Recombinant Mucins with Tailored Glycosylation as Bacterial Toxin Inhibitors

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Happy is the man who finds wisdom and

the man who gains understanding

Proverbs 3:13

To my Parents, P.M Cherian and Alice Cherian

who have always put me above their own wants and needs

&

the reason of what I become today!

ABSTRACT

Multivalent carbohydrate-based ligands that can inhibit biomedically important protein–carbohydrate interactions have therapeutic potential. One of the important targets for therapeutic intervention is the binding processes mediated through the interactions of bacterial toxins with cell-surface receptors. Inhibition of these interactions has the potential to prevent the toxins from reaching their site of action, and thus, averting the subsequent toxin effects. Even though, multivalent inhibitors that engage in multiple weak interactions can enhance the overall binding interaction, it has been observed that tailoring of specific ligands based on the functional carbohydrate receptor can greatly improve the binding strength of inhibitors.

In this thesis, we have engineered the CHO cell line to produce the recombinant mucin-type fusion protein with tailored glycosylation by expressing P-selectin glycoprotein ligand-1/mouse immunoglobulin G2b (PSGL-1/mIgG2b) together with glycosyltransferases that are known to mediate the biosynthesis of specific carbohydrate determinants. PSGL-1/mIgG2b, which we have proposed as a versatile inhibitor of protein–carbohydrate interactions, consist of the extracellular part of P-selectin glycoprotein ligand-1(PSGL-1) fused to the Fc part of mouse IgG2b. The high density expression of *O*-linked glycans in the mucin part of PSGL-1/mIgG2b provides the scaffold for multivalent display of bioactive carbohydrate determinants, making it suitable as an inhibitor of carbohydrate-binding bacterial toxins, microbial adhesins, viral surface proteins, and antibodies.

In paper I and IV, genetically engineered CHO cells were used to produce PSGL-1/mIgG2b carrying the functional carbohydrate receptors of Shiga toxin 1 and 2 (Stx1 and Stx2) and *C. difficile* toxin A, respectively. The blood group P1 determinant generated in multiple copies on PSGL-1/mIgG2b by the expression of pigeon α4GalT and the core 2 enzyme (C2β6GnT1) bound with high avidity to both Stx1 and Stx2. In Paper IV, PSGL-1/mIgG2b expressing terminal Galα1,3Gal was shown to bind *C. difficile* toxin A and to inhibit its cytotoxic and hemagglutinating properties.

In paper II and III, PSGL-1/mIgG2b was used as a probe to understand the *O*-glycan biosynthesis pathways in CHO cells. The expression of various *O*-glycan core chain glycosyltransferases aided in defining their *in vivo* glycan specificities and their potential competition with the endogenous CHO glycosylation machinery. In paper II, small-scale transient transfections were

employed to analyze the effects of *O*-glycan core enzymes, ST6GAL1 and CHST4 on the *O*-glycome repertoire of PSGL-1/mIgG2b. Using these data, in paper III, a panel of recombinant mucins carrying terminal α2,3- or α2,6-linked sialic acid on defined *O*-glycan core saccharide chains was produced by generating stable CHO cell lines. Owing to the pathobiological significance of sialylated glycans, these recombinant mucins will be an important tool for determining the fine *O*-glycan binding specificity of sialic acid-specific microbial adhesins and lectins.

In conclusion, we have recreated the enzymatic pathways involved in the biosynthesis of specific target carbohydrate determinants on defined *O*-glycan chains in CHO cells. Using a mucin-type scaffold has allowed us to create high affinity, multivalent carbohydrate ligands and inhibitors of bacterial toxins.

Keywords: *O*-glycans, mucin, bacterial toxin

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SAMMANFATTNING PÅ SVENSKA

Ett första steg i en infektion utgörs av bakteriers eller virus bindning till cellytan. Denna vidhäftning förmedlas ofta av att proteiner på smittämnet binder till sockermolekyler på cellytan. Detsamma gäller bakterietoxiner som vid många bakterieinfektioner är den faktor som orsakar vävnadsskadan. Arbetet i denna avhandling har kretsat kring att i så kallade cellfabriker producera (d.v.s. rekombinant produktion) mucinliknande glykoproteiner (sockerbärande proteiner) som skulle kunna användas för att hämma framför allt bakterietoxiners bindning till cellytan och därigenom minska deras skadeverkningar. De toxiner som studerats utgörs av de Shiga-liknande toxinerna, som bildas av vissa Escherichia coli stammar och som kan orsaka en njursjukdom vi kallar hemolytiskt uremiskt syndrom, och toxin A från Clostridium difficile, som bidrar till att orsaka en svår tjocktarmsinflammation. Genom att ge de rekombinant producerade mucinliknande glykoproteinerna speciella egenskaper, bland annat genom att de bär flera kopior av specifika socker, kan nya mer effektiva hämmare av bakterietoxiners effekt produceras.

Som cellfabrik har använts en cellinje som heter CHO. Fördelen med denna är att dess förmåga att producera olika sockerstrukturer noga karaktäriserats. Genom att sedan föra in det genetiska materialet för de enzym (glykosyltransferaser) som bygger upp önskade sockerstrukturer, kan CHO cellen fås att göra ett mucinliknande protein (PSGL-1/mIgG2b) bärande just de sockerstrukturer som behövs för att hämma de bakterietoxiner vi studerat.

I arbete I och IV har vi genetiskt modifierat CHO celler så de producerar PSGL-1/mIgG2b bärande de sockerstrukturer i flera kopior som hämmar Shigaliknande toxin 1 och 2 (Stx1 and Stx2) samt C. difficile toxin A. Sockerstrukturen som förmedlar bindning till de förstnämnda toxinerna utgörs av ett blodgruppsantigen känt som P1 och skapades i CHO celler genom att uttrycka två glykosyltransferaser, varav ett från duva. Ett mucin med sockerstrukturen Galα1,3Gal band C. difficile toxin A och hämmade dess toxiska effekter på celler och förmåga att klumpa ihop röda blodkroppar från kanin.

I arbete II och III, användes PSGL-1/mIgG2b som ett verktyg för att bättre förstå hur olika enzymer påverkar bildningen av specifika sockerstrukturer när de uttrycks i CHO celler. En förutsättning för dessa studier är den sofistikerade masspektrometriska metodik som användes för att karaktärisera sockerstrukturerna. Denna kunskap användes sedan för att generera en hel

uppsättning av cellfabriker (olika stabila cellinjer) som producerar vårt mucinliknande protein med olika, väl definierade sockerstrukturer på. Dessa proteiner förväntas bli mycket viktiga när det gäller att kartlägga andra bakteriers, virus och bakterietoxiners bindning till specifika sockerstrukturer.

Sammanfattningsvis har detta avhandlingsarbete bidragit till att kartlägga de biosyntetiska reaktionsvägar som används för att bygga upp specifika sockerstrukturer i CHO celler. Genom att använda ett mucinliknande protein och uttrycka det i dessa CHO celler, har vi på detta protein i flera kopior lyckats återskapa de sockerstrukturer som behövs för att producera effektiva hämmare av bakterietoxin. Hämmare som kan tänkas få en terapeutisk betydelse.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Maria Cherian, R**., Gaunitz, S., Nilsson, A., Liu, J., Karlsson, N.G., and Holgersson, J. Shiga-like toxin binds with high avidity to multivalent O-linked blood group P1 determinants on mucin-type fusion proteins. Glycobiology 2014; 24, 26-38.

II. Liu, J., Jin, C., **Maria Cherian, R**., Karlsson, N.G., and Holgersson, J. O-glycan repertoires on a mucin-type reporter protein expressed in CHO cell pools transiently transfected with O-glycan core enzyme cDNAs. J Biotechnol 2015; 199, 77-89.

III. **Maria Cherian, R.**, Jin, C., Liu, J., Karlsson, N.G., and Holgersson, J. A panel of recombinant mucins carrying a repertoire of sialylated O-glycans based on different core chains for studies of glycan binding proteins. Biomolecules 2015; 5, 1810-1831.

IV. **Maria Cherian, R.,** Jin, C., Liu, J., Karlsson, N.G., and Holgersson, J. Recombinant mucin-type fusion proteins with Galα1,3Gal substitution as *C. difficile* toxin A inhibitors. Manuscript

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ABBREVIATIONS

Asn	Aspargine
CDAD	Clostridium difficile associated disease
CHO	Chinese Hamster Ovary cells
CROPs	Combined repetitive oligopeptides
C1 β3GalT1	Core 1 β1,3galactosyltransferase
C2 β6GnT1	Core 2 β1,6-*N*-acetylglucosaminyltransferase
C3 β3GnT6	Core 3 β1,3-*N*-acetylglucosaminyltransferase 6
ELISA	Enzyme-linked immuno sorbent assay
ER	Endoplasmic reticulum
ETEC	Enterotoxigenic *E. coli*
extC1 β3GnT3	Extended core 1 β1,3-*N*-acetylglucosaminyltransferase
FMT	Fecal microbiota transplantation
Fuc	Fucose
FUT1,H	α1,2-fucosyltransferase-1
FUT2, Se	α1,2-fucosyltransferase-2
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GalNAcT	*N*-acetylgalactosyltransferase
GalT	Galactosyltransferase
Gb3	Globotriaosylceramide

Glc	Glucose
GlcNAc	*N*-acetylglucosamine
HEK	Human embryonic kidney cells
Hex	Hexose
HUS	Hemolytic uremic syndrome
IA	Immunoadsorption
LacdiNAc	*N, N'*-diacetyllactosamine
LacNAc	*N*-acetyllactoseamine
LC	Liquid chromatography
LR	Long repeats
Man	Mannose
MAL-I	Maackia amurensis lectin I
MAL-II	Maackia amurensis lectin II
MS	Mass spectrometry
MUC	Mucin
NA	Neuraminidase
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
OST	Oligosaccharyltransferase
P1	Galα1,4Galβ1,4GlcNAc
PAA	Poly(acrylic acid)amide

Pk	Galα1,4Galβ1,4Glc
ppGalNAcT	UDP-GalNAc-polypeptide-*N*-acetylgalactosaminyltransferase
PSGL-1/mIgG2b	P-selectin glycoprotein ligand-1/mouse IgG2b
RBD	Receptor Binding Domain
RNAi	Interfering RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
Sia	Sialic acid
SNA	Sambucus nigra bark lectin
SPR	Surface plasmon resonance
SR	Short repeats
STEC	Shiga toxigenic *Escherichia coli*
Stx	Shiga toxin
Stx1	Shiga toxin 1
Stx2	Shiga toxin 2
TcdA	Toxin A
TcdB	Toxin B
Thr	Threonine
Type 1	Galβ1,3GlcNAc
Type 2	Galβ1,4GlcNAc
VNTR	Variable number tandem repeats

1 INTRODUCTION

Glycobiology, in its broadest sense, is the study of the role of carbohydrates in cellular life. Carbohydrate metabolism and chemistry was of prominent interest in the early 20th century. During the mid-20th century, the scientific interest in carbohydrates was limited as they were considered only as a source of energy or as structural materials, and they were believed to lack any other biological activities. However, the development of new technologies in the 1980's provided opportunities to study the structure and function of glycans revealing their biological importance and paving the way to a new horizon of medical science - Glycobiology.

1.1 Fundamentals of glycobiology

The surface of every cell is decorated with a diverse array of glycans - known as the glycocalyx - that defines the molecular frontier of the whole organism. Glycans that are one of the four fundamental building blocks of life, play critical roles in many physiological and pathological cell functions because of their prominence, abundance and structural variations. Glycans are involved in many biological interactions that help in cell adhesion, trafficking and signaling (Figure 1). They are significant determinants of self/non-self and

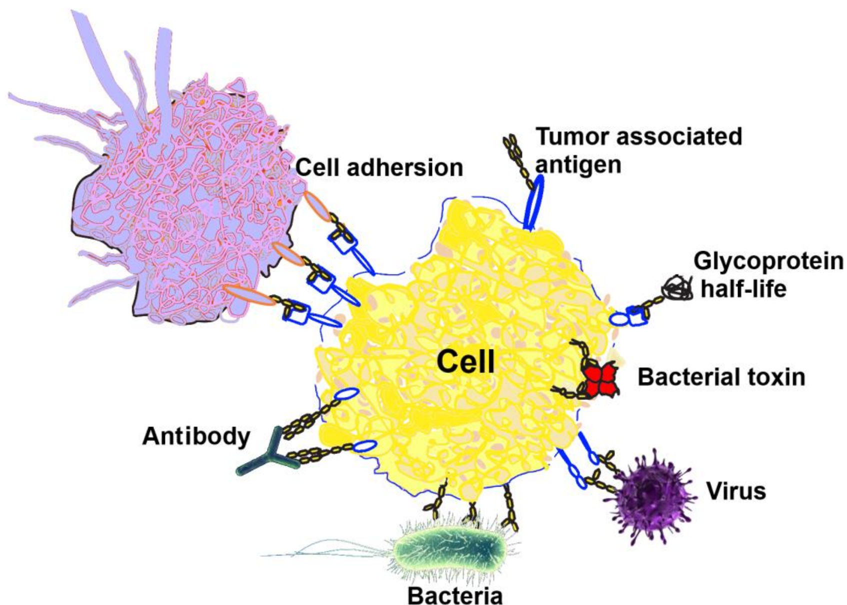

Figure 1. *A schematic drawing illustrating protein-carbohydrate interactions at cell surface mediating cell-cell binding, cell-microbe (bacterial, viral and bacterial toxin) interactions and cell-antibody binding. Modified from (Holgersson,et al., 2005)*

play key roles in host-pathogen interactions (Gustafsson & Holgersson 2006; Springer & Gagneux 2013). Glycans can be either found as free saccharides or in most cases attached to cell surface and extracellular proteins and lipids, then known as glycoconjugates. These glycoconjugates can be glycoproteins, glycolipids or proteoglycans. The enzymatic process that covalently attaches the glycans to non-carbohydrate moieties by glycosidic linkages is known as glycosylation (Lis & Sharon 1993).

1.2 Protein glycosylation

Glycosylation is one of the major types of post-translational modification that proteins can undergo and 50% of all human proteins are glycosylated (Kobata 2004). Modification of the protein through enzymatic glycosylation is determined by the structure of the protein backbone and the carbohydrate attachment site. It is also the most diverse modification due to the structural variation of the attaching carbohydrates, which not only differ in sequence and chain length, but also in anomeric configuration (α or β), position of linkages and branching sites (Dwek 1995). Further structural diversification may occur by covalent attachment of sulfate, phosphate, acetyl or methyl groups to the sugars. The central event in the biosynthesis of a glycoprotein is the formation of a sugar-amino acid bond that determines the nature of the carbohydrate units that are subsequently added, which in turn influences the biological activity of the protein. There are 13 different monosaccharides and eight amino acids that can make up sugar-peptide linkages leading to *N*- and *O*-glycosylation, *C*-mannosylation, phosphoglycation and glypitation (Spiro 2002). Two of the most abundant forms of glycosylation occurring on proteins, which are either secreted or membrane-bound, are *N*- and *O*-linked glycosylation. *N*-linked glycans are usually attached via a *N*-acetylglucosamine (GlcNAc) to Aspargine (Asn) and *O*-linked glycans can be variously attached to Ser or Thr via fucose, glucose, mannose, xylose and other sugars including *N*-acetylgalactosamine (GalNAc) which is found in the most frequent *O*-glycan, the mucin-type *O*-glycan (Lis & Sharon 1993).

1.2.1 *N*-glycosylation

N-glycans are classified into three types; high mannose (oligomannose), complex and hybrid type *N*-glycans (Figure 2). In eukaryotes, the glycosylation process is initiated by the synthesis of a core unit Man5-GlcNAc2, built on a lipid anchor (dolichyl pyrophosphate) on the cytosolic side of the endoplasmic reticulum (ER) membrane. The lipid linked oligosaccharide is then re-oriented to the luminal side of the ER membrane, where it is extended to a Glc3-Man9-GlcNAc2 sequence. This precursor

structure of *N*-glycans, synthesized through the stepwise addition of monosaccharides by various glycosyltransferases in the ER, is conserved in all eukaryotic cells. Proteins that are translocated to the ER lumen and having the consensus sequence (N-X-S/T) serve as acceptors for an oligosaccharyltransferase (OST), the central enzyme in the pathway of *N*-linked protein glycosylation. The acceptor substrate of *N*-glycosylation is an asparagine residue present within the consensus sequence N-X-S/T and it has been reported that OST shows a preference for N-X-T sites over N-X-S (tyrosine over serine). Proline is not tolerated in the second position. The transfer of the oligosaccharide to the acceptor polypeptides occurs en bloc resulting in the synthesis of homogeneous glycoproteins. After being covalently linked to proteins, the *N*-glycans are further modified in the late ER and Golgi producing diversity in the *N*-glycans. The processing is possibly determined by the function of the glycan structures and the compartment where they are localized, resulting in a species- or even cell type-specific diversity of *N*-linked glycans (Aebi 2013; Schwarz & Aebi 2011).

