Reinl et al. BMC Proceedings 2013, 7(Suppl 6):P84
http://www.biomedcentral.com/1753-6561/7/S6/P84

BMC Proceedings

POSTER PRESENTATION Open Access

Golgi engineering of CHO cells by targeted integration of glycosyltransferases leads to the expression of novel Asn-linked oligosaccharide structures at secretory glycoproteins

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From 23rd European Society for Animal Cell Technology (ESACT) Meeting: Better Cells for Better Health
Lille, France. 23-26 June 2013

Background and novelty

N-glycans constitute an important information carrier in protein-driven signaling networks. Amongst others, N-glycans contribute to protein folding quality, adjust protein turnover and operate as address label for targeting proteins to specific cells and tissues [1]. Hence, the composition of N-glycans attached to recombinant glycoprotein therapeutics is vital for in-vivo therapeutic efficacy and strongly depends on the choice of the expression host [2,3]. Due to absence or silencing of glycosyltransferase genes homologue to human enzymes, biotechnologically used cell lines are limited by their intrinsic glycosylation machinery and produce host specific glycoforms.

Cetuximab, a therapeutic chimeric mouse/human monoclonal antibody (IgG1), is N-glycosylated both at the CH2-domain (Asn299) and at the VH-domain (Asn88) (Figure 1A). Sold under the trade name Erbitux®, Cetuximab is expressed from a murine myeloma cell line and targets the human EGF receptor [4], which is overexpressed in about 1/3 of all human cancers. The antibody is highly decorated with the αGal-epitope (Galα1-3Galβ1-4GlcNAc) which has been shown to result in fatal allergic/hypersensitivity response in several patients [5].

The design of new quality-optimized and functionally improved biopharmaceuticals with properties conferred by host cell unrelated N-glycans requires a rational Golgi engineering strategy. Here, we apply GET, a system that enables the positioning of a desired catalytic glycosyltransferase activity into a favorable localization within the intracellular glycosylation machinery, to suspension CHO cells developed to secrete suitable amounts (200 μg/ml) of Cetuximab as a model glycoprotein. The presented Golgi engineering project aims in the extension of the intrinsic glycosylation repertoire enabling CHO cells to produce new human-type glycosylation motifs as indicated in Figure 1A: (i) GalNAcβ1,4GlcNAc-R (LacdiNAc, LDN),(ii) GlcNAc in β1,4 linkage to central mannose residue (bisecting GlcNAc, bGN), (iii) Galβ1,4(Fucα1,3)GlcNAc-R (Lewis^X, Le^X) and (iv) NeuAcα2,3Galβ1,4 (Fucα1,3)GlcNAc-R (Sialyl-LewisX, sLe^X). To assemble (ii) and (iv), we co-express GnT3 and FT7. As shown earlier, the latter enzyme catalyzes fucosylation exclusively of (iv). Therefore, we included in our study a variant of FT6 that is targeted to the early Golgi compartment with the aim to additionally generate structure (iii) [6,7]. The uncommon LDN motif (i) which is e.g. detected on lutropin is assembled by human B4GalNT3 [8,9]. We analyze oligosaccharides released from the products of genetically engineered CHO cells based on the resolution of single glycosylation sites of VH- and CH2- glycopeptides by quantitative NP-HPLC-FLD and use our comprehensive oligosaccharide standard library to identify novel oligosaccharide motifs.

Experimental approach

Cloning of human glycosyltransferases and engineering of $_{VAR}$FT6 [7] as well as construction of pGET expression plasmids encoding either the heavy and light chain of Cetuximab or the glycosyltransferase cDNAs was done acc. to standard DNA technologies. A stable clone with Cetuximab titers of 200 μg/ml and doubling times

