Available online at www.sciencedirect.com
ScienceDirect
Biochimie 90 (2008) 1279–1290
BIOCHIMIE
www.elsevier.com/locate/biochi

Research paper

Human fucosyltransferase IX: Specificity towards *N*-linked glycoproteins and relevance of the cytoplasmic domain in intra-Golgi localization

C. Brito [a], S. Kandzia [b], T. Graça [b], H.S. Conradt [b], J. Costa [a,c,*]

[a] *Instituto de Tecnologia Química e Biológica, Avenida da República, 2780-157 Oeiras, Portugal*
[b] *GlycoThera GmbH, Inhoffenstrasse 7, D-38124 Braunschweig, Germany*
[c] *Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal*

Received 7 December 2007; accepted 5 March 2008
Available online 19 March 2008

Abstract

The α3-fucosyltransferase IX (FUT9) catalyses the transfer of fucose in an α3 linkage onto terminal type II (Galβ4GlcNAc) acceptors, the final step in the biosynthesis of the Lewis^x (Le^x) epitope, in neurons. In this work, FUT9 cloned from NT2N neurons and overexpressed in HeLa cells (FUT9wt), was found to efficiently fucosylate asialoerythropoietin (asialoEPO), and bovine asialofetuin, but not sialylated EPO. Analysis by HPAEC-PAD and MALDI/TOF-MS revealed predominantly mono-fucosylation by FUT9wt of type II di-, tri- and tetraantennary *N*-glycans with proximal fucose, with and without *N*-acetylactosamine repeats from asialoEPO. Minor amounts of difucosylated structures were also found. The results suggested that FUT9 could fucosylate Le^x carrier-glycoproteins in neurons. Furthermore, FUT9wt was found to be activated by Mn^{2+} and it was capable of synthesizing Le^a, although to a lesser extent than Le^x and Le^y. *In vivo*, HeLa cells transfected with FUT9wt expressed *de novo* Le^x, as detected by immunofluorescence microscopy. FUT9 was found to be a *trans*-Golgi and *trans*-Golgi network (TGN) glycosyltransferase from confocal immunofluorescence co-localization with the markers of the secretory pathway β4-galactosyltransferase (*trans*-Golgi and TGN) and TGN-46 (TGN). Deletion of the cytoplasmic domain caused a shift to the *cis*-Golgi, thus suggesting that information for intra-Golgi localization is contained within the cytoplasmic domain.

Keywords: Fucosyltransferase IX; Lewis^x; *N*-Glycans; Erythropoietin; Golgi localization

1. Introduction

Glycosyltransferases (GTs) mediate the transfer of monosaccharide residues from nucleotide sugar donors to glycoconjugates along their transport in the secretory pathway. α3-Fucosyltransferases (α3-FUTs) catalyse the transfer of fucose in an α3 linkage, onto terminal Galβ4GlcNAc motifs in the oligosaccharide chains, the final step in the biosynthesis of the Lewis^x (Le^x) epitope. FUT3, FUT4, FUT5, FUT6, FUT7 and FUT9 constitute the human α3/4-FUT family (reviewed in [1]), presenting the type II membrane topology characteristics of Golgi resident GTs: a short N-terminal cytosolic tail, a single transmembrane domain, a flexible region adjacent to the transmembrane domain called stem and a large C-terminal globular catalytic domain, facing the lumen of the Golgi [2]. FUT10 and FUT11 are possible α3/4-FUTs based on their homology with

Abbreviations: AF, asialofetuin; AP-1, adaptor protein 1; BFA, brefeldin A; CNS, central nervous system; dHex, deoxyhexose; EPO, erythropoietin; FUT9, fucosyltransferase IX; FUT, fucosyltransferase; GT, glycosyltransferase; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPAEC-PAD, high pH anion exchange chromatography with pulsed amperometric detection; LacNAc, *N*-acetylactosamine; Le^a, Lewis^a; Le^x, Lewis^x; Le^y, Lewis^y; MALDI/TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MTOC, microtubule organizing centre; sLacNAc, sialyl-*N*-acetylactosamine; sLe^x, sialyl-Lewis^x; TFA, trifluoroacetic acid; TGN, *trans*-Golgi network; TX-100, Triton X-100.

* Corresponding author. Laboratory of Glycobiology, Instituto de Tecnologia Química e Biológica, Avenida da República, Apartado 127, 2781-901 Oeiras, Portugal. Tel.: +351 214 469 437; fax: +351 214 411 277.
E-mail address: jcosta@itqb.unl.pt (J. Costa).

0300-9084/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2008.03.002

other FUTs [3], however their activity has not been validated [4]. The α3/4-FUT family members share four conserved peptide motifs in their catalytic domains: the α3-FUT motifs I and II [5]; motif III, required for folding and catalytic activity [6,7] and the acceptor binding motif, involved in enzyme specificity [8].

FUT9 is the most recently characterized member of the α3/4-FUT family [9]. Its sequence presents the lowest homology with the other FUTs and is the only one highly conserved among species [9–11], which indicates that it has been under strong selective pressure during evolution, suggesting that it is responsible for an essential biological activity. FUT9 exhibits a unique site-specificity, generating Le^x determinants more efficiently than all the other α3-FUTs [12]. It is also able to synthesize the $Lewis^y$ (Le^y) epitope but not $sialyl\text{-}Lewis^x$ (sLe^x); poor or no activity has been found with type I-based acceptors [13,14].