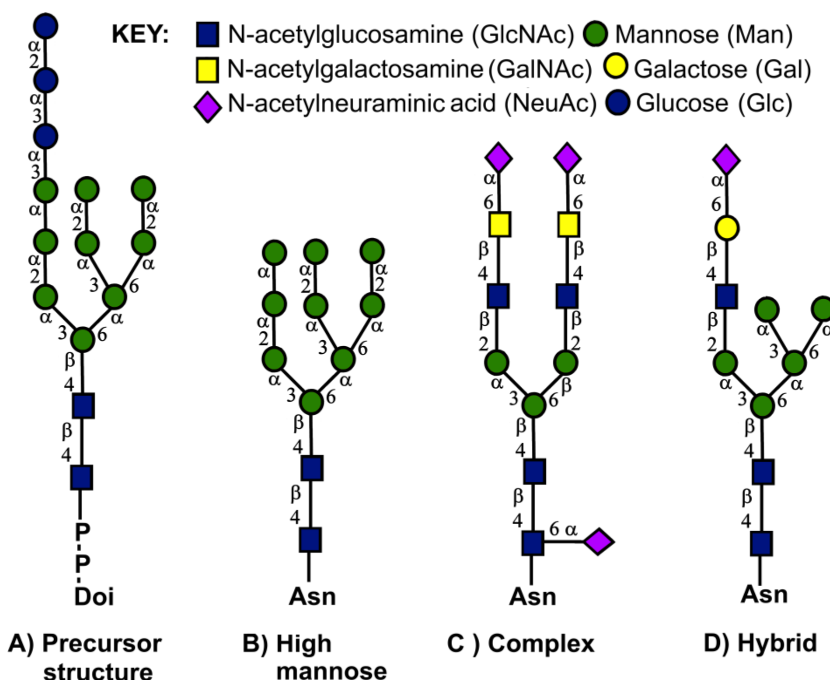

Figure 2. *The major N-glycan types found in mammals*

1.2.2 *O*-glycosylation

O-glycans are synthesized in the ER and Golgi (or in the cytosol in case of *O*-GlcNAc glycans) by stepwise enzymatic transfer of monosaccharides. In contrast to the *N*-glycosylated sites, *O*-glycosylation sites do not reside in a known amino acid sequence. *O*-glycans are attached to the hydroxyl groups in serine and threonine residues and include *O*-linked GlcNAc, mannose, fucose, and GalNAc (Van den Steen et al. 1998). The most abundant form of *O*-linked glycosylation in higher eukaryotes is that formed by the addition of GalNAc to serine or threonine, also termed as mucin-type *O*-linked glycosylation. The other types are rare or restricted to certain species, tissues or proteins. *O*-linked GlcNAc is a reversible modification that competes with phosphorylation in the activation/deactivation of cytosolic and nuclear proteins, while *O*-linked fucose is seen in the epidermal growth factor domains of human blood coagulation factors VII and IX (Bjoern et al. 1991; Nishimura et al. 1992; Wells & Hart 2003). *O*-linked mannose is a characteristic of yeast proteins and *O*-linked xylose is seen mostly in proteoglycans (Roden et al. 1985; Herscovics & Orlean 1993). The mucin type *O*-linked GalNAc glycosylation has been found on almost all phyla of the animal kingdom and even in higher plants (Hang & Bertozzi 2005).

Mucin-type *O*-glycosylation

O-GalNAc glycosylation is designated as 'mucin-type' because mucins are heavily *O*-glycosylated proteins that carry clusters of GalNAc-based glycans in their repetitive Ser- and Thr-rich peptides. The initiating step of this glycosylation is the addition of the monosaccharide *N*-acetylgalactosamine (GalNAc) from UDP-GalNAc to the hydroxyl groups in serine and threonine residues; a reaction catalyzed by a large family of up to 20 different polypeptide GalNAc-transferases (ppGalNAc-Ts) (Bennett et al. 2012). These enzymes are differentially expressed over tissue and time and the diversity of ppGalNAc-Ts influences the density and site occupancy of the mucin-type *O*-glycosylation (Hang & Bertozzi 2005). Subsequently a stepwise enzymatic elongation by specific glycosyltransferases produces several core structures, which are further elongated or modified by acetylation, fucosylation, sialylation, sulfation, and polylactosamine-extension. The Tn antigen is represented by the innermost GalNAc; a determinant enriched in different cancers (Springer 1997; Springer 1984; Desai 2000; Springer et al. 1975). The *O*-linked glycans in a glycoprotein comprise three main regions: the core region, which include the innermost two or three sugars of the glycan chain adjacent to the peptide, the backbone region formed by the uniform elongation that contributes to the length of the glycan chains, and the terminal region, which exhibits a high degree of

structural complexity and make up the biologically important carbohydrate determinants (Hanisch 2001). The structural variability of *O*-linked glycans of the mucin-type already starts at the level of their core structure. At least eight different types of core structures have been reported so far to occur in mammalian glycoproteins. All are based on the core-α-GalNAc residue, which can be substituted at C3, C6, or at both positions with additional monosaccharides. The biosynthetic pathways of core 1-4 and extended core 1 *O*-glycans are shown in Figure 3.

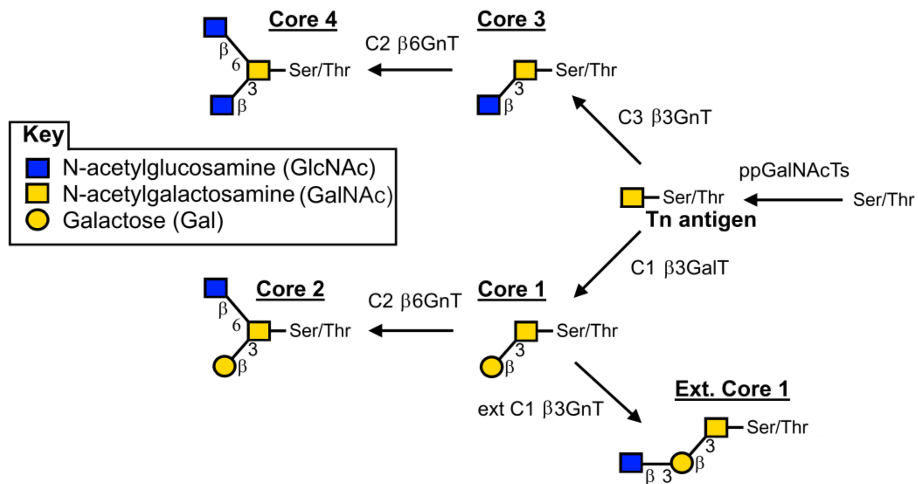

Figure 3. *Synthesis of O-glycan core structures, core 1 to core 4 and extended core 1*

The most common is the core 1 or T antigen structure catalyzed by the core 1 β1,3galactosyltransferase (T-synthase or C1 β3GalT1 or B3GALT1), which adds galactose in a β1,3-linkage to the GalNAc residue (Ju et al. 2002). To days' date, only a single C1 β3GalT1 has been reported and it is expressed ubiquitously. The core 2 structure is produced by the addition of an *N*-acetylglucosamine (GlcNAc) in a β1,6 linkage to the GalNAc of the core 1 structure by a β1,6-*N*-acetylglucosaminyltransferase (C2 β6GnTs or GCNT1)(Bierhuizen & Fukuda 1992) . There are three C2 GnTs in mammals, two of which catalyze the formation of the core 2 structure (C2 β6GnT1 and C2 β6GnT3) and one that can catalyze biosynthesis of either the core 2 or core 4 structure (C2 β6GnT2) (Schwientek et al. 2000; Yeh et al. 1999). When a GlcNAc instead of a Gal is transferred in a β1,3-linkage to the innermost GalNAc, a core 3 structure is formed. Core 3 is synthesized by the enzyme β1,3-*N*-acetylglucosaminyltransferase 6 (C3 β3GnT6 or B3GNT6) which competes with the C1 β3GalT1 (Iwai et al. 2002). The core 3 structure may serve as a substrate for the core 4 enzyme reaction, where GlcNAc is

added in a β1,6-linkage to GalNAc similar to the synthesis of the core 2 structure. The core 3 and core 4 exhibits a more restricted, organ-characteristic expression compared to the most abundant core 1 and core 2 core structures (Hanisch 2001). In addition to these core structures, core 5-8 *O*-glycans also exist, but are rather rare. The core *O*-glycan structures are then further modified or extended by other Golgi-resident glycosyltransferases to produce complex *O*-linked glycans that are involved in a variety of biological processes (Van den Steen et al. 1998; Hang & Bertozzi 2005; Jensen et al. 2010; Tian & Ten Hagen 2009; Tran & Ten Hagen 2013). Both the C3 and C6 branch of the core GalNAc can principally serve as substitution sites for chain elongation, however, the C6-branch is generally preferred. The backbone region is often formed by addition of the repetitive disaccharide element Galβ1,4GlcNAc (lactosamine or type 2 chain) to the core structure. Other types of backbone structures that can occur in *O*-linked glycans are listed in Table 1. Further elongation and termination have

Table 1. *Structural elements of mucin-type O-glycans. The major core structures with their common backbone extensions are listed. With respect to the peripheral structures, only a few examples are shown due to the great diversity of known structures at the non-reducing terminal of O-linked glycans.*

Peripheral Structures		Backbones	Cores	
Blood group O	Fucα1,2Gal		Core1 or T antigen	Galβ1,3GalNAcα Ser/Thr
Blood group A	GalNAcα1,3 (Fucα1,2)Gal	Type 1 chain-Galβ1,3GlcNAc	Core 2	Galβ1,3(GlcNAcβ1,6) GalNAcαSer/Thr
Blood group B	Gal α1,3 (Fucα1,2)Gal		Core 3	GlcNAcβ1,3GalNAcα Ser/Thr
Blood group Lewis^a	Galβ1,3 (Fucα1,4) GlcNAc		Core 4	GlcNAcβ1,3(GlcNAc β1,6)GalNAcαSer/Thr
Blood group Lewis^b	Fucα1,2Gal β1,3(Fucα1,4) GlcNAc	Type 2 Chain-Galβ1,4GlcNAc (neo-*N*-acetyllactosamine)	Core 5	GalNAcα1,3GalNAcα Ser/Thr
Blood group Lewis^x	Galβ1,4 (Fucα1,3) GlcNAc		Core6	GlcNAcβ1,6GalNAcα Ser/Thr
Blood group Sialyl-Lewis^x	Siaα2,3Gal β1,4(Fucα1,3) GlcNAc		Core7	GalNAcα1,6GalNAcα Ser/Thr
		LacdiNAc - GalNAc β1,4 GlcNAc	Core 8	Galα1,3GalNAcα Ser/Thr
			Extended Core 1	GlcNAcβ1,3Galβ1,3 GalNAcαSer/Thr

been shown to occur with the addition of other sugars such as galactose, *N*-acetylglucosamine, fucose and sialic acid, creating extended linear or branched structures. These glycan structures can define various antigenic determinants, for example Lewis-type antigens and blood group determinants (Hanisch 2001).

Functions of *O*-linked glycans

O-linked glycosylation has a prominent effect on the protein structure in terms of secondary, tertiary (elongation of the protein backbone), and quaternary structure (Van den Steen et al. 1998). Proteins with *O*-linked glycosylation may adopt a ‘bottle brush’-like structure conferring an elongated structure to the peptide backbone. A typical example is the P-selectin glycoprotein ligand-1 (PSGL-1). The presence of *O*-linked glycans on proteins in the cell membranes may extend them several hundred nanometers out from the cell surface, thus shielding the cell sterically from invading pathogens. *O*-linked glycans that are terminated with sialic acids also provide negative charge repulsion between cells, which could alter the biophysical properties of cellular interactions (Varki & Gagneux 2012). *O*-glycans are also important for the stability of glycoproteins and confers protease and heat resistance to the mucins and mucin-type proteins (Van den Steen et al. 1998). They are also crucial elements in carbohydrate-protein interactions and play key roles in many important recognition events like selectin binding in leukocyte circulation, fertilizing spermatozoan-oocyte interactions, immunological recognition of antigens, glycoprotein clearance and signal transduction (Benoff 1997; Barthel et al. 2007; Varki 1993; Varki 2008). *O*-linked glycans can also influence the activity of hormones and cytokines (Van den Steen et al. 1998; Chamorey et al. 2002). Mucins that are characterized by high-density *O*-glycan substitution can act as decoys for carbohydrate binding bacteria, their toxins and viruses, thus protecting the host from pathogen colonization and infection (Varki 1993). For example, cell surface mucin, mucin 1 (Muc1) act as releasable decoy molecules that display an array of targets for microbial adhesion and contribute to host defense against *Campylobacter jejuni* infection in the gastrointestinal tract (McAuley et al. 2007). In addition, alterations of *O*-linked glycosylation are also associated with cancer and other autoimmune diseases, which suggest that *O*-glycans may become important biomarkers of disease and malignancy (Jensen et al. 2010; Tran & Ten Hagen 2013; Varki 1993; Tarp & Clausen 2008).

Mucins and mucin-type fusion proteins

Mucins are glycoproteins rich in *O*-linked, mucin-type glycans. They have the characteristic mucin domains rich in proline/threonine/serine motifs (PTS domains) that are found in variable number tandem repeats (VNTR) and may be represented up to 10-100 times in the polypeptide chain (Hang & Bertozzi 2005). The number of tandem repeats influences the mucin glycosylation and the length of the oligosaccharide. The multiple *O*-linked glycans confer an elongated structure to the peptide backbone which is reflected in a number of physicochemical properties of the mucins. It appears that prolines contribute to the extended formation of mucins and mucin-type protein, as the classic α-helix formation requires hydrogen bonds and the nitrogen of prolines lack hydrogen atoms (Cid et al. 1986). The formation of typical tertiary structures is precluded by the steric interactions between the peptide-linked GalNAc residue and adjacent amino acids in the peptide core (Live et al. 1996; Coltart et al. 2002). Moreover, the hydrophobic interactions that promote protein folding cannot overcome the strong hydrophilic interactions of the carbohydrates. Therefore the *O*-glycans induce the mucin peptide core to adopt a stiff random coil conformation that prevents folding into a globular structure (Hanisch 2001). The ability of mucins to form gels is attributed to its larger solution size that form intertangled networks at lower concentration. The carbohydrate constitute more than half of the total weight of these heavily glycosylated glycoproteins (Davies et al. 2012). *O*-glycans that extend out from the mucin protein core are intimately associated with the external environment. The mucins found as components of mucus gel layers at mucosal surfaces throughout the body play roles in protection as part of the defensive barrier on an organ and tissue specific basis. The inner, adherent mucus adjacent to the epithelial cells provides a bacteria-free environment, while the outer layer harbors bacterial populations (Hang & Bertozzi 2005; Varki 1993; Hilkens et al. 1992; Hansson 2012; Johansson et al. 2013).

1.3 Protein-carbohydrate interactions

The “glycocode” encoded in the cell-surface glycans and exogenous soluble glycans in the extracellular matrix are interpreted by a plethora of lectins and other glycan-binding receptors, which translate them into cellular activity. These interactions play a crucial role in human health and diseases such as growth regulation, tumor cell adhesion, cell migration and host-pathogen recognition. Carbohydrates interact with protein partners belonging to a number of protein families including enzymes like glycosyltransferases, antibodies, lectins and transporters. Lectins are ubiquitous glycan-binding proteins that can recognize and bind specific carbohydrate structures. Unlike

antibodies and enzymes, they are non-immune origin and lack catalytic activity. Various examples of lectin-carbohydrate interactions and its biological role have been extensively reviewed (Varki 1993). Despite the importance of these interactions, an individual protein-carbohydrate binding is typically quite weak and not very specific. Nature obtains strong and specific recognition through multiple protein-carbohydrate interactions, a phenomenon known as multivalency.