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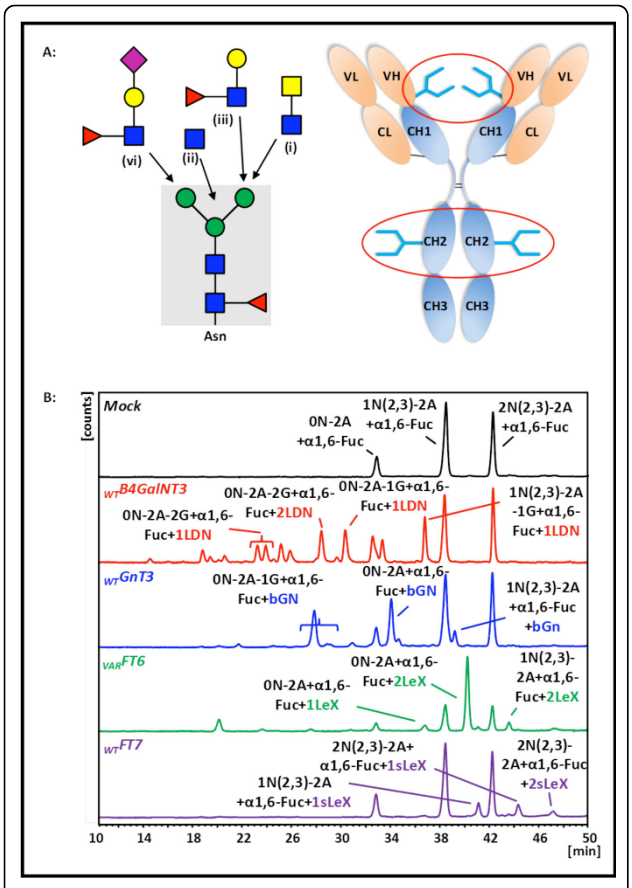

Figure 1 (A) Non-reducing terminal oligosaccharide motifs attached to N-glycans of specific human glycoproteins (left side). Scheme of model glycoprotein Cetuximab with CH2- and VH-domain N-glycans (right side). **(B)** NP-HPLC-FLD elution profiles of 2-AB labeled oligosaccharides from VH-domain of Cetuximab after co-expression of the indicated glycosyltransferases.

of 25 hours was selected after transfection of pGET-Cetuximab in CHO cells. This clone was either mock- or co-transfected with pGET plasmids encoding the indicated glycosyltransferases. After shake flask subcultivation for 72 h Cetuximab was purified from supernatants, digested and applied to RP-HPLC peptide mapping. CH2- and VH-domain glycopeptides were separated and oligosaccharides were enzymatically released. After 2-AB labeling, the isolated oligosaccharides were subjected to quantitative NP-HPLC-FLD and ESI-TOF-MS and MS/MS analysis. Oligosaccharide structures were unambiguously identified in comparison to GlycoThera's reference standard oligosaccharide library.

Results and discussion

n combination with our site specific and quantitative micro glycan structure analysis we provide a modular system (GET) for the customized assembly of novel CHO unrelated oligosaccharide motifs. As exemplified for VH-domain, the NP-HPLC-FLD elution profiles of 2-AB labeled oligosaccharides after heterologous co-expression of Cetuximab and the indicated glycosyltransferases are shown in Figure 1B. Quantitative results of all oligosaccharide structures are given in Figure 2. The Mock-transfected control approach reveals the intrinsic glycosylation repertoire of our stable CHO cell clone. Cetuximab is decorated with agalactosylated (35,5%), mono- (50,0%) and di-galactosylated (10,1%) diantennary complex-type N-glycans containing proximal α1,6-linked fucose at the CH2-domain. VH-domain N-glycans consist of neutral (13,8%), mono- (50,3%) and di-sialylated (35,8%) oligosaccharide structures. Whereas N-glycans from the market product Erbitux® produced in SP2/0 cells are extensively decorated with Galα1,3Gal and NeuGc (data not shown), those allergenic structures are not detected in Cetuximab N-glycans from our CHO cell clone.

The heterologous co-expression of wildtype B4GalNT3, GnT3 and FT7 and genetically modified FT6 results in the formation of the uncommon LacdiNAc motif (ca. 40%), the Lewis^X and di-Lewis^X structures (ca. 50%) and Sialyl-Lewis^X (ca. 15%) almost exclusively on oligosaccharides from the VH-domain. Relevant modification of both VH-domain (ca. 40%) and CH2-domain glycans (ca. 30%) is only achieved by GnT3 catalyzed attachment of bisecting GlcNAc. In addition, glycosyltransferase co-expression leads to charge state reduction of oligosaccharides by depletion of suitable acceptors for endogenous sialyltransferases. The strongest reduction in the content of neuraminic acid at VH-domain was observed by co-expression of $_{VAR}$FT6 (ca. 55% reduction) and $_{WT}$B4GalNT3 (ca. 50% reduction).

As a conclusion, Golgi engineering endows CHO cells to assemble significant amounts of LacdiNAc, bisecting GlcNAc, Lewis^X and Sialyl-Lewis^X to Cetuximab N-glycans (Figure 1B and Figure 2). Therefore, our glycosylation engineering strategy provides a tool to produce tailored N-glycosylation variants with defined structural motifs. As demonstrated, the tailored addition of bisecting GlcNAc to CH2-domain N-glycans increases ADCC of an αCD20 therapeutic mAB [10]. We therefore assume that the presented structural motifs exhibit novel therapeutic properties (ADCC, CDC, tissue specificity, serum half-life). Our strategy represents a relevant basis for the development of biotherapeutics and biobetters with potentially improved pharmacokinetics, pharmacodynamics, safety properties and in vivo therapeutic efficacy.

