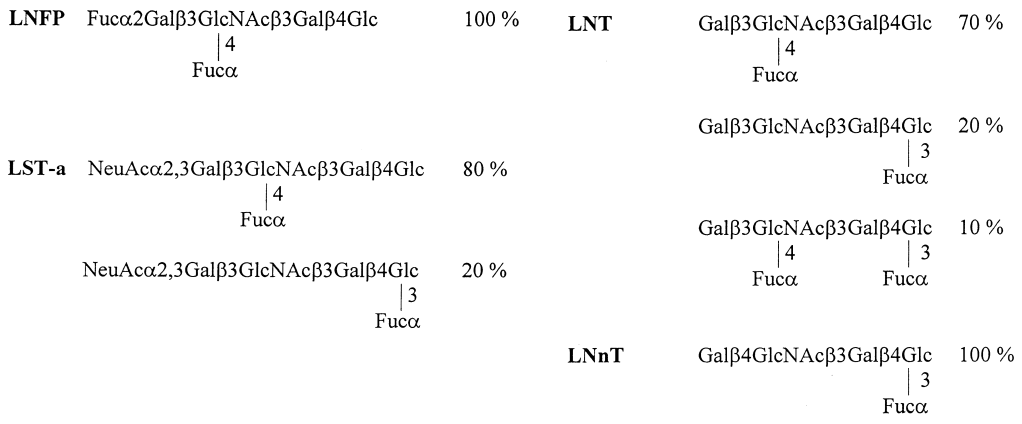

Fig. 3. Fucosylation of the acceptors LNFP, LST-a, LNT and LNnT by S2FT3T2. The reaction products and their proportions were determined by MALDI/TOF-MS, ESI/MS-MS and methylation analysis.

3. Results and discussion

BHK cells were transfected with plasmides encoding the Golgi form of Fuc-TIII with a tag at their carboxyl terminus, FT3T2 and S2FT3T2, respectively (Fig. 2). After transfection, cells were selected with 0.8 μg ml^{-1} puromycin hydrochloride. A high producing clone of S2FT3T2 was obtained. The S2FT3T2 was localized intracellularly by immunofluorescence by using an antibody produced against the catalytic domain of Fuc-TIII, and a high staining level was obtained in accordance with the high rate of enzyme production. Preliminary results suggest that the enzyme is detected in the endoplasmic reticulum and Golgi apparatus.

For the purification of S2FT3T2, confluent recombinant monolayers were grown for 4 weeks in DMEM containing 2% fetal calf serum, the medium being harvested every 2 or 3 days. The enzyme was purified from 1.61 of culture supernatant by affinity chromatography on a GDP-Fractogel column. The purified S2FT3T2, with an activity of 33 units l^{-1} using the type I methoxycarbonyloctyl glycoside as a substrate, was used for the fucosylation of small oligosaccharides and glycoproteins.

The specificity of the S2FT3T2 towards the small oligosaccharide acceptors LNFP, LNT, LNnT, LST-a and SLN was analysed after incubations of 2, 4 and 21 h. The formation of the fucosylated products was monitored by HPAEC-PAD and the molecular masses of the products were determined by MALDI/TOF-MS. In order to identify the linkage position of fucose residues, after reduction and permethylation the oligosaccharides were analysed by collision induced decomposition mass spectrometry (CID) and by methylation analysis (Fig. 3). For LNT, it was found that S2FT3T2 transferred Fuc to either the O-4 position of GlcNAc (70%), to the O-3 position of Glc (20%) or to both positions (10%). In LNnT all the Fuc was found to be attached to the O-3 position of Glc. With LNFP Fuc was transferred to the O-4 position of GlcNAc. Eighty per cent of the product obtained with LST-a contained Fuc at the O-4 position of GlcNAc and 20% was modified at the O-3 position of Glc. These results are in agreement with those

Table 1
Activity of S2FT3T2 vs soluble form of fucosyltransferase VI (SFT6) with glycoproteins as acceptors

Glycoprotein	SFT6	S2FT3T2
	[^{14}C]-fucose incorporation (nmol/nmol glycoprotein)	
Antithrombin III (CHO cells)[a]	4	0.005
II-4 receptor (CHO cells)[b]	n.d.	0.005
Erythropoietin (BHK cells)[c]	6	$<$ 0.001
β-Trace protein (BHK cells)[d]	0.3	$<$ 0.001
IL-2 mutant 41 (BHK cells)[e]	0.25	n.d.
Fetuin (bovine)[f]	0.02	n.d.
Asialofetuin (bovine)	0.02	0.03
Thyroglobulin (bovine)	n.d.	$<$ 0.001
β-Trace protein (human)	n.d.	0.1

Major terminal structures of N-linked oligosaccharides.
[a]Four sites — bi-tri- and tetraantennary, terminal α2,3-NeuAc;
[b]Six sites — di-tri- and tetraantennary; terminal α2,3-NeuAc;
[c]Three sites — tetraantennary with one or two repeats; terminal α2,3-NeuAc;
[d]Two sites — diantennary; terminal α2,3-NeuAc;
[e]Di-, tri- and tetraantennary; terminal α2,3-NeuAc;
[f]Three sites — di- and triantennary.

described for enzyme preparations purified from human milk and the medium of A431 cells (Johnson et al., 1992, 1993). No fucosylation was detected in the trisaccharide SLN when incubated under identical conditions.