1.3.1 Importance of multivalency

Multivalency is the key principle in nature for achieving strong and reversible interactions that are important in recognition, adhesion and signaling processes (Reynolds & Pérez 2011; Reynolds & Pérez 2011; Fasting et al. 2012; Mammen et al. 1998). This high affinity binding is achieved by the simultaneous binding of multiple ligands on one biological entity, to multiple receptors on the other. It permits not only the high affinity of the interaction between proteins and glycans, but also causes biologic activity, such as agglutination. Several examples of multivalent interactions occur in nature like adhesion of the influenza virus to the surface of a bronchial epithelial cell and adherence of P-fimbrial filaments of the uropathogenic *E. coli* to multiple copies of the Pk antigen (Mammen et al. 1998). The high avidity of these multivalent interactions is attributed to two distinct mechanisms namely steric stabilization and entropically enhanced binding. The first factor is related to the decrease in the disassociation $k_{\mathrm{off}\ \mathit{value}}$ (k_{d}) instead of increase in association $k_{\mathrm{on}\ \mathit{value}}$ (k_{a}). During a multivalent interaction, if one bond is disassociated, due to the decreased off rates, the remaining bonds keep the unbound ligand and receptor in close proximity which facilitates re-binding (Reynolds & Pérez 2011; Dam et al. 2002). Similarly, in case of inhibitors that interfere with protein-carbohydrate interactions, the large backbone structure can sterically hinder the receptor from reaching its ligand as well as shield a large part of the existent receptors without even binding to them. An example of this kind of steric hindrance is the mucins in the gastrointestinal tract that protect the cells from invading pathogens. In case of the second mechanism, instead of unfavorable entropy in monovalent interactions due to the conformational restrain of the carbohydrate ligand, in multivalent interactions the entropy cost upon binding of the first ligand is smaller than the monovalent interaction. This is because the restriction of carbohydrate flexibility has already been induced by the backbone carrier. Therefore, the enthalpy gain resulting from the multivalent interaction is not compensated for by entropy cost, resulting in an increased change in the free energy (ΔG) and hence a higher affinity (Fasting et al. 2012; Mammen et al. 1998; Holgersson et al. 2005).

1.3.2 Inhibition of protein-carbohydrate interaction

Protein-carbohydrate interactions mediate the first contact between the microbe, bacterial toxin, antibody or cell and the host, thus being involved in several medical conditions like inflammation, cancer, and infectious diseases. Interference with these recognition events by functional mimics of carbohydrates could thus be used to alter signal transmission, or to prevent the onset of diseases. Nature also uses this approach; soluble glycans, such as human milk oligosaccharides and mucins, capture and aid in removal of microbes (Newburg 2000; Andersson et al. 1986). Therefore, the development of inhibitors for biologically important interactions has attracted much attention over recent years. This class of glycan-based therapeutics offers a suitable alternative to antibiotics or antivirals by acting as competitive inhibitors for the cellular receptor, thereby arresting and eliminating the microbe. Glycan-based therapeutics is advantageous as microbes may be less prone to develop resistance to this class of molecules, because in many cases glycan-binding plays an essential part in its pathogenic strategy. The glycan-based drugs may also suffer less from phenotypic and genotypic drifts than vaccine and monoclonal antibody-based therapies (Seeberger & Werz 2007; Kulkarni et al. 2010).

1.4 Designing a glycan-based inhibitor

Important factors to be considered for the development of a potent inhibitor that can compete with nature to inhibit unwanted protein–carbohydrate interactions are: the primary structure of the recognizing glycan, its presentation and density on the ligand backbone, and the nature of the ligand. The primary structure of the glycan remains the most important factor in determining pathogen/host interactions. Within a pathogen family, the binding preferences of different variants can be different with the internal glycans exerting their influence in the recognition process (Kulkarni et al. 2010). Therefore, for the design of an effective ligand it is important to engineer the entire glycan sequence including the internal glycan chains recognized by the pathogen or its released toxins.

Another important factor that should be considered during the design of an inhibitor is the multivalent nature of the pathogen receptors. The molecules that mediate adherence of bacteria and viruses to their target cells are present in multiple copies (Mammen et al. 1998). In order to competitively inhibit these multivalent interactions, the concept of a multivalent inhibitor was developed that could inhibit the adherence of pathogen to the cell surface. Due to the complexity of protein–carbohydrate interactions, monovalent

inhibitors are usually ineffective even if the binding activity of the inhibitor has been structurally optimized. A variety of molecules can be used as a backbone for the multivalent presentation of the ligand, for example the use of polyacrylamide polymers (PAA), dendrimers, nanoparticles, liposomes, neoglycoconjugates and glycoproteins (Kulkarni et al. 2010; Imberty et al. 2008; Bovin 1998).

Recognition of the glycan can also be influenced by how it is displayed on the ligand backbone. Glycans adopt several thermodynamically stable conformations, and the ability of a glycan to adopt the conformation needed for receptor recognition can be influenced by adjacent residues that orient the binding determinants in the appropriate conformation (Das et al. 2001; Xu et al. 2009). In case of the natural ligands, the existence of inner core chains influences the presentation of the specific determinant (Lofling & Holgersson 2009). Attaching glycans to a solid surface can limit the number of conformations, and it has been well established that glycans-on-a-surface exhibit different binding affinities towards the same protein than free glycans-in-solution (Lundquist & Toone 2002; Corbell et al. 2000).

The antigenicity of the carrier of the carbohydrate determinants is also an important factor, for example the early blood group A and B trisaccharide columns used to remove anti A and B blood group antibodies were not successful due to the brittle and bio-incompatible nature of silica (Bensinger et al. 1981; Blomberg et al. 1993). Furthermore, a polyclonal anti-carbohydrate antibody response may appear as if it recognizes the particular carbohydrate determinant in a core chain-independent manner, but may in fact consist of several different antibody specificities recognizing the determinant in a core chain-dependent manner. Therefore, an inhibitor carrying a specific carbohydrate ligand on several different inner core saccharide chains can be more efficient in blocking all potential antibody specificities (Holgersson et al. 2005).

1.4.1 Recombinant mucin-type fusion protein (PSGL-1/mIgG2b): A versatile inhibitor

One of the ways to create powerful inhibitors of protein–carbohydrate interactions is to rely on natural ligands that could be engineered to carry the desired carbohydrate epitope for each interaction. Many of the natural ligands are based on mucin-type proteins (MUC1, MUC5B, MUC5AC, PSGL-1, CD43, etc.). PSGL-1 is a mucin-type glycoprotein and is the high-affinity receptor for P-selectin, which is expressed on activated endothelial cells and platelets (Moore et al. 1995). The interaction between PSGL-1 and its

receptor P-selectin facilitates tethering and rolling of leukocytes along the vascular endothelium at sites of inflammation. PSGL-1 is a membrane-bound protein with an extracellular domain rich in serines, threonines and prolines. It has a highly extended structure with an extracellular domain about 50 nm long that allows it to protrude from the cell surface, and its high *O*-glycan chain substitution makes it ideal for attracting carbohydrate binding receptors (Li et al. 1996; Spertini et al. 1996; Moore 1998).

We have developed a mucin-type fusion protein by genetically fusing the extracellular part of PSGL-1 to the Fc part of mouse IgG2b (Figure 4). The frequent *O*-glycosylation of the mucin part of PSGL-1/mIgG2b supports a multivalent display of the carbohydrate determinant. PSGL-1/mIgG2b is mainly expressed as a dimer when produced in glyco-engineered cell lines, has an approximate molecular weight of 250 – 350 kDa depending on the glycosylation, and has the capacity to carry 106 *O*-glycans and 6 *N*-glycans per molecule (Gustafsson & Holgersson 2006; Liu et al. 2003; Löfling et al. 2002; Lindberg et al. 2013). Thus, the large size and the elongated shape of this protein makes it suitable as an inhibitor of carbohydrate-binding bacterial adhesins, toxins, antibodies, and viral surface proteins.

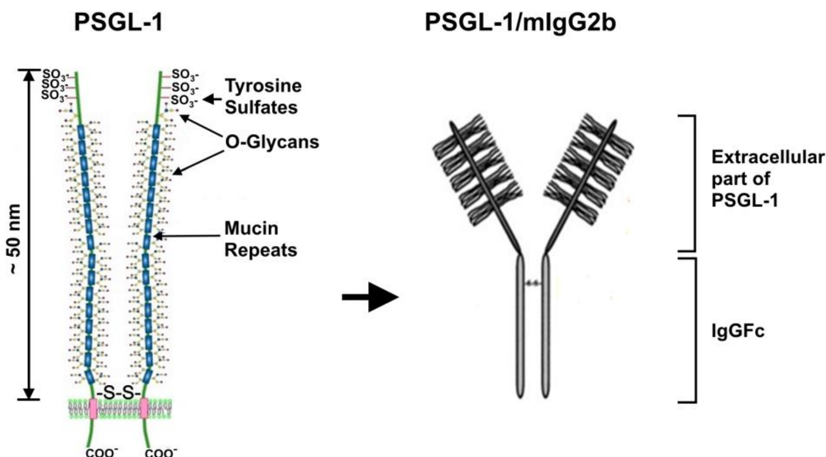

Figure 4. *The extracellular part of PSGL-1 was genetically fused to the Fc portion of mouse IgG2b to form a recombinant mucin-type fusion protein, PSGL-1/mIgG2b (modified from Varki A et al, Essentials of Glycobiology, edition 2, 2009 and Liu et al. 2003.*

Theoretically, mucin-based, glycan-multivalent ligands can also be used in diagnostics and aid in distinguishing between closely related microbes or toxins differing only in carbohydrate binding specificity. For example, the avian and human influenza virus that preferentially bind α2,3- and α2,6-

linked sialic acids, respectively. Different mucin-based glycoforms can also help in elucidating the specific binding motifs of certain glycan-binding proteins. In addition, the glyco-engineering of several different inner *O*-glycan chains on PSGL-1/mIgG2b can influence the conformation of the outer carbohydrate determinant, thereby providing the diversity for determining the specificity of various glycosyltransferases and carbohydrate-binding proteins (Lofling & Holgersson 2009; Liu et al. 2003; Lindberg et al. 2013).

In this thesis PSGL-1/mIgG2b was used as a multivalent carrier of specific carbohydrate ligands of bacterial toxins; Shiga-like toxins and *C. difficile* toxin A (Paper I and IV). In paper II and III, PSGL-1/mIgG2b was used as a reporter protein to assess the core chain specificity of sialyl- and sulfotransferases involved in the biosynthesis of bioactive carbohydrate determinants by generating stable and transient Chinese Hamster ovary (CHO-K1) transfectants.

Multifunctionality of PSGL-1/mIgG2b; Practical applications

The practical applications of recombinant mucin immunoglobulin fusion proteins have been widely studied in our laboratory using several strategies. One of the preliminary applications was as a superior adsorber that can be used in a pre-transplant extracorporeal immunoadsorption device to remove anti-pig antibodies responsible for the complement-mediated destruction of pig endothelial cells. The mucin-type fusion protein was expressed in COS cells together with the porcine α1,3 galactosyltransferase to generate a Galα1,3Gal-substituted PSGL-1/mIgG2b (Liu et al. 1997). In a similar way we have also generated an efficient adsorber of ABO antibodies by expressing blood group determinants at high density on our fusion protein produced in CHO cells. In comparison to a commercial blood group A trisaccharide covalently linked to macroporous glass beads, the fusion protein was an efficient adsorber of anti-blood group A antibodies in human blood group O serum (Löfling et al. 2002). Owing to the clinical utility of blood group A and B substituted PSGL-1/mIgG2b as substrates in enzyme-linked immunosorbent assays or as affinity matrices in immunoadsorption columns, a repertoire of stable CHO-K1 cells secreting mucin-type fusion proteins carrying blood group A or B determinants on defined *O*-glycan core saccharide chains has been generated (Lindberg et al. 2013). Many biologically important carbohydrate determinants have been successfully expressed multivalently on the fusion protein, like sialyl-Lewis x (SLe^x), sialyl-Lewis A (SLe^a), Lewis A (Le^a), and Lewis B (Le^b) (Lofling & Holgersson 2009; Löfling et al. 2008; Holgersson & Löfling 2006).

The efficacy by which this mucin-based potential inhibitor can interfere with protein-carbohydrate interactions has been investigated in many of our studies. The hemagglutinin of the H5N1 avian influenza strain was shown to bind with high avidity to PSGL-1/mIgG2b carrying mostly sialylated core 1 and sialylated lactosamine (Gaunitz et al. 2014). In this thesis, the data of Paper I shows that Shiga-like toxin binds to PSGL-1/mIgG2b carrying multiple copies of the blood group P1 determinant, making it a potential inhibitor of this bacterial toxin. In paper IV the ability of the fusion protein carrying Galα1,3Gal determinants to neutralize the cytopathic, cytotoxic and hemagglutination properties of *C. difficile* toxin A is demonstrated.

PSGL-1/mIgG2b has also been used as a reporter protein for studies on protein glycosylation. We have directed the expression of this mucin-type immunoglobulin fusion protein in various mammalian host cell lines like CHO-K1, HEK-293, COS-7m6 as well as in insect (Sf9 and HI-5) and yeast (*Pichia pastoris*) cells. Novel *O*-glycans with phosphocholine and sulfate substitutions were identified in insect cell lines (Gaunitz et al. 2013). Similarly, PSGL-1/mIgG2b expressed in *Pichia pastoris* carried *O*-glycans mainly comprised of α-linked mannoses that bound mannose-specific receptors with high apparent affinity and can be a potent targeting molecule for these receptors *in vivo* (Gustafsson et al. 2011; Ahlén et al. 2012). Transient expression of PSGL-1/mIgG2b with selected glycosyltransferases can provide important information about the specificity of expressed glycosyltransferases (paper II). It can also be used to predict the biosynthetic pathways of *O*-glycans, define the core chain specificities of various glycosyltransferases and the potential competition between them (Lofling & Holgersson 2009; Holgersson & Löfling 2006). PSGL-1/mIgG2b carrying a repertoire of *O*-glycan core structures (core 1-4 and extended core 1) and harbouring terminal α2,3- and α2,6-linked sialic acid was generated in glyco-engineered CHO-K1 cells (paper IV). These fusion proteins can be important reagents for determining the fine *O*-glycan binding specificity of sialic acid-specific microbial adhesins and mammalian lectins.

1.5 Importance of glycosylation in recombinant therapeutic proteins

Glycoproteins account for more than two thirds of the available therapeutic proteins in the market today. Glycosylation has a huge impact on the biological activity of glycoproteins and should be carefully controlled during manufacturing to achieve optimized therapeutic efficacy. The carbohydrate moiety influences the thermal stability, solubility, bioavailability, clearance

and pharmacokinetics of the therapeutic glycoprotein (Sola & Griebenow 2009; Li & d'Anjou 2009). Glycosylation can also improve the Fc effector function of recombinant antibodies, leading to increased ADCC (antibody dependent cellular cytotoxicity) activity (Jefferis 2009). Terminal capping by sialic acid of glycoproteins can prevent their degradation by masking the exposed galactose and *N*-acetylglucosamine residues that are recognized by the asialoglycoprotein receptors, thus conferring longer *in vivo* circulatory half-life (Morell et al. 1971). Therefore, in order to increase drug efficacy, decrease immunogenicity and increase the circulatory half-life of recombinant biopharmaceuticals, engineering of the host cell glycosylation phenotype is required (Sinclair & Elliott 2005; Elliott et al. 2003; Solá & Griebenow 2010).

1.5.1 Glyco-engineering

Glyco-engineering offers great potential for the generation of glycoprotein therapeutics with reduced side effects and enhanced activity. Many efforts have been made in recent years to establish *in vivo* and *in vitro* glyco-engineering technologies for efficient production of homogeneous therapeutic glycoproteins (Sinclair & Elliott 2005). Dependent on the species, cell type and physiological status of the production host, the glycosylation pattern on recombinant glycoproteins can differ significantly (Dicker & Strasser 2015). Different expression systems like mammalian cells, insect cells, yeast and plants have been utilized for the industrial production of biopharmaceutical products. However, mammalian cell culture is currently the dominant system for the production of biopharmaceuticals because of its capacity for proper assembly, folding and post-translational modifications such as glycosylation of proteins (Lim et al. 2010). Among the mammalian cells, including mouse myeloma cells, mouse fibroblast cells, human embryonic kidney 293 cells, baby hamster kidney cells, CHO cells are the most widely employed mammalian cell line (Griffin et al. 2007).

We have utilized many glyco-engineered cell lines for tailoring specific glycan sequences on PSGL-1/mIgG2b. Suitable host cell lines are transfected with plasmids encoding glycosyltransferases, which support the rational design of glycans carried in a multivalent fashion on PSGL-1/mIgG2b. The production of PSGL-1/mIgG2b with tailored glycosylation has been a successful strategy in our laboratory. The carbohydrate structures expressed on the fusion protein will vary depending on the host cell repertoire of endogenous glycosyltransferases and the glycosyltransferases expressed as a result of glycosyltransferase cDNA transfection. In the studies presented

here, we have employed CHO-K1 for the production of tailored PSGL-1/mIgG2b.