CHO cell line	Mock		$_{WT}$B4GalNT3		$_{WT}$GnT3		$_{VAR}$FT6		$_{WT}$FT7	
Oligosaccharide structure	CH2	VH	CH2	VH	CH2	VH	CH2	VH	CH2	VH
0N-2A-2G+α1,6-Fuc	35,5	/	41,0	2,8	24,1	/	28,2	/	34,1	/
0N-2A-2G-α1,6-Fuc+1β1,4-GalNAc (1LDN)	/	/	/	2,8	/	/	/	/	/	/
$Man_5GlcNAc_2$	/	/	1,1	/	/	/	12,4	8,8	/	/
0N-2A-2G+α1,6-Fuc+1β1,4-GlcNAc (bGN)	/	/	/	/	9,8	1,0	/	/	/	/
0N-2A-2G+α1,6-Fuc+1β1,4-GalNAc (1LDN)	/	/	1,7	9,1	/	/	/	/	/	/
0N-2A-1G+α1,6-Fuc	50,0	/	41,7	8,2	33,1	/	34,4	/	49,9	/
0N-2A-1G+α1,6-Fuc+1β1,4-GlcNAc (bGN)	/	/	/	/	19,6	16,7	/	/	/	/
0N-2A-2G+α1,6-Fuc+2β1,4-GalNAc (2LDN)	/	/	/	8,8	/	/	/	/	/	/
0N-2A-1G+α1,6-Fuc+1β1,4-GalNAc (1LDN)	/	/	/	10,1	/	/	/	/	/	/
0N-2A-1G+α1,6-Fuc+1α1,3-Fuc (1Le^X)	/	/	/	/	/	/	4,4	/	/	/
0N-2A+α1,6-Fuc	10,1	13,8	8,0	8,7	8,1	6,9	6,4	4,9	10,6	12,7
1N(2,3)-2A-1G+α1,6-Fuc	/	/	/	5,6	/	/	/	/	/	/
0N-2A+α1,6-Fuc+1β1,4-GlcNAc (1bGN)	/	/	/	/	1,2	15,9	/	/	/	/
1N(2,3)-2A-1G+α1,6-Fuc+1β1,4-GlcNAc (1bGN)	/	/	/	/	/	2,1	/	/	/	/
0N-2A+α1,6-Fuc+1α1,3-Fuc (1Le^X)	/	/	/	/	/	/	2,2	4,3	/	1,1
1N(2,3)-2A-1G+α1,6-Fuc+1β1,4-GalNAc (1LDN)	/	/	/	9,7	/	/	/	/	/	/
1N(2,3)-2A+α1,6-Fuc	/	50,3	/	18,4	/	28,7	/	17,2	/	42,1
1N(2,3)-2A+α1,6-Fuc+1β1,4-GlcNAc (1bGN)	/	/	/	/	/	4,7	/	/	/	/
0N-2A+α1,6-Fuc+2α1,3-Fuc (2Le^X)	/	/	/	/	/	/	0,9	40,9	/	/
1N(2,3)-2A+α1,6-Fuc+1α1,3-Fuc (1sLe^X)	/	/	/	/	/	/	/	1,6	/	5,7
2N(2,3)-2A+α1,6-Fuc	/	35,8	/	14,9	/	22,5	/	13,2	/	29,7
1N(2,3)-2A+α1,6-Fuc+2α1,3-Fuc (1Le^X/1sLe^X)	/	/	/	/	/	/	/	4,3	/	/
2N(2,3)-2A+α1,6-Fuc+1α1,3-Fuc (1sLe^X)	/	/	/	/	/	/	/	/	/	5,9
2N(2,3)-2A+α1,6-Fuc+2α1,3-Fuc (2sLe^X)	/	/	/	/	/	/	/	1,9	/	2,8

Figure 2 Amount of oligosaccharide structures detected on CH2- and VH-domain of Cetuximab after heterologous glycosyltransferase co-expression (given in% peak area values after integration of NP-HPLC-FLD chromatograms)

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Published: 4 December 2013

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doi:10.1186/1753-6561-7-S6-P84
Cite this article as: Reinl *et al.*: Golgi engineering of CHO cells by targeted integration of glycosyltransferases leads to the expression of novel Asn-linked oligosaccharide structures at secretory glycoproteins. *BMC Proceedings* 2013 **7**(Suppl 6):P84.