The glycoproteins bovine asialofetuin, native fetuin and bovine thyroglobulin, human β-trace protein, recombinant human antithrombin III and recombinant human IL-4 receptor from CHO cells, were tested as acceptors for S2FT3T2 by determination of [^{14}C]-fucose incorporation (Table 1). Fucosylated glycoproteins were subjected to SDS-PAGE and following subsequent autoradiography radiolabelled bands were detected at migration positions corresponding to the molecular masses of the untreated glycoproteins. Only very small incorporation of fucose was achieved with S2FT3T2 for the glycoproteins tested. No fucosylation of bovine fetuin or IL-2 mutant 41 from BHK cells were detected. However, we have observed that among the glycoprotein acceptors studied, asialofetuin is the best substrate for S2FT3T2. The oligosaccharides from asialofetuin were characterized in detail in order to determine to which position Fuc was transferred. Three major oligosaccharide peaks were obtained by HPAEC-PAD for the glycan mixture from unmodified asialofetuin: biantenary, triantennary 2,4-branched, and triantennary 2,4-branched with one Galβl-3GlCNAc antenna, in a ratio of 10:55:35. The glycan mixture from in vitro fucosylated asialofetuin produced a new peak at the expenses of triantennary 2,4-branched with one Galβl-3GlcNAc antenna. Further, methylation analysis and MALDI/TOF-MS results indicated that the N-linked oligosaccharide of asialofetuin with one type **I** antenna is modified by S2FT3T2 with fucose linked to position 4 of GlcNAc in the type **I** motif.

The same glycoprotein acceptors were incubated in similar conditions with a soluble form of fucosyltransferase VI which is known to transfer Fuc onto sialylated and non-sialylated type II acceptors with the formation of Le^x and sLe^x structures. The recombinant proteins from BHK or CHO cells were in general much better acceptors for SFT6 that for S2FT3T2 (Table 1). This is due to the fact that terminal glycosylation of the CHO and BHK cells consists mostly of type II determinants which are good substrates for Fuc-TVI but not for Fuc-TIII.

From the results obtained we concluded that S2FT3T2 preferentially synthezises Le^a structures in oligosaccharides and glycoproteins. This enzyme constitutes a useful tool for the synthesis in vitro of molecules containing oligosaccharide structures with the Le^a determinants.

We have also constructed stable BHK cell lines expressing the Golgi FT3T2. Preliminary results on immunofluorescence microscopy indicated that the FT3T2 was detected in the Golgi apparatus. These new geneticaly engineered cells can be used for the production of natural glycoproteins or glycolipids with peripheral focose. Since the animal cells most widely used for glycoprotein production, CHO and BHK, do not have the capability to form the Lewis structures since they lack the required active fucosyltransferases, these newly constructed cells provide a valuable tool for the in vivo synthesis of natural therapeutical glycoproteins with the Lewis determinants.

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References

Costa, J., Grabenhorst, E., Nimtz, M., & Conradt, H.S. (1997). J. Biol. Chem., *272*, 11613–11621.

Hakomori, S. (1964). J. Biochem., *55*, 205–207.

Johnson, P.H., Donald, A.S.R., Feeney, J., & Watkins, W.M. (1992). Glycoconjugate J., *9*, 251–264.

Johnson, P.H., Donald, A.S.R., & Watkins, W.M. (1993). Glycoconjugate J., *10*, 152–164.

Kukowska-Latallo, J.F., Larsen, R.D., Nair, R.P., & Lowe, J.B. (1990). Genes Dev., *4*, 1288–1303.

Lowe, J.B., Stoolman, L.M., Nair, R.P., Larsen, R.D., Berhend, T.L., & Marks, R.M. (1990). Cell, *63*, 457–484.

Lowe, J.B. (1994). In M. Fukuda & O. Hindsgaul (Eds.), *Molecular glycobiology* (pp. 163–205). New York: IRL Press.

Munro, S. (1991). EMBO J., *10*, 3577–3588.

Nimtz, M., Noll, G., Paques, E.-P., & Conradt, H.S. (1990). FEBS Lett., *271*, 14–18.

Nimtz, M., Martin, W., Wray, V., Klöppel, K.-D., Augustin, J., & Conradt, H.S. (1993). Eur. J. Biochem., *213*, 39–56.