1.5.2 CHO-K1

Cell lines derived from Chinese hamster ovary (CHO) cells are widely used for the production of recombinant protein therapeutics such as monoclonal antibodies, hormones, cytokines, and blood products (Birch & Racher 2006; Jayapal et al. 2007; Omasa et al. 2010; Zhu 2012). The foremost clinically approved recombinant protein produced in CHO cells was tissue plasminogen activator (Kaufman et al. 1985). The glycosylation phenotype of CHO-K1 cells is well characterized and they often produce glycoforms similar to those produced in humans. One exception is that they do not produce the bisecting GlcNAc branch of *N*-glycans, which is found on 10% of human IgG glycoforms and is catalyzed by the ALG13 gene (Vishwanathan et al. 2015; Xu et al. 2011). The presence of bisecting GlcNAc enhances the antibody dependent cellular cytotoxicity (ADCC) of recombinant IgG, which is a desirable feature for some therapeutic antibodies (Kaufman et al. 1985; Umaña et al. 1999). CHO cells do not express ST6Gal1 and ST6Gal2, and are thereby unable to transfer sialic acid in an α2,6-linkage. This is in contrast to human cells, which contain glycans carrying a mixture of α2,3- and α2,6-linked sialic acids. They also lack CHST7 and CHST13 activity; enzymes involved in sulfation. With regard to fucosylation, CHO-K1 express fucosyltransferase 8 (FUT8) that add α1,6-linked fucose to the core pentasaccharide of *N*-linked glycans, and the protein-*O*-fucosyl transferases POFUT1 and POFUT2. They also exhibit considerably lower levels of Neu5Gc sialylation compared to murine cell lines due to lack of CMAH activity (Xu et al. 2011). Further, CHO cells appear to have insufficient enzymatic machinery to produce glycan structures with terminal Galα1,3Gal (αGal) determinants (Xu et al. 2011). The *O*-glycosylation capacity of CHO-K1 cells is limited unless genetically engineered. Proteins expressed in CHO-K1 cells have revealed that simple mono- and disialylated core 1 *O*-glycans dominate the *O*-glycan repertoire (Olson et al. 2005).

Bio-engineering of CHO-K1

Several strategies have been employed to increase the productivity of recombinant proteins in host cells including manipulation of culture media by developing serum-free or chemically defined media and optimization of process control methods, such as the fed-batch processes (Lim et al. 2010). Cellular engineering is another alternate means for creating more robust bioprocesses and higher production. Many approaches to alter metabolic

pathways in CHO-KI cells have been taken, including silencing or over-expressing individual genes in a metabolic pathway and modifying the expression of entire groups of genes using microRNAs. Strategies for gene silencing include interfering RNA (RNAi) and gene targeting, often employing a variety of nucleases such as zinc finger nucleases, homing endonucleases (or meganucleases), and transcription activator-like effector nucleases (Datta et al. 2013). Most of these approaches have engineered cells to reduce lactate production, resist apoptosis and to improve glycosylation. Some of the examples of CHO cell bioengineering are listed in Table 2.

Table 2. *Cell engineering strategies employed in CHO-K1 cells to increase the productivity of recombinant proteins.*

Engineering	Mechanism of action	Effects	Reference
Cell Metabolism	Over expression of Pyruvate carboxylase – enhanced conversion of pyruvate to oxaloacetate	Reduced glucose consumption and production of the metabolic waste, lactate	(Cockett et al. 1990; Fogolín et al. 2004)
	Over expression of Glutamine synthase-enable conversion of glutamate to glutamine	Eliminate need for glutamine and reduced lactate and ammonia accumulation	
Cell cycle	Expression of $P27^{KIP1}$ (effector gene that induces cell cycle arrest) coupled to recombinant gene of interest.	G1 phase arrest and increased productivity of recombinant protein	(Fussenegger et al. 1997)
Protein Secretion	Over expression of ER chaperones like BiP and ERp57	Facilitates folding and assembly of proteins in ER and catalyses the formation of disulfide bonds respectively leading to improved protein secretion	(Hwang et al. 2003; Borth et al. 2005)
Apoptosis Resistance	Over expression of anti-apoptotic genes like Bcl-x_l and EIB-19K	Increased apoptosis resistance under nutrient and serum deprived conditions, increased cell viability and higher protein yields	(Figueroa Jr. et al. 2007; Chiang & Sisk 2005)
Glycosyation	Over expression of ST6Gal that transfers sialic acid to galactose in an α2,6 linkage	Introduces the presence of human like α2,6 linked sialic acid	(Bragonzi et al. 2000; Monaco et al. 1996; Minch et al. 1995)

	Antisense knock down of CMP-sialic acid hydroxylase that converts NeuAc to NeuGc	Decreased proportion of NeuGc residues, which are potentially immunogenic in humans.	(Wong et al. 2006)
	Over expression of CMP-sialic acid transporter that transport it from cytosol to golgi	Increased recombinant protein sialylation that improve the circulatory half life	(Umaña et al. 1999)
	Over expression of GnTIII That generate bisecting GlcNAc	Increased proportion of bisecting GlcNAc, resulting in recombinant antibodies with enhanced ADCC	(Imai-Nishiya et al. 2007; Kanda et al. 2006)
	siRNA knockdown of Fut8 resulting in α1,6 linked fucose	Antibodies with defucosylated structures and enhanced ADCC	(Imai-Nishiya et al. 2007; Kanda et al. 2006)

1.6 Bacterial toxins

Many pathogenic bacteria produce toxins that play key roles in many infectious diseases. They can range from peptides to complex high molecular weight proteins and lipopolysaccharides. However, the majority of the toxins that play significant roles in the pathogenesis of diseases are proteins and have enzymatic activity. In some cases, the toxin itself is directly accountable for the majority of the symptoms of the disease, for example tetanus, anthrax and diphtheria. In others, the toxin is one of the virulent factors that play a contributory role to the disease process (Henkel et al. 2010; Montecucco et al. 1994).

Protein toxins can be classified into various groups based on their overall structure and mode of action. The toxin can act at the plasma membrane level, where they interfere with the signaling pathway or alter the membrane permeability. For example the *C. perfringens* α-toxin is a phospholipase C which hydrolyses membrane phospholipids (Titball et al. 1999). Others are intracellularly acting toxins where they enzymatically modify a specific cytosolic target. The latter kind of toxins generally have an AB toxin structure, where the B-subunit binds to a cell surface receptor and promotes translocation of the enzymatically active A-subunit into the cell (Clark et al. 2007; Menestrina et al. 1994). Cell intoxication in this case, is a four-step process composed of binding, internalization, membrane translocation and enzymatic modification of a cytosolic target. The receptor binding domain of

the B-subunit mostly recognizes specific oligosaccharides displayed on the surface of the host cells (Menestrina et al. 1994). The toxins are also delivered into the target cell with the aid of type III secretion systems that direct the formation of a molecular syringe which injects toxins from the bacterium into the host cell cytosol, such as described in *Yersinia pestis*, *Salmonella enterica* and *Pseudomonas aeruginosa* (Hauser 2009). Toxicity is usually attributable to the consequent proliferation of T-cells and the overproduction of cytokines.

1.6.1 Structure of bacterial toxins

Bacterial toxins that enter their target cells by binding to specific oligosaccharides on the cell membrane can have different structural organizations (Montecucco et al. 1994; Clark et al. 2007). One group of toxins which includes Shiga toxins, cholera toxin and pertussis toxin are characterized by an oligomeric B subunit composed of a pentameric disc-shaped protomer with a central cavity (Sixma et al. 1993; Sixma et al. 1991). Even though the individual binding domain has a low affinity binding site for the receptor glycans, high affinity cell associations are accomplished by the pentavalent binding. The catalytic domain, that has little protein–protein contacts with B, is linked via a linker peptide that penetrates into the central hole of the B oligomer (Merritt et al. 1994). In these toxins, it is not easy to identify the membrane translocating domain (Figure 5A). Another group of toxins including diphtheria toxin, difficile toxin and tetanus and botulinum neurotoxins are organized in three domains: catalytic domain translocating domain and the receptor binding domain (Montecucco & Schiavo 1993). The

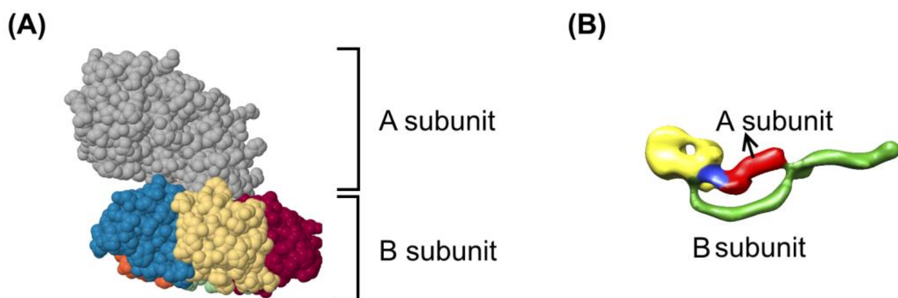

Figure 5. *Two different structures of bacterial toxins with intracellular targets. These toxins contain a catalytic A subunit linked to a B subunit, responsible for cell binding and penetration. (A) is the space filling 3D model of shiga holotoxin with five B subunits forming a pentameric ring. The A subunit is linked via the C-terminal helix and four β sheets. Source; PDB accession number1DM0. (B)3D construction of difficile toxin A comprising the catalytic A subunit (red) and the B subunit containing autoprotease (blue), delivery (yellow) and binding (green) domains.*

B subunit is comprised of the carboxy-terminal receptor binding domain and an amino-terminal domain involved in membrane translocation. The A subunit is linked to the B subunit via a peptide loop and an inter-chain disulfide bond (Figure 5B). Inhibition of the binding of the receptor binding domain to its cognate host receptor has the potential to prevent the translocation of the A subunit and thus preventing its enzymatic activity (Montecucco et al. 1994). Thus, interference with the binding of the toxin is a promising therapeutic strategy.

1.6.2 Multivalent inhibitors of bacterial toxins

Inhibitors of bacterial toxins can be designed to target different stages in the intoxication process, such as preventing the binding of the toxin to cell membrane receptors, preventing its translocation across the cell membrane, blocking its interaction with the intracellular target molecule and also by inhibiting its catalytic activity (Montecucco et al. 1994). According to many studies, interference with the first protein-carbohydrate interaction on the host cell surface is the most promising and feasible strategy that would prevent entry of the toxin into the cell (Zopf & Roth 1996). In addition, microbes are unlikely to develop resistance to such agents as the glycan recognition is tied to the biology of the toxin and is less susceptible to variation (Paton et al. 2010).

A number of studies have employed synthetic oligosaccharides corresponding to a specific receptor determinant in order to competitively inhibit toxin binding. However, such free oligosaccharides have low affinities for the protein toxins and in case of toxins released by enteric pathogens like *Vibrio cholerae*, Shiga toxigenic *Escherichia coli* (STEC), enterotoxigenic *E. coli* (ETEC), *Clostridium difficile* etc, the digestive enzymes present in the small intestine may cleave these free oligosaccharides making them ineffective (Paton et al. 2010). Therefore, effective toxin inhibitors are usually comprised of specific glycan epitopes displayed multivalently on scaffolds. The major multivalent bacterial toxin inhibitors include glycopolymers, glycodendrimers and tailored glycoclusters (Branson & Turnbull 2013).

- Polymers are used to organize multiple copies of the toxin ligand, and their relative ease of synthesis and variability of structure and length are beneficial for its use as a scaffold. For example, a polylysine scaffold carrying GM1 oligosaccharides was a more effective inhibitor of Cholera toxin than soluble GM1 oligosaccharides (Schengrund & Ringler 1989). The studies of polymer-based bacterial toxin inhibitors

have shown that inhibitors with higher density of ligands can decrease the overall binding due to steric hindrance of unbound ligands. Therefore, spacing of the ligand corresponding to the binding site dimensions can improve the effectiveness of the ligands (Polizzotti & Kiick 2006; Polizzotti et al. 2007).

- Glycodendrimers are characterized as highly branched 'dendrons' or 'wedges' that originate from a central multifunctional core unit and terminate in many reactive groups around their peripheries, which constitute the sites to which the bioactive saccharides are usually attached (Turnbull & Stoddart 2002; Chabre & Roy 2010). Most of the studies have used PAMAM or polypropylene imines as cores for dendrimers. Potential bacterial toxin inhibitors based on glycodendrimers have been synthesized for Cholera toxin, Vero toxin from *E. coli*, Shiga toxin etc (Thompson & Schengrund 1998; Thompson & Schengrund 1997).

- Templated assembly is another strategy for inducing protein dimerization whereby two pentameric serum amyloid P component proteins (SAP) were fused by simple divalent proline derivatives to form a face to face dimer. SAP harbors a pentameric structure similar to certain bacterial toxins and their binding sites are suitably spaced for a divalent ligand to bridge the binding sites in both proteins (Pepys et al. 2002). An inhibitor for Cholera toxin was constructed using this strategy (Liu et al. 2005).

1.7 Shiga toxins

Shiga and Shiga-like toxins produced by *Shigella dysenteriae* and enterohaemorrhagic *Escherichia coli* (EHEC) strains, respectively can cause serious complications during infection. Infection by these organisms has been associated with the ingestion of contaminated food and water or contact with infected humans or animals (Tarr et al. 2005; Bergan et al. 2012). The infection causes dysentery and haemorrhagic colitis, which may further lead to the life-threatening disease, hemolytic uremic syndrome (HUS), which is characterized by thrombocytopenia, hemolytic anemia, acute renal failure and various degrees of complications in central nervous system (Melton-Celsa et al. 2012; Nathanson et al. 2010; Petruzziello-Pellegrini & Marsden 2012). These infections have become an increasing threat to human health, especially in children and elderly (Melton-Celsa et al. 2012).

1.7.1 Toxin structure and mode of action

Shiga-like toxins are divided into two groups (Stx-1 and Stx-2) on the basis of the degree of sequence identity shared with Stx. Stx-1 have a higher sequence identity compared to Stx-2 (Jackson et al. 1987). These toxins consist of an enzymatically active A fragment and five B subunits responsible for binding to cellular receptors. Both subunits are released into the bacterial periplasm, where they assemble non-covalently into the holotoxin. The molecular weight of the intact toxin is about 70 kDa. The B subunits form a pentameric ring with a central pore in which the C-terminus of the A fragment is anchored. The A fragment contains A_1 and A_2 subunits that are joined by an internal disulfide bond. The A_1 fragment inactivates the 60S ribosomal subunit by removing one adenine from the 28S RNA, which inhibits protein synthesis (Fraser et al. 1994; Stein et al. 1992; Garred et al. 1997).

1.7.2 Cellular receptors

The receptor for most of the Shiga toxins is the neutral glycosphingolipid globotriaosylceramide (Gb3; also known as CD77 or the Pk blood group antigen) (Lindberg et al. 1987; Lingwood 1993). One variant toxin, Stx2e also binds to Gb4, which contains an additional terminal β1,3-linked *N*-acetylgalactosamine residue (DeGrandis et al. 1989). The crystal structure of the Stx1 B subunit complexed with a trisaccharide receptor analogue of Gb3 has revealed the existence of three trisaccharide-binding sites per B subunit monomer, which accounts for a strong multivalent binding to the cell surface with up to fifteen bound Gb3 molecules (Ling et al. 1998) (Figure 6).

In humans, Gb3 (Pk) expression is mostly observed in the kidney epithelium and endothelium, microvascular endothelial cells in intestinal lamina propria, platelets, and in subsets of B lymphocytes (Bergan et al. 2012). It is also

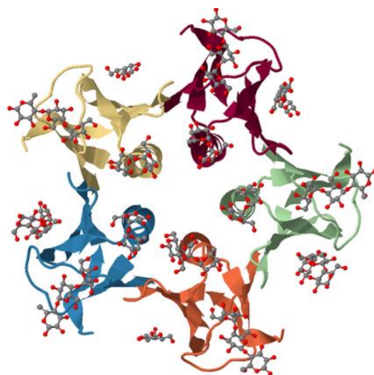

Figure 6. *A 3D model of the Stx1 B pentamer complexed with the Gb3 analogue (PK-MCO). Each B subunit can bind up to three ligands, accounting for 15 binding sites. Source Protein data bank, accession number 1BOS*

expressed in a subset of cells in the peripheral and central nervous system (Ren et al. 1999). However, kidneys are the major organs affected in diarrhea-associated hemolytic uremic syndrome. Human kidneys contain a series of Gb3 subspecies which vary in ceramide hydrocarbon chain length (C16–C24) and their degree of hydroxylation. All these factors and the membrane environment, including cholesterol levels were found to be essential for the toxin recognition process (Trachtman et al. 2012).