FUT9 is mainly expressed in the central nervous system (CNS), in both developing and mature brain of human, rat and mouse [9,10,13]. The $FUT9^{-/-}$ mouse showed disappearance of Le^x in the brain, concomitant to increased anxiety-like behaviours [15]. In human neurons in culture FUT9 is the α3-FUT responsible for the synthesis of Le^x [16]. Thus, it is probable that FUT9 also synthesizes Le^x in human CNS similarly to that found in the mouse. Furthermore, the abundant neutral complex-type *N*-glycans with terminal Le^x on one arm detected in human brain [17] may be products of FUT9 action.

The structure of the oligosaccharides present on glycoconjugates depends on the sequential action of Golgi resident glycosidases and GTs. The ordered, although overlapping, localization of GTs in the Golgi apparatus is a key factor in the *in vivo* regulation of the glycosylation profile of the nascent glycoconjugate. Several studies have implicated the transmembrane domain and flanking regions of GTs in Golgi localization (reviewed in [18,19]). The transmembrane domain of GTs, e.g., β4-galactosyltransferase [20] or FUT3 [21], has been shown to be required for localization in the Golgi apparatus. On the other hand, the cytosolic domain has been shown to play a role in intra-Golgi localization of α2-FUT [22], FUT3 [21], sialyltransferase 2 and *N*-acetylgalactosaminyltransferase [23]. Particularly, a serine residue from the cytoplasmic domain has been shown to be important for α2-FUT functional localization [22].

In the present work, FUT9 was found to efficiently fucosylate asialoglycoproteins, namely, human recombinant asialoerythropoietin, with synthesis of peripheral monofucosylated di-, tri- and tetraantennary *N*-glycans with proximal fucose, with and without *N*-acetylactosamine (LacNAc) repeats, as revealed by HPAEC-PAD and MALDI/TOF-MS analysis. Furthermore, FUT9wt localized in the *trans*-Golgi and *trans*-Golgi network (TGN) of HeLa cells and the cytoplasmic tail played an important role in the regulation of intra-Golgi localization of the enzyme.

2. Materials and methods

2.1. Cell culture

NTERA-2/cl.D1 cells were cultured and differentiated into NT2N neurons essentially as described previously [16,24]. NT2N neurons were cultured in Dulbecco's modified Eagle's medium-High Glucose (Gibco) with 5% foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, and 10 μM uridine, on surfaces coated with 10 μg/ml poly-D-lysine (Sigma) and 0.26 mg/ml Matrigel (BD Biosciences). HeLa cells were grown in DMEM-HG with 10% FBS (Gibco) and P/S. All cells were grown in a 5% CO_2 incubator, at 37 °C.

2.2. Cloning of human FUT9 from NT2N neurons

Total RNA was extracted from 5×10^6 NT2N neurons using the RNeasy extraction kit (Qiagen), according to the manufacturer's protocol. Two micrograms of total RNA were used in reverse transcription with the primer 5′-TTA ATT CCA AAA CCA TTT CTC TA-3′ (FUT9-rev): after 5 min at 65 °C, the reaction was performed at 37 °C, for 50 min, using 1 unit of Moloney murine leukaemia virus reverse transcriptase (Invitrogen), and terminated by heating at 70 °C, for 15 min. The resulting cDNA was used to amplify two fragments of human FUT9 by polymerase chain reaction (PCR). The 25 μl reaction mixtures contained 1 μl of cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM primers and 2.5 U Taq DNA polymerase (Invitrogen). As primer pairs 5′-ATG ACA TCA ACA TCC AAA GGA ATT C-3′ (FUT9-fwd) with 5′-GCT TTT CCG TGA TGT AAT CCT TGT G-3′ (FUT9α3-rev) and 5′-GAT TCA GAT ATC CAA GTG CCT TAT G-3′ (FUT9α3-fwd) with FUT9-rev were used. PCR reactions were carried out in the following conditions: initial denaturation at 94 °C for 5 min; 25 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s and elongation at 68 °C for 1 min; final elongation at 68 °C for 7 min. The PCR products were cloned into the mammalian expression vector pcDNA3.1/V5-HisTOPO (Invitrogen). A mixture of the two plasmids was used as template in a second PCR reaction where the complete human FUT9 coding sequence was amplified. The primers used were FUT9-fwd and FUT9-rev with a mixture of Taq DNA polymerase (Invitrogen) and Pfu Turbo DNA polymerase (Stratagene) as described above. The amplified fragment (FUT9wt) was cloned in the pcDNA3.1/V5-HisTOPO vector (Invitrogen) and in the PCRScriptCam vector (Stratagene). Automatic DNA sequencing confirmed FUT9 sequence [9].

2.3. Plasmid construction

The mutants FUT9dcyt, FUT9d6, FUT9S/A and FUT9wt with a V5 and His-tag were produced by PCR-based site directed mutagenesis using the PCRScriptCam-FUT9 vector as template. The V5 epitope was added at the C-terminus of FUT9 for detection purposes.

All V5-tagged mutants were constructed using the reverse primer FUT9V5-rev (5′-ATT CCA AAA CCA TTT CTC TA-3′). The forward primers were FUT9dcyt-fwd (5′-ATG CGC CCA TTT TT-3′), FUT9d6-fwd (5′-ATG AAA GGA ATT CTT-3′) and FUT9S/A-fwd (5′-ATG ACA gca ACA gcc AAA GGA A-3′).