In mammals, studies suggest that both Pk and P1 determinants are synthesized by the same α4GalT/Gb3 synthase that adds a galactose to lactosylceramide and paragloboside, respectively (Iwamura et al. 2003; Thuresson et al. 2011). But lactosylceramide is considered to be the favored acceptor as Pk can be synthesized even at low enzyme levels (Figure 7) (Thuresson et al. 2011). Unlike human α4GalT (Gb3 synthase or A4GALT) which acts on glycosylceramides, pigeons have an α4GalT which is capable of transferring a Gal residue to β galactosides on glycoproteins (Suzuki & Yamamoto 2010). This enzyme was employed in Paper 1, for the engineering of P1 blood group determinants on our recombinant mucin type fusion protein.

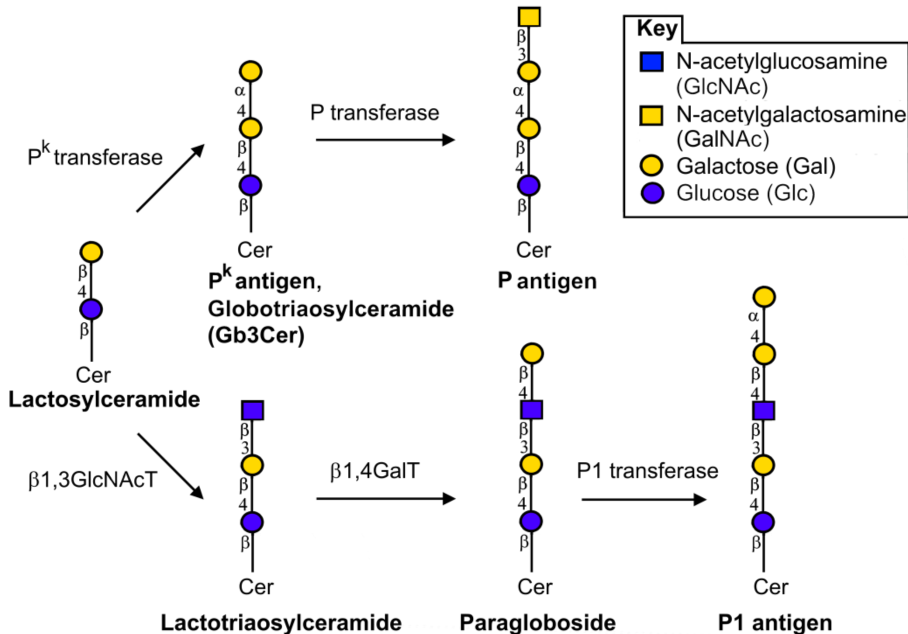

Figure 7. *Schematic representation of the biosynthesis of antigens of the human P blood group system. Recent evidence suggests that the enzyme adding α1,4Gal in the P^k and P1 structures is the same α1,4GalT glycosyltransferase and that it can act on different substrates. Gb3Cer (P^k antigen) is the native receptor of Shiga toxins.*

1.7.3 Shiga toxin inhibitors

Many multivalent inhibitors have been developed to prevent the binding of Shiga toxin to its host cell surface receptors. Some of them are discussed in this section. Polyacrylamide-based glycopolymers with varying degrees of Gb3 trisaccharide substitution have been used to neutralize Stx-1 (Dohi et al. 1999). One of the inhibitors was SYNSORB Pk, an orally delivered compound composed of an inert silicon-based matrix, Chromosorb-P, to which the trisaccharide was covalently linked. This failed to demonstrate any significant reduction of the severity of HUS (Takeda et al. 1999). Another versatile inhibitor was the STARFISH; an oligovalent, water-soluble inhibitor with two carbohydrate ligands arranged at the end of each arm of a pentavalent glucose core (Kitov et al. 2000). It was found that following subcutaneous administration of toxin and inhibitor, Starfish protected mice from the toxic effects of Stx-1 but not Stx-2 (Mulvey et al. 2003). Improvement in STARFISH, led to the development of another derivative termed DAISY that had slightly longer linker spacing between the oligosaccharide groups. It could partially protect mice from a challenge with both Stx-1 and Stx-2 (Mulvey et al. 2003). Super Twig was another multivalent inhibitor based on a glycodendrimer structure composed of carbosilane. It contains six Pk trisaccharides and protected the mice from purified toxins, Stx-1 and Stx-2 (Nishikawa et al. 2002). Promising results were obtained in studies using monoclonal antibodies specific for both Stx1 and Stx2 (Mukherjee et al. 2002a; Mukherjee et al. 2002b). However, to date no licensed drug against Shiga toxin is approved. In Paper I we have demonstrated the high avidity binding of Stx-1 and Stx-2 to PSGL-1/mIgG2b carrying multiple copies of the blood group P1 epitope.

1.8 *Clostridium difficile* toxins

Clostridium difficile is an anaerobic, spore-forming, gram-positive bacillus that is a common cause of hospital-acquired and antibiotic-associated diarrhea (Depestel & Aronoff 2013). The microbiotas of patients who receive antibiotics or chemotherapeutics become disrupted, compromising their colonization resistance and thereby making them susceptible to *C. difficile* infection (Hoffman 1994; Goldman et al. 1994). *C. difficile* is mostly acquired from an exogenous source, either from an infected individual or indirectly from a contaminated environment. *C. difficile*, evades the immune response, multiplies in the colon and produces toxins A and B. The organism is isolated mainly from fecal specimens obtained from neonates and the elderly. Even though it is often asymptomatic in neonates, in the elderly it is often associated with symptoms of diseases ranging from mild diarrhea to

serious diarrhea, with or without pseudomembranous colitis (Poutanen & Simor 2004). It can also cause life-threatening complications such as toxic megacolon, perforation and peritonitis. *C. difficile* infection, which are collectively known as *C. difficile* associated disease (CDAD) can be recurrent due to repeated recurrences of the manifestation (Pruitt & Lacy 2012; Poxton et al. 2001; Bartlett 2006).

1.8.1 Pathogenesis and virulence factors

When an individual is exposed to *C. difficile* spores, spores germinate and vegetative forms multiply resulting in colonization of the organism through the surface proteins (Vedantam et al. 2012). *C. difficile* can adhere to the mucus layer covering the enterocytes and penetrate the mucus layer with the aid of *C. difficile* surface layer proteins. Then they adhere to the enterocytes by means of its multiple adhesins. This results in the first phase of the pathogenic process and the virulence factors implicated in this step are; flagella composed of flagellin-Fli C and the flagellar cap protein-Fli D, associated with cell and mucus attachment (Tasteyre et al. 2001), proteolytic enzymes such as the cysteine protease, Cwp84 (Janoir et al. 2007), a cell-surface protein with adhesive properties (Cwp 66) (Waligora et al. 2001), a fibronectin-binding protein, Fbp68 (Hennequin et al. 2003) and a para-crystalline surface-layer decorated with cell wall proteins that mediates interaction with epithelial cells (Calabi et al. 2002). The second phase of the pathogenic process is the production of toxins. *C. difficile* produces three secreted protein toxins: TcdA, TcdB, and the binary toxin CDTab. The role in pathogenesis is unclear for CDTab which is an actin-specific ADP-ribosyltransferase produced in only few strains. (Perelle et al. 1997). Toxin A (TcdA) and toxin B (TcdB) are the main virulence factors which damage the human colonic mucosa (Pruitt & Lacy 2012).

1.8.2 Roles of *C. difficile* TcdA and TcdB

TcdA and TcdB are homologous AB toxins of 308 and 270 kDa respectively, with 49% identity and 63% similarity. They can inactivate the Rho/Ras superfamily of GTPases by glucosylation (Voth & Ballard 2005). These GTPases regulate a number of vital cellular processes including cell division, migration, cell-cell adhesion, cytokinesis, secretion, and maintenance of the cytoskeleton. In cell cultures, toxin treatment causes a retraction of cell processes and rounding of the cell body due to the disassembly of filamentous F-actin (Pruitt & Lacy 2012; Von Eichel-Streiber et al. 1996). TcdB is 100-10,000 times more potent in terms of cell rounding, also known as its cytopathic effect (Tucker et al. 1990). The action of toxins also leads to

cell death, referred to as cytotoxicity, through a number of different mechanisms including p53-dependent and p53-independent apoptosis, caspase-dependent and -independent apoptosis, as well as necrosis (Gerhard et al. 2008; Nottrott et al. 2007). TcdA is referred to as an enterotoxin that causes hemorrhage and fluid secretion in the intestines of rodents and causes extensive damage to the epithelial lining of the intestine, whereas TcdB causes minimal or no intestinal pathology. Therefore, the two toxins have distinctive properties both in animals and on cells. In case of CDAD, the toxins cause disruption of the barrier function by opening the tight junctions of the colonic epithelium through the inactivation of the Rho GTPases that regulate tight junction complexes. This increases the colonic permeability resulting in watery diarrhea, which is a typical feature of CDAD (Lyerly et al. 1988; Lyerly et al. 1985; Lyerly et al. 1982).

1.8.3 Structure of the toxins

The functional domains of the toxins include an N-terminal glucosyl transferase domain which holds the cytotoxic activity of the toxin, a cysteine auto protease domain, a hydrophobic pore-forming domain that is responsible for the translocation of the toxin into the cytosol, and a receptor-binding domain (RBD) (Schirmer & Aktories 2004; Just et al, 2000; Aktories & Just 1995). The RBD consists of highly repetitive structures termed as combined repetitive oligopeptides (CROPs) that are made up of 19–24 amino acid short repeats (SRs) and 31 amino acid long repeats (LRs), which together form the binding motif that interacts with glycans on the surface of the host cells. TcdA CROPs contain 32 SRs and 7 interspersed LRs, whereas it is considerably shorter in TcdB with 19 SRs and 4 LRs (von Eichel-Streiber & Sauerborn 1990).

1.8.4 Receptor binding

Various oligosaccharides have been shown to bind TcdA, of which Galα1,3Galβ1,4GlcNAc is the most extensively studied binding motif (Krivan et al. 1986). However, this trisaccharide sequence is not found on human cells, where the native ligand has not been definitely identified. Human I, Lewis X and Lewis Y antigens as well as a human glycosphingolipid that carries a common type 2 chain (Galβ1,4GlcNAc) can bind to TcdA (Tucker & Wilkins 1991; Teneberg et al. 1996). Two proteins, rabbit sucrose-isomaltase and human gp96, have been shown to serve as receptors for TcdA. The TcdA binding to sucrose-isomaltase was inhibited by galactosidase treatment, but the identity of the sugar on this ligand is unknown (Pothoulakis et al. 1996; Na et al. 2008). The co-crystal structure of

Figure 8. *3D model of the C-terminal fragment of C. difficile toxin A (TcdA-f2) bound to a synthetic derivative of the Galα1,3Gal receptor. The structure reveals seven putative binding sites for the entire receptor binding domain of TcdA. Source: Protein data bank, accession number 2G7C.*

the larger fragment (f2) of the TcdA CROPs complexed with a Galα1,3Galβ1,4GlcNAc derivative has revealed carbohydrate receptor binding junctions that are formed between the LRs and SRs of TcdA (Figure 8). The model suggests seven binding sites for TcdA and four sites for TcdB implying a multivalent binding along an extended binding domain (Ho et al. 2005; Greco et al. 2006).

1.8.5 Treatment options

An early resolution of the diarrheal symptoms in CDAD may be accomplished by withdrawing or changing the antibiotic therapy that triggered the disease. Oral metronidazole, vancomycin, or fidaxomicin treatment is the antibiotic recommendations for curing CDAD (Leffler & Lamont 2009; Johnson 2009). Active and passive vaccination methods are being studied in various animal models and are now in phase 3 clinical trials. Parenteral administration of a *C. difficile* toxoid vaccine might protect high-risk individuals against CDAD by virtue of an antibody response (Kotloff et al. 2001). Another innovative treatment involves the reconstitution of a protective intestinal flora via the application of vital bacteria. Probiotics like *Lactobacilli* species or *Saccharomyces boulardii* can be used to replace the pathogenic *C. difficile* flora (Verna & Lucak 2010). Fecal microbiota transplantation (FMT) is another strategy that has been increasingly utilized as a treatment for patients with recurrent CDAD in order to restore all the organisms that include the normal colonic flora by importing the colonic microbiome of a healthy person (Kelly et al. 2012). However, till now, a satisfactory treatment option for CDAD is still in question. In paper IV, we have shown that our recombinant mucin-type fusion protein, PSGL-1/mIgG2b, carrying Galα1,3Gal determinants can neutralize TcdA.

2 AIMS

The overall aim of this thesis was to produce recombinant mucin-type fusion protein (PSGL-1/mIgG2b) with tailored bacterial toxin or viral receptors.

Specific aims:

I. Produce a multivalent potential inhibitor of Shiga toxins by glyco-engineering CHO cells to express PSGL-1/mIgG2b carrying the blood group P1 determinant.

II. Analyze the *O*-glycan core chain biosynthesis and to characterize the core chain specificity of human ST6Gal I and CHST4 in CHO cells by transient expression of the glycosyltransferases.

III. Generate stable glyco-engineered Chinese hamster ovary (CHO) cells that secrete PSGL-1/mIgG2b carrying terminal α2,3- or α2,6-linked sialic acid on defined *O*-glycan core saccharide chains.

IV. Assess the ability of PSGL-1/mIgG2b carrying Galα1,3Gal determinants to neutralize TcdA-mediated hemagglutination of rabbit erythrocytes, and the cytopathic and cytotoxic properties of TcdA.

3 METHODOLOGICAL CONSIDERATIONS

The detailed description on the materials and methods utilized in this thesis is provided in the respective papers. Here, a brief discussion of the methodology is provided.

3.1 Cell culturing

For the generation of stable CHO-K1 cell lines, multiple cDNAs were transfected into adherently growing CHO-K1 cells cultured in the presence of serum. The high-producing single cell clones were then sequentially adapted to serum-free medium for large scale production of the fusion protein. Sequential adaptation to serum-free medium decreases the risk of gene expression being lost (Doyle & Griffiths 1998). Furthermore, besides being a potential source of infectious agents, serum supplementation makes the purification and further downstream processing more difficult. In terms of glycosylation, serum-free cultures were reported to result in a higher level of terminal sialylation and proximal fucosylation (Gawlitzek et al. 1995).

For large scale culturing of stable transfectants, the cells were maintained in a single cell suspension culture in shaker flasks rotated at 100 rpm in 37°C with 5% CO_2. However, the levels of glucose, glutamine and pH were not monitored and therefore it was difficult to reach the high cell densities obtained in Wave bioreactors and other industrial systems (Clincke et al. 2013). The glycosylation of recombinant protein can be affected by the culturing conditions (Li et al. 2010; Hossler et al. 2009). Therefore, the cell culture conditions as well as other parameters (oxygen concentration, accumulation of ammonia) influencing the productivity and glycosylation of PSGL-1/mIgG2b must be optimized for any commercial purposes.

3.2 Stable and transient transfection

In this thesis we have generated a repertoire of stable (Paper I, III and IV) and transient (Paper II) CHO-K1 cell transfectants for the production of a recombinant fusion protein, PSGL-1/mIgG2b, with tailored *O*-glycan substitution. The backbone of CDM8 vectors carrying the cDNA of PSGL-1/mIgG2b and different glycosyltransferase cDNAs has either the EF1α or CMV-IE promoter driving the expression of the transgene. All transfections were done with the Lipofectamine 2000 reagent (Invitrogen). Even though,

transient transfection experiments have gained popularity over the past few years due to the ease in analyzing the expression of the transgene, for large-scale production stable transfection is preferred. In paper II, we employed small-scale transient transfections to analyze the fine *in vivo* specificity of various glycosyltransferases and their impact on the *O*-glycome repertoire of CHO-K1 cells. In this case, transient transfections were advantageous as we could obtain small amounts of protein in less time and it was possible to analyze a large number of clones in order to investigate the glycosylation capacity of the transfected glycosyltransferases. In contrast to the episomal transcription of the plasmid taking place following transient transfections, the selection of stable transfectants relies on the presence of a selection marker that is used for selective growth of cells that have integrated the plasmid into their genomes. As the process of chromosomal integration is a random event, it can sometimes result in gene silencing, result in different levels of fusion protein expression or varied glycosylation of the fusion protein in the generated clones due to integration-dependent “position effects” (Dalton & Barton 2014). Therefore, the highest-producing cell clone expressing the desired carbohydrate determinant must be selected for the production of the secreted fusion protein.

3.3 Purification and quantification of PSGL-1/mIgG2b

The purification of the mucin-type protein is described in detail in the respective papers. Large-scale purifications were done by affinity chromatography using protein A columns followed by gel filtration, and small-scale purification was done on goat anti-mouse IgG (whole molecule) agarose beads. The purification of PSGL-1/mIgG2b on the protein A column appears to be glycosylation-dependent because some glycoforms of the fusion protein bind poorly this affinity matrix (unpublished observation). A second size-dependent purification step, gel filtration, is used to remove proteins co-purified with PSGL-1/mIgG2b on the affinity chromatography column.

The concentration of PSGL-1/mIgG2b produced by different cell lines was determined using a sandwich enzyme-linked immunosorbent assay (ELISA). The coating antibody for capturing the fusion protein was an affinity-purified, polyclonal goat anti-mouse IgG(Fc) antibody and for detection, an HRP-conjugated form of the same antibody was employed. A dilution series of IgG2b antibody with known concentration was used to derive the concentration of the fusion protein based on its optical density. However, it

should be considered that there are many factors that can influence the ability of these antibodies to recognize the fusion protein. The mIgG2b Fc part linked to PSGL-1 can have different conformation as compared to the Fc portion of the intact antibody. The altered glycosylation of the Fc portion can also influence the recognition (Gustafsson et al. 2011). In certain cases, the longer and branched *O*-glycans produced in glyco-engineered CHO-K1 cell lines may sterically hinder the detecting antibodies from reaching the Fc portion of the fusion protein. Therefore, the concentration of the PSGL-1/mIgG2b as determined by the ELISA is only used as guidance and is not taken as the definite concentration.

3.4 Characterization of PSGL-1/mIgG2b and its carbohydrate determinants using Western blotting

Antibodies recognizing the PSGL-1 and Fc parts as well as terminal carbohydrate determinants on the mucin-type fusion protein were employed in Western blot experiments in all the papers in order to confirm expression. In paper I and IV, anti-P1 and anti-Galα1,3Gal were used to detect the presence of blood group P1 and Galα1,3Gal terminal determinants, respectively. In paper II and III, lectins specific for defined carbohydrate determinants were used. Lectin recognition of a particular carbohydrate determinant depends on its presentation; type of linkage to the penultimate sugar residue, the core chain carrying the determinant, and sometimes the protein backbone on which the glycan is situated (Ambrosi et al. 2005). For example, the MAL-1 lectin requires the type 2 chain for optimal binding and can recognize this chain even if terminated with an α2,3-linked sialic acid. The MAL-2 lectin on the other hand requires α2,3-linked sialic acid for binding, but the binding affinity for α2,3-linked sialic is core chain dependent with relatively lower affinity for core 2 and 4 chain structures despite abundant α2,3 sialylation. These observations are clearly demonstrated in paper II and paper IV.

3.5 LC-MS analysis of *O*-linked glycans

In order to determine the composition, sequence and linkage information of *O*-glycans released from PSGL-1/mIgG2b, we have used liquid chromatography - mass spectrometry. In contrast to *N*-linked glycans which can be released from the protein backbone following enzymatic cleavage with PNGase-F, *O*-linked glycans are chemically released using strong alkali

under reducing conditions in the so called β–elimination reaction (Carlson 1968). This can be done in solution or following immobilization of the glycoprotein on PVDF membranes (Schulz et al. 2002; Karlsson et al. 2004). The analysis is performed on a LTQ ion-trap mass spectrometer which is coupled to a porous graphitized column that can separate glycans based on size and isomeric structure. The separated glycans are analyzed in the negative ion mode and the MS^n spectra are manually interpreted for structural assignment. With the help of UniCarb-DB, a reasonably quick MS2 spectral intensity comparison for annotating the structure can be done (Hayes et al. 2011). In this thesis, we have employed LC-MS to characterize the *O*-glycans released from our recombinant mucin-type fusion protein (PSGL-1/mIgG2b) produced in the glyco-engineered transient and stable CHO-K1 cell lines. In paper I and paper IV, the blood group P1 determinant and the Galα1,3Gal carbohydrate determinant on a core 2 *O*-glycan core structure were identified and in paper II and paper III, LC-MS was used to detect the major *O*-glycan core structures (core 1-4 and extended core 1). This technique was also used to differentiate the type 1 (Galβ1,3GlcNAc) or type 2 (Galβ1,4GlcNAc) outer chains and terminal α2,6-or α2,3- sialic acid substitution. The LC-MS analysis requires small amounts of starting material and is proved to be robust and sensitive in this thesis.

3.6 The Biacore biosensor - surface plasmon resonance

SPR is a label-free technology, capable of measuring real-time quantitative binding affinities and kinetics of interactions between molecules. We have employed this technique in paper I to measure the interaction between the mucin-type fusion protein and Shiga like toxins. In the Biacore instrument, one of the binding components, the ligand (Shiga toxin) is immobilized on a sensor chip surface and the other component, analyte (PSGL-1/mIgG2b carrying blood group P1 determinants) in solution is flowed over the sensor surface at different concentrations in a number of different injection cycles. The binding interaction is detected using an optical method that measures small changes in refractive index at the sensor surface. The change in angle of reflected light is proportional to the mass of the analyte at the surface. This change is translated to a response signal and is plotted in a graph called sensorgram with binding levels depicted as response units (RU) (Jason-Moller et al. 2006).

The sensorgram generated from an SPR experiment shows the main stages of the injection cycle. The curve depicting the binding process starts with a

baseline which represents no response shift. Due to the analyte-ligand complex formation, a positive slope (associate phase) is generated and as more and more analyte is bound, an equilibrium is reached where the curve reaches the steady state. When analyte is no longer injected the analyte ligand complexes starts to decay causing the disassociation of the analyte from the surface resulting in the disassociation phase. In order to reuse the ligand surface for an additional injection cycle, all bound analyte must be removed by a regeneration buffer that restores the base line (Rich & Myszka 2000). The affinity and kinetics of the ligand-analyte interaction were calculated according to a 1:1 binding model. More details on the determination of kinetic constants are provided in paper I and also in other references (Drescher et al. 2009; Karlsson et al. 1994).

3.7 Laser scanning confocal microscopy

The laser scanning microscope scans objects point by point by a finely focused laser beam. The principle for this distinct kind of microscopy was developed by Marvin Minsky in 1953 and towards the end of 1980s development of lasers for confocal microscopy became readily available (Rino et al, 2009). The key feature of confocal microscopy is its ability to increase the contrast of images and produce blur-free images and thereby improving their quality. Unlike a conventional fluorescence microscope, the confocal microscopy system has

- Point light source for illumination
- Point light focus within the specimen
- Pinhole at the image detecting plane
- Minimal blurring and sharper image due to the rejection of out-of-focal plane signal
- Higher contrast due to increase in signal-to-noise ratio achieved by minimizing interference from lateral stray light
- Improved resolution due to optical sectioning
- Higher Z resolution make confocal pictures more crisp and clear

In this thesis, the ability of our recombinant mucin type fusion protein carrying Galα1,3Gal *Cl. difficile* toxin A receptors to inhibit the toxin induced cytopathicity in CHO cells was analyzed using confocal microscopy. Bacterial toxins like *Cl. difficile* toxin, have a complex effect on cultured cells, especially on the morphology of cells (Fiorentini & Thelestam 1991). We used confocal microscopy to visualize these signs of a cytopathic effect like retraction and rounding of intoxicated CHO cells and polarization of the

nucleus to cell wall. Confocal imaging can resolve the complex three dimensional cell structures, for example the cytoskeletal fibers in the cytoplasm. It also gives greatly enhanced images of biological structures and more wealth of details compared to conventional fluorescence microscopy. This technique made it possible for us to visualize the difficile toxin A effects on CHO cells and also the impact of our recombinant mucin type fusion protein on the damaging effects of the toxin.

4 RESULTS AND DISCUSSION

4.1 Producing PSGL-1/mIgG2b with tailored glycosylation

This thesis focuses on the production of recombinant mucin-type fusion proteins (PSGL-1/mIgG2b) carrying specific carbohydrate determinants. PSGL-1/mIgG2b, developed by combining the extracellular part of PSGL-1 and the Fc portion of mouse IgG2b, has 6 potential *N*- and 106 potential *O*-glycosylation sites. This enables the multivalent presentation of specific carbohydrate determinants on the mucin-type scaffold. The glycosylation pattern of this fusion protein depends on the host cell used for its production and can be further modified by glyco-engineering the host cell. In all the papers, we have used CHO-K1 host cells for the production of PSGL-1/mIgG2b. Besides expressing the P-selectin glycoprotein ligand-1/mouse immunoglobulin G2b cDNA (PSGL-1/mIgG2b), plasmids encoding specific glycoysltransferases are expressed in CHO-K1 cells to tailor the desired carbohydrate determinant (Figure 9).

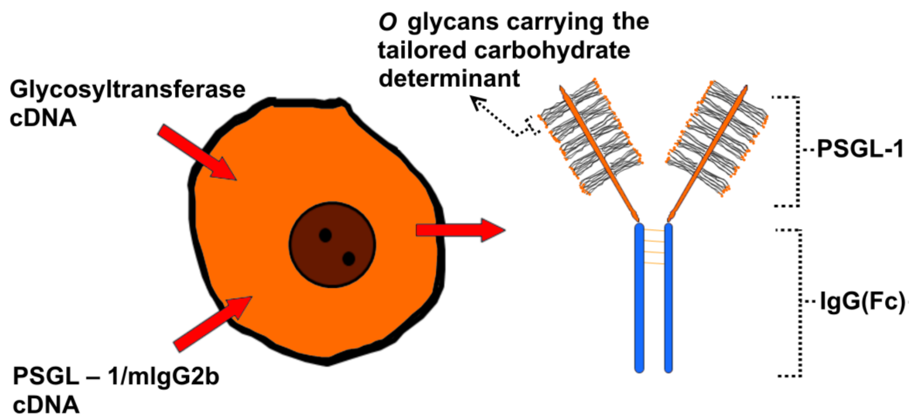

Figure 9. *Production of recombinant mucin-type fusion protein PSGL-1/mIgG2b with tailored carbohydrate determinants. The glycosylation of the mucin-type protein is dependent on the endogeneous glycosylation machinery of the host cell line and the transfected glycosyltransferase cDNAs.*

In paper I and IV, the functional carbohydrate receptors of Shiga toxin 1 and 2 (Stx1 and Stx2) and *C. difficile* toxin A were tailored by expressing pigeon α4GalT and porcine α3GalT respectively. The biological significance of these recombinant mucin type proteins carrying the bacterial toxin receptor

mimetics was evaluated by determining its binding strength (paper I) and also by assessing its ability to neutralize the biological properties of toxin like hemagglutination, cytopathic effects and cytotoxicity (paper IV). The *O*-glycan biosynthesis pathways in CHO cells was extensively studied by transiently (paper II) and stably (paper III) expressing the *O*-glycan core chain glycosyltransferases (C2 β6GnT1, C3 β3GnT6, extended C1 β3GnT3). The core chain specificity of human ST6GAL1 and CHST4 was assessed in paper II by transient expression and the potential competition between the endogenous and the expressed glycosyltransferases was also studied. In paper III, we have generated a repertoire of PSGL-1/mIgG2b with terminal α2,3- or α2,6-linked sialic acid on defined *O*-glycan core 1-4 and extended core 1 structures carrying either type 1 (Galβ3GlcNAc) or type 2 (Galβ4GlcNAc) outer chains in order to study the core chain-dependent binding activity of carbohydrate-binding proteins. The information about the different stable and transient CHO-K1 cell lines generated in this thesis is given in Table 3. Even though the cells are engineered to support the biosynthesis of certain defined *O*-glycan target structures, the cells will produce an array of different *O*-glycan structures including alternative target structures and various non-sialylated precursor saccharides. Competition between endogenous and exogenously expressed glycosyltransferases for the same precursor chain may explain *O*-glycan heterogeneity.

Table 3. *CHO transfections performed with designations of cell clones or bulk-selected cell populations and their respective target structures. CHO-K1 cells with their endogenous glycosylation machinery and the transfected P-selectin glycoprotein ligand-1/mouse IgG2b Fc and glycosyltransferase cDNAs generate the desired carbohydrate determinant. In the name designation, C stands for CHO-K1, P for PSGL-1/mIgG2b, followed by the expected O-glycan core structures and (B) denotes bulk-selected cell populations.*

Type of Transfection	Transfected glycosyl transferase cDNAs	Clone Designations	Tailored Carbohydrate determinant
Stable	C2 β6GnT1 + α4GalT	C-PP1	NeuAcα2,3Galβ1,3(Galα1,4Galβ1,4 GlcNAcβ1,6)GalNAc
Stable	C2 β6GnT1 + α3GalT	C-PGC2	NeuAcα2,3Galβ1,3(Galα1,3Galβ1,4 GlcNAcβ1,6)GalNAc
Stable	C2 β6GnT1	CP-C2	NeuAcα2,3Galβ1,3(NeuAcα2,3Galβ1,4 GlcNAcβ1,6) GalNAc
Stable	C3 β3GnT6 + β3GalT5	CP-C3-T1	NeuAcα2,3 Galβ1,3GlcNAcβ1,3GalNAc

Stable	C3 β3GnT6 + C2 β6GnT1 + β3GalT5	CP-C4-T1	NeuAcα2,3Galβ1,3GlcNAcβ1,3 (NeuAcα2,3Galβ1,3GlcNAcβ1,6)GalNAc
Bulk -Stable	C3 β3GnT6	CP-C3(B)	NeuAcα2,3 Galβ1,4GlcNAcβ1,3GalNAc
Bulk -Stable	C2 β6GnT1 + C3 β3GnT6	CP-C4(B)	NeuAcα2,3Galβ1,4GlcNAcβ1,3 (NeuAcα2,3Galβ1,4GlcNAcβ1,6) GalNAc
Stable	Ext C1 β3GnT3	CP-extC1	NeuAcα2,3Galβ1,4GlcNAcβ1,3Galβ1,3GalNAc
Bulk -Stable	C2 β6GnT1 + ST6Gal1	CP-C2-ST6(B)	NeuAcα2,3Galβ1,3 (NeuAcα2,6Galβ1,4GlcNAcβ1,6)GalNAc
Bulk -Stable	C3 β3GnT6 + β3GalT5+ ST6Gal1	CP-C3-T1-ST6(B)	NeuAcα2,6Galβ1,3GlcNAcβ1,3GalNAc
Bulk -Stable	C3 β3GnT6 + C2 β6GnT1 + β3GalT5 + ST6Gal1	CP-C4-T1-ST6(B)	NeuAcα2,6Galβ1,3GlcNAcβ1,3 (NeuAcα2,6Galβ1,3GlcNAcβ1,6)GalNAc
Bulk -Stable	B3GNT6 + ST6Gal1	CP-C3-ST6(B)	NeuAcα2,6Galβ1,4GlcNAcβ1,3GalNAc
Bulk -Stable	C3 β3GnT6 + C2 β6GnT1 + ST6Gal1	CP-C4-ST6(B)	NeuAcα2,6Galβ1,4GlcNAcβ1,3 (NeuAcα2,6Galβ1,4GlcNAcβ1,6)GalNAc
Bulk -Stable	Ext C1 β3GnT3 + ST6Gal1	CP-extC1-ST6(B)	NeuAcα2,6Galβ1,4GlcNAcβ1,3Galβ1,3 GalNAc
Transient	C2 β6GnT1 + CHST4		NeuAcα2,3Galβ1,3 (NeuAcα2,3Galβ1,4(6S)GlcNAcβ1,6) GalNAc
Transient	C3 β3GnT6 + CHST4		NeuAcα2,3Galβ1,4(6S)GlcNAcβ1,3 GalNAc
Transient	Ext C1 β3GnT3 + CHST4		NeuAcα2,3Galβ1,4(6S)GlcNAcβ1,3 Galβ1,3GalNAc

4.2 Characterization of PSGL-1/mIgG2b carrying tailored glycosylation

PSGL-1/mIgG2b purified by protein A affinity chromatography and gel filtration or by the use of anti-mouse IgG agarose beads is a protein of 250-350 kDa under non-reducing conditions and 100-180 kDa under reducing conditions as assessed by SDS-PAGE (Figure 10). The expected molecular weight of a non-glycosylated PSGL-1/mIgG2b dimer is about 117 kDa,

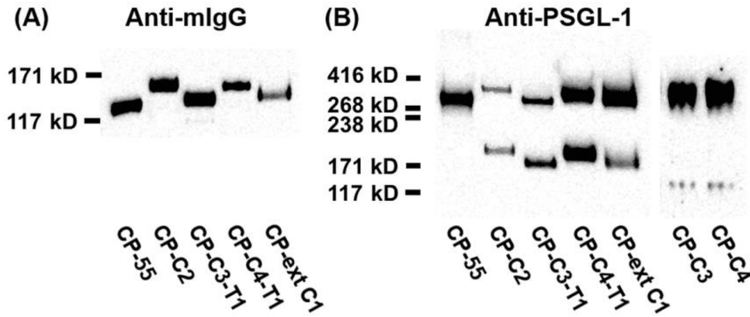

Figure 10. *SDS-PAGE and Western blot analysis of purified PSGL-1/mIgG2b carrying α2,3-sialylated O-glycan core structures. For Western blot analyses, 0.2 µg of protein were loaded and analyzed on SDS-PAGE under reducing (A) or non-reducing (B) conditions.After blotting, membranes were probed with anti-mIgG Fc (A) and anti-PSGL-1 (B). This figure is reproduced from paper III*

which is much less than the apparent molecular weight of the native fusion protein indicating abundant glycosylation. Full length fusion protein detection and size estimation were confirmed by staining with anti-IgG (Fc) (Figure 10A) and anti-CD162 (which recognizes PSGL-1, Figure 10B). The presence of complex glycans for example, core 2, core 4 and extended core 1 structures, on the fusion protein was suggested by an increase in size of PSGL-1/mIgG2b in the Western blot analysis.

The presence of the glyco-engineered glycan structure on the mucin type scaffold was confirmed by staining with specific antibodies against the carbohydrate determinant. The expression of blood group P1 and Galα1,3Gal determinants on PSGL-1/mIgG2b purified from C-PP1 (paper1) and C-PGC2 (paper IV) was verified using anti-P1 and anti-Galα1,3Gal antibodies. In paper II and III, three biotinylated lectins, MAL-1, MAL-2 (both from *Maackia amurensis*) and SNA (*Sambucus nigra* bark lectin) were used to detect Galβ1,4GlcNAc (type 2) with or without α2,3-linked sialic acid, glycan structures with terminal α2,3-linked sialic acid and α2,6-linked sialic acid, respectively.

4.3 Shiga-like toxin binds with high avidity to multivalent *O*-linked blood group P1 determinants on mucin-type fusion proteins (Paper I)

The binding of Shiga-like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2) to PSGL-1/mIgG2b carrying multiple copies of the blood group P1 determinant

was investigated with Western blotting and the biosensor Biacore. Chinese hamster ovary K-1 (CHO-K1) cells were stably transfected with linearized plasmids encoding the PSGL-1/mIgG2b fusion protein, the core 2 β1,6-N-acetylglucosaminyltransferase (GCNT1 or C2β6GnT-1) and the pigeon α1,4-galactosyltransferase (A4GALT or α4GalT). The α4GalT glycosyltransferase cDNA was cloned from pigeon liver. The sequential action of the endogenous glycosyltransferases in CHO-K1 (C1 B3GalT, B4GalT and ST3GAL1 or 4) and the stably expressed glycosyltransferases (GCNT1 and A4GALT) generates the blood group P1 determinant on a core 2 *O*-glycan core structure - Siaα2,3Galβ1,3 (Galα1,4Galβ1,4GlcNAcβ1,6)GalNAcol. The MS/MS data generated from LC-MS suggested that one of the major ions in the composite spectrum, m/z 1202.4 represented a core 2 structure terminated by sialic acid on one of the branches and a hexose (Hex) on the other, i.e. a tentative Galα4Galβ4GlcNAcβ6(NeuAcα3Galβ3)GalNAcol *O*-glycan. The other major ions were m/z 1040.3 and m/z 1331.3/665.32−, which represent tentative *N*-acetyllactosamine on core 2 with one or both chains terminated with sialic acid. These structures confirm the successful transfection of the C2β6GnT-1 (core 2 enzyme) cDNA, which together with an endogenous β1,4-galactosyltransferase (β4GalT) support the biosynthesis of a single type 2 *N*-aceyllactosamine unit.

4.3.1 PSGL-1/mIgG2b carrying the blood group P1 determinant binds Stx1 and Stx2

The interaction of C-PP1-produced PSGL-1/mIgG2b and Shiga-like toxins was evaluated by Western blotting and SPR assay. The binding of Shiga-like toxins (Stx1 and Stx2) to PSGL-1/mIgG2b carrying P1 determinants was compared to the binding to Pk and P1 neo-glycoconjugates. In Western blotting, strong binding of Stx1 to Pk-HSA and C-PP1 was observed, while only weak binding was observed to P1-BSA. The much strong binding of C-PP1 can be owed to the multivalent expression of the P1 epitope on the mucin scaffold. However, Stx2 did not bind to any of the glycoconjugates in Western blotting. The interaction of the Shiga-like toxins with the mucin type fusion protein and Pk-HSA was further investigated by the Biacore assay. In this system, Stx1 or Stx2 was immobilized on a CM5 dextran chip and Pk-HSA or C-PP1 were flowed over the surface and the binding was measured in real time on a Biacore 2000 instrument and plotted in a graph or sensorgram (Figure 11). The masses of C-PP1 produced PSGL-1/mIgG2b and Pk-HSA were 300 kDa and 73.5 kDa, respectively, and this is considered important as the response signal is directly proportional to the mass of the injected analyte. The dissociation equilibrium constant (KD value) was estimated by plotting the response at the end of the injections (550–600 s)

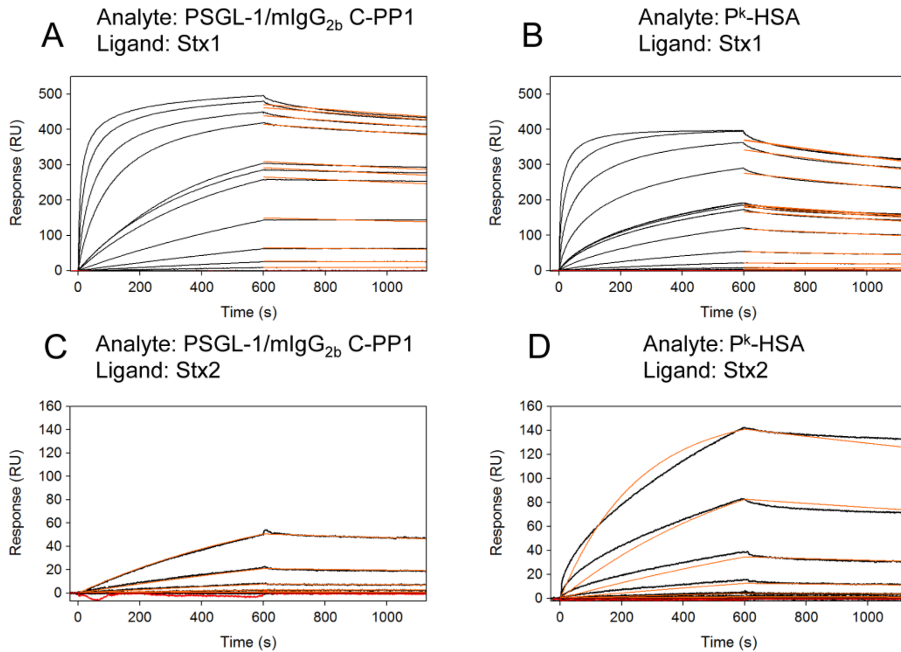

Figure 11. Biacore sensorgrams showing the binding of C-PP1 produced PSGL-1/mIgG2b and Pk-albumin to immobilized Stx1 (A and B) and Stx-2 (C and D). Curves are overlaid with the simulated disassociation curves based on a 1:1 binding model (orange). This figure is reproduced from paper I.

against the analyte concentration using a simple 1:1 model fit available in the Scrubber 2 software. Both Pk-HSA and C-PP1 KD values to Stx1 were in the low nanomolar range (39 nM and 16 nM, respectively). The shape of the binding- and dissociation curves and the low KD values lead us to suggest that the binding of Pk albumin and C-PP1 to Stx1 is multivalent.

In case of Stx2, the binding was considerably lower in comparison to Stx1. The binding curves had another shape and did not reach equilibrium. The KD of C-PP1 binding to Stx2 was 25 times weaker (379 nM) compared to Stx1, while the KD value of Pk-HSA binding to Stx2 was 3 times weaker (101 nM) than binding to Stx1. Comparing the kinetic constants of all the analyte-ligand pairs, it becomes apparent that the main difference in the KD values could be explained by the slow association (ka) of Stx2 compared to Stx1, as the dissociation (kd) values were similar. It should be noted that different KD values have been reported for Stx1 and Stx2 depending on the analysis method used (Kitov et al. 2000; Mulvey et al. 2003; Gallegos et al. 2012; Flagler et al. 2010). In this Biacore assay, C-PP1 produced PSGL-1/mIgG2b, Pk-HSA and Stx1 have multiple-binding sites that are not accounted for in

the employed 1:1 binding model. However, there is no binding model that includes as many possible binding sites as we have in our analyte-ligand combinations. Further, the exact valency of the analytes and immobilized ligand is not known in our system. The calculated values are, therefore, estimations of avidity and most suitable for comparisons between C-PP1 PSGL-1/mIgG2b and Pk-HSA. However, through this study we could show that, C-PP1 is a good binding partner of Stx1 but binds with lower affinity to Stx2.

A toxin inhibitor that can act in an early onset of the disease, could potentially prevent the toxin from entering the blood stream and subsequent systemic effects (Karmali et al. 1983). However, this may be difficult to accomplish considering the large surface area to be covered, accessibility problems and route of administration. A Shiga toxin inhibitor that is designed to prevent the toxin action in the vascular system by inhibiting the toxin binding to kidney microvascular endothelial cells may be more efficient in terms of preventing HUS. In animal models of STEC infections, oral treatment with linear acrylamide polymers substituted with highly clustered Gb3 trisaccharides bound to both Stx1 and Stx2 and decreased the disease symptoms (Watanabe et al. 2006). However, in case of a monovalent inhibitor of Shiga toxin, Synsorb-Pk, notably failed in clinical trials (Takeda et al. 1999). Other possible applications of PSGL-1/mIgG2b expressing the P1 epitope include the inhibition of P-fimbriated uropathogenic *E coli*, *Pseudomonas aeruginosa* (PA-I lectin), and *Staphylococcus aureus* (enterotoxin B) as the P1 determinant is utilized as the receptor for these microbes and toxins (Bock et al. 1985; Gilboa-Garber et al. 1994; Tikkanen et al. 1995; Tikkanen et al. 1996; Chatterjee et al. 1995).

4.4 Recombinant mucin-type fusion proteins with Galα1,3Gal substitution as *C. difficile* toxin A inhibitors (Paper IV)

In this paper, we investigated the capability of PSGL-1/mIgG2b carrying Galα1,3Galβ1,4GlcNAc determinants to bind and inhibit *Clostridium difficile* toxin A (TcdA). This recombinant mucin-like fusion protein was produced by a glyco-engineered stable CHO-K1 cell line, designated as C-PGC2 (Liu et al. 2005). PSGL-1/mIgG2b carrying the Galα1,3Gal carbohydrate determinant was characterized by SDS-PAGE and Western blot analyses using anti-IgG(Fc), anti-CD162 and anti-Galα1,3Gal antibodies. LC-MS analysis was also used to characterize the by β-elimination released, non-derivatized *O*-glycans of C-PGC2-produced PSGL-1/mIgG2b. A

comparison between the *O*-glycans released from PSGL-1/mIgG2b produced in C-PGC2 and C-PP1, which carry terminal Galα1,4Gal sequences, was done using LC-MS in order to differentiate *O*-glycans with Galα1,4Gal and Galα1,3Gal terminals, respectively. It was noted that the base peaks containing terminal α1,4Gal had a shorter retention time than those terminated in α1,3Gal. Both the *O*-glycans were sensitive to α-galactosidase treatment, indicating the presence of terminal αGal on *O*-glycans at m/z 911 and 1202. In the MS3 spectra, Galα1,3Gal terminated *O*-glycans yielded high intensity ^{0,2}A-H2O (m/z 263) ions, but lacked ^{0,2}A (m/z 281) and ^{2,4}A (m/z 221) fragmentation ions of the penultimate Gal residue. These cross-ring cleavages can be used as diagnostic ions to distinguish Galα1,3Gal from Galα1,4Gal.

4.4.1 C-PGC2 - a novel cell-based model for TcdA cytotoxicity

The cytopathic effects of TcdA on wild type CHO-K1 and glyco-engineered C-PGC2 cells expressing the Galα1,3Gal *C. difficile* toxin A receptor were compared in this study. It was evident from the cytopathic assay (cell rounding assay) that the expression of the Galα1,3Gal determinant increased the TcdA sensitivity of CHO-K1 cells (Figure 12). The cytopathic effects of TcdA on C-PP1 cells resembled those seen on wild type CHO-KI cells. This confirms that the increased TcdA sensitivity of C-PGC2 cells is due to the expression of the Galα1,3Gal toxin receptor on their cell surface. The

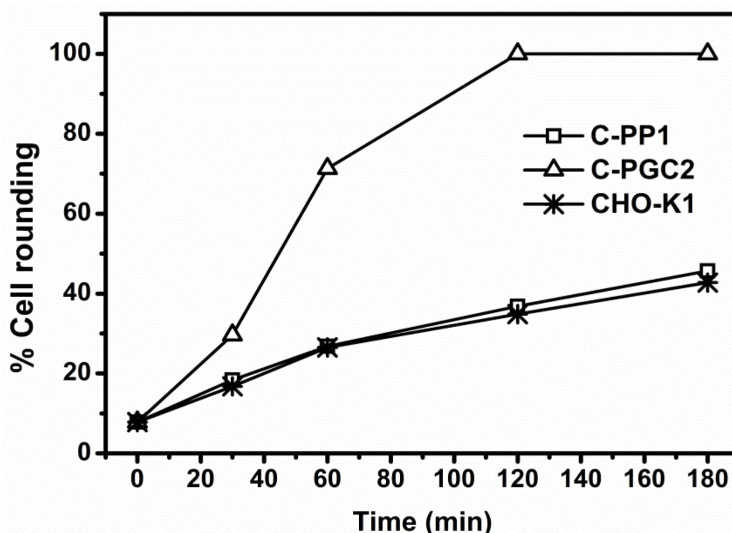

Figure 12. *Sensitivity of C-PGC2, CHO-K1 and C-PP1 cells to the cytopathic effect of 3.3 nM C. difficile toxin A. At the times indicated, cells were counted and the cytopathic effect was estimated by calculating the number of rounded cells as a percentage of the total number of cells.*

CPGC2 cell line which was 20-fold more sensitive to TcdA compared to the wild-type CHO-K1 is proposed as a novel cell-based model for TcdA cytotoxicity and neutralization assays.

4.4.2 Inhibition of TcdA mediated hemagglutination of rabbit erythrocytes

The binding of C-PGC2 -produced PSGL-1/mIgG2b to TcdA of *C. difficile* was initially confirmed by Western blotting. TcdA exhibits many patho-biological functions and one of them is its interaction with Galα1,3Galβ1,4GlcNAc sequences on rabbit erythrocyte membranes, leading to crosslinking and subsequent hemagglutination (Clark et al. 1987). The ability of the fusion protein to neutralize TcdA-mediated hemagglutination of rabbit erythrocytes was evaluated by a hemagglutination inhibition assay. PSGL-1/mIgG2b substituted with Galα1,3Gal determinants could completely inhibit hemagglutination at a concentration of 500 nM, making it a high efficiency inhibitor of the hemagglutination property of TcdA. Hemagglutination and enterotoxicity mediated by TcdA seem to be solely dependent on the interaction of the CROPs region that serves as the receptor binding domain of TcdA (Fiorentini & Thelestam 1991). The complete neutralization of the hemagglutination of rabbit erythrocytes mediated by TcdA suggests that the Galα1,3Galβ1,4GlcNAc determinant is the main receptor for TcdA CROPs.

4.4.3 Inhibition of TcdA mediated cytopathicity and cytotoxicity

However, this was not the case for the cytopathic and cytotoxicity neutralization assays where PSGL-1/mIgG2b carrying the Galα1,3Galβ1,4GlcNAc determinant could only partially neutralize the

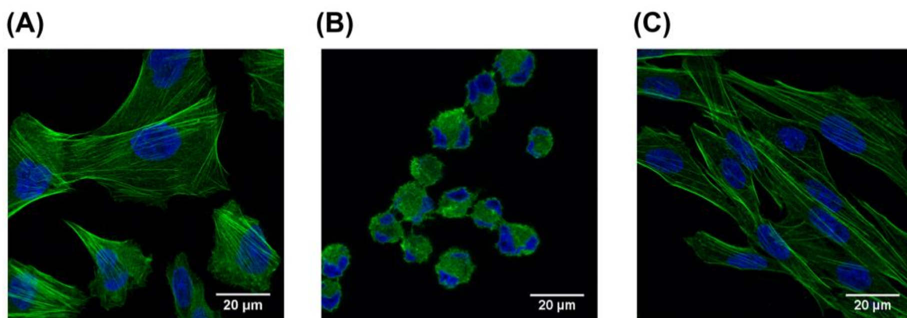

Figure 13. *Galα1,3Gal-substituted PSGL-1/mIgG2b neutralizes the cytopathic effect of C. difficile toxin A. Confocal staining of CHO-K1 filamentous actin (F-actin) staining using Alexa fluor 488-conjugated phalloidin; native CHO-K1 (A), CHO-K1 cells after exposure to a 100% cytopathic dose (1.6 nM) of toxin A for 18 hours (B), and CHO-K1 cells cultured under same conditions but exposed to TcdA pre-incubated with Galα1,3Gal-substituted PSGL-1/mIgG2b (C).This figure is reproduced from paper IV.*

TcdA effects. The cytopathic effect characterized by the destruction of the actin cytoskeleton was assessed by staining the actin filaments of CHO-K1 cells by confocal microscopy following staining with Alexa fluor 488-labelled phalloidin (Figure 13). Exposure to TcdA at a concentration of 1.6 nM completely disrupted normal F-actin organization in native CHO-K1 cells. The nuclei were polarized with an irregular shape. While exposure to TcdA pre-incubated with the fusion protein carrying Galα1,3Gal determinants exhibited a cytotonic effect due to the decreased TcdA potency. The results of the MTT based cytotoxicity assay also correlated with the cytopathic assay as the fusion protein exhibited only a partial neutralization with 34% cell survival in CHO-K1 and 48% cell survival in C-PGC2 cells (Figure 14).

These results indicates that TcdA can display its cytopathic action in the absence of the CROPs region suggesting that an additional binding activity may be encoded in the region preceding the C-terminal repeats and which can bind to alternate cellular receptors other than the Galα1,3Galβ1,4GlcNAc determinant. Many studies also supports this hypothesis by showing that removal the CROPs from TcdA only reduces the cytopathic potency and cannot completely inhibit the cytotoxic properties (Teichert et al. 2006; Olling et al. 2011). Even though we have provided the evidence for the usefulness and efficacy of PSGL-1/mIgG2b in blocking the CROPs region of TcdA in this study, further investigations are required to provide strong evidence for preventing the toxic effects seen on the large intestine during a *C. difficile* infection.

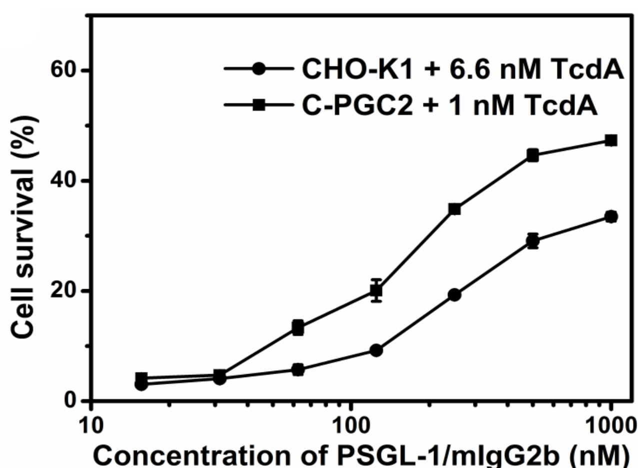

Figure 14. *The inhibition of C. difficile toxin A cytotoxicity by Galα1,3Gal-substituted PSGL-1/mIgG2b as assessed by the MTT assay. The capability of Galα1,3Gal-substituted PSGL-1/mIgG2b to neutralize TcdA (1 nM TcdA on C-PGC2 and 6.6 nM on CHO-K1) was analyzed in the MTT assay following co-incubation of the TcdA and the fusion protein assay. The cytotoxicity assay was performed after 72 hours of TcdA incubation in both experiments. Each data point represents the mean ± SEM of the three separate experiments.This figure is reproduced from paper IV.*

4.5 *O*-glycan repertoires on a mucin-type reporter protein expressed in CHO cell pools transiently transfected with *O*-glycan core enzyme cDNAs

The recombinant mucin-type fusion protein (PSGL-1/mIgG2b) was used as a probe to study and predict the biosynthetic pathways of *O*-glycans and the potential competition between glycosyltransferases involved in *O*-glycan core chain biosynthesis in CHO-K1 cells. The repertoire of *O*-glycans on PSGL-1/mIgG2b following transient expression of cDNAs encoding the enzymes responsible for extended core 1, core 2 and core 3 *O*-glycan biosynthesis was characterized. The core chain specificity of human ST6GAL1 and CHST4 was also assessed by co-transfection of plasmids encoding these enzymes. Liquid chromatography–mass spectrometry and Western blotting were utilized to map the *O*-glycome of the expressed mucin-type fusion protein. The *O*-glycans released from purified PSGL-1/mIgG2b protein produced in the CHO-K1 cells without any co-transfection of other glycosyltransferases is mostly dominated by mono- and disialylated core 1 *O*-glycans [Neu5Acα2,3Galβ1,3GalNAcol, 51% and Neu5Acα2,3Galβ1,3(Neu5Ac2,6)GalNAcol, 30%]. A low GCNT1 activity was also observed in CHO cells as trace amounts of core 2 *O*-glycans were detected in the LC-MS analysis (Figure 15A). Co-expression of CHST4 together with PSGL-1/mIgG2b did not result in any detectable sulfation. Similarly, no Neu5Acα2,6Gal-residues were identified on PSGL-1/mIgG2b expressed in CHO cells that were transiently co-transfected with ST6GAL1.

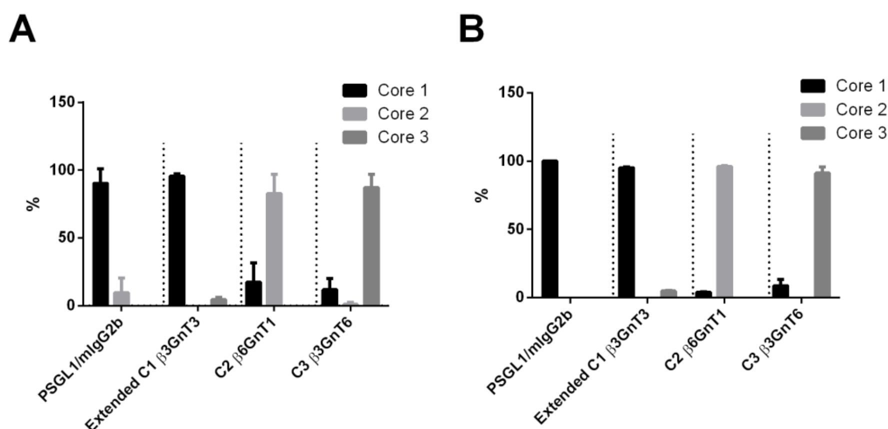

Figure 15. *Core chain distribution in O-glycans derived from PSGL1/mIgG2b transiently expressed in CHO cells with the co-expression of extended B3GNT3, GCNT1 or B6GNT1. The A panel represents the distribution of O-glycan core chains on PSGL-1/mIgG2b without the co-expression of ST6Gal I and the B panel with ST6Gal I co-expression. Each structure was quantified with base peak intensity of LC–MS data.*

This reconfirms that ST6GAL1 is unable to act on core 1 (Galβ1,3GalNAcα) *O*-glycans.

4.5.1 Transient expression of the extended core 1 enzyme

When the B3GNT3 enzyme was co-transfected into CHO-K1 cells, 38% of the core 1 *O*-glycans were extended by a β1,3-linked GlcNAc. Strong competition between the B3GNT3 and the endogenous ST3GAL (1 or 4) was observed as the amount of sialylated core 1 structures was decreased by 27% upon co-expression of B3GNT3. The extended core 1 *O*-glycan core structure was further elongated by an endogenous β1,4-galactosyltransferase (β4GalT) activity and a subsequent α2,3-sialyltransferase activity. Low amounts of core 3 *O*-glycans were also detected in this clone, which may be due to the activation of B3GNT6 in CHO cells following transient expression of extended B3GNT3. Another hypothesis would be that B3GNT3 has indeed a weak B3GNT6 activity. In fact, it has the highest homology to the core 3 enzyme among the β3-glycosyltransferase family members (Narimatsu et al. 2006). Additionally, the poly-LacNAc synthase, B3GNT2, was active following transient expression of the extended core 1 enzyme. Poly-LacNAc - a scaffold for further fucosylation, sialylation and sulfation - was more frequently sulfated following co-transfection of CHST4 because of the presence of more internal GlcNAc residues available for sulfation. The MECA-79 epitope (Galβ1,4(6S)GlcNAcβ1,3Galβ1,3GalNAc) was the predominant sulfated structure as revealed by the LC-MS analysis, which was also confirmed by Western blots. ST6GAL1 had its highest activity on extended core 1 *O*-glycans and it decreased the level of α2,3-sialylation and the overall degree of Neu5Ac substitution. This was not observed when co-expressed with other *O*-glycan core enzymes.

4.5.2 Transient expression of the core 2 enzyme

CHO co-transfected with GCNT1 supported high expression of core 2 *O*-glycans in CHO-K1 cells. The newly synthesized core 2 branches carry the terminal type 2 chain due to the endogeneous activity of β4GalT. No poly-LacNAc extension was observed on the core 2 branch. Only a low degree of sulfation was detected on the C6 branch of core 2 *O*-glycans when CHST4 was co-transfected. Co-expression of ST6GAL1 capped the core 2 *O*-glycans with α2,6-linked sialic acid, but it did not alter the level of α2,3 sialylation or the overall level of Neu5Ac substitution.

4.5.3 Transient expression of the core 3 enzyme

Co-transfection of B3GNT6 (core 3 *O*-glycan synthase) with PSGL-1/mIgG2b resulted in high amounts of core 3 *O*-glycans on the fusion protein; amounts accounting for up to 86% of the total *O*-glycans (Figure 15). There is a competition between the endogenous B3GALT (C1 β3GalT) and the transiently expressed B3GNT6 for the same acceptor, resulting in decreased amounts of core 1 *O*-glycans. Similar to the extended core 1 *O*-glycans, core 3 *O*-glycans were either terminated by α2,3-sialylation or were further elongated with additional LacNAc units. CHST4 had the highest activity on core 3 *O*-glycans. The predominant sulfated structure was Galβ1,4(6S)GlcNAcβ1,3GalNAc of m/z 667. Upon co-transfection with ST6GAL1, some of the core 3 chains were terminated by α2,6-linked sialic acids. In contrast to what was observed on the extended core 1 structure, the ST6GAL1 activity on core 3 increased the level of Neu5Ac and Neu5Gc substitution.

In conclusion, employing relatively small-scale transient transfection experiments we were able to investigate the core chain specificity of sialyl- and sulfotransferases involved in the biosynthesis of bioactive carbohydrate determinants. It is important to determine the fine *in vivo* specificity of a particular glycosyltransferase in order to recreate the biologically important carbohydrate determinants on defined *O*-glycan chains. Even though all the glycosyltransferase sequences used in the study were of human origin, the experimental model can be also used to compare the glycosyltransferases from different species in the context of *O*-glycan core chain specificity.

4.6 A Panel of recombinant mucins carrying a repertoire of sialylated *O*-glycans based on different core chains for studies of glycan binding proteins

Because of the pathobiological significance of sialylated glycans in general and as recognition motifs in a large number of microbial-host interactions in particular (Varki 2008), we aimed to develop a tool box based on our recombinant mucin-type fusion protein substituted with a diverse selection of sialylated *O*-glycans. A panel of stable CHO-K1 cell lines and bulk-selected cell populations was engineered that supported the expression of a repertoire of *O*-glycan core structures (core 1-4 and extended core 1) presenting terminal α2,3- and α2,6-linked sialic acid on type 1 (Galβ1,3GlcNAc) or type 2 (Galβ1,4GlcNAc) outer chains. Four single cell-cloned stable CHO

cell lines secreting PSGL-1/mIgG2b carrying the extended core 1 (CP-ext C1), core 2 (CP-C2), core 3 type 1 (CP-C3-T1), and core 4 type 1 (CP-C4-T1), and nine bulk-selected stable cell populations including the core 3 (CP-C3), core 4 (CP-C4) and all the ST6GAL1 transfectants were glyco-engineered. The mucin-type fusion protein produced from all the cell lines and populations were characterized for their *O*-glycan distribution and were subjected to lectin staining using MAL-1, MAL-2 and SNA. The major assumption that was made based on the LC-MS analysis of the major *O*-glycan core structures (core 1-4 and extended core 1) was that the B3GNT6 converts core 1 to core 3 more efficiently than GCNT1 converts core 1 to core 2.

4.6.1 PSGL-1/mIgG2b carrying *O*-Glycans extended with type 1 outer core chains

The introduction of the human B3GALT5 together with B3GNT6 generated core 3 *O*-glycans with a type 1 (Galβ3GlcNAc) outer core chain. This was also confirmed by MAL-1 Western blotting, which recognizes the type 2 chain (Galβ4GlcNAc) with or without α2,3-linked sialic acid and therefore revealed reduced staining following B3GALT5 expression. Because of the competition between β3GalT5 and the endogenous β4GalT enzyme(s), a mixture of type 1 and 2 outer core chains was obtained. We also noted that there was a core chain-dependent recognition of the type 2 chain (Galβ4GlcNAc) with or without α2,3-linked sialic acid by MAL-1 as the type 2 chain on core 2 stained weaker than the type 2 chain on core 3. Using negative-ion mode LC-MS/MS, type 2 and 1 chains were distinguished by the presence or absence, respectively, of the diagnostic ions arising from a cross-ring cleavage of GlcNAc ($^{0,2}A_{GlcNAc}$-H2O and $^{0,2}A_{GlcNAc}$). It was also noted that the type 2 chain-containing *O*-glycans eluted earlier on the graphitized carbon column. In case of the sialylated type 1 and type 2 *O*-glycans, they were only distinguished by virtue of their different retention times on the LC column.

4.6.2 PSGL-1/ mIgG2b carrying α2,3- and α2,6-sialylated *O*-glycan core structures

For the detection of terminal α2,3- and α2,6-linked sialic acids, we used MAL-2 and *Sambucus nigra* agglutinin (SNA), respectively. As for the MAL-1 staining, we observed a core chain-dependence of MAL-2 binding as it appeared to bind less well to core 2 and 4 *O*-glycans despite them having abundant α2,3-sialylation. All the stable clones lacking ST6GAL1 were found to be negative with SNA staining, confirming that the CHO cells only

have ST3GALT activity. The presence of α2,6-sialylated *O*-glycans was determined by two criteria: (1) the presence of specific fragmentation ions of α2,6-linked sialic acid in the MS/MS spectra (Thomsson et al. 2012) and (2) shorter retention times on the graphitized carbon column. Our LC-MS/MS results also confirmed that the human ST6GAL1 cannot use the type 1 outer chain as an acceptor as no α2,6-sialylation was detected on *O*-glycans of PSGL-1/mIgG2b expressed in CP-C3-T1-ST6 and CP-C4-T1-ST6. However, the endogenous ST3GAL(s) activity(ies) in CHO cells supports terminal α2,3-sialylation on all *O*-glycan core structures, both on type 1 and type 2 outer chains. The binding specificity of sialic acid-specific microbial adhesins and mammalian lectins can be changed by many factors such as the types of sialic acid, their linkages to the underlying carbohydrate chain, the nature of the underlying monosaccharides, the absence or presence of concomitant fucosylation and/or sulfation, and the types of linkages (1,3 or 1,4) between inner monosaccharide moieties of the carbohydrate chain (Varki 2008; Varki 1997; Gagneux et al. 2003). Therefore, it is important to carefully characterize the specificities of these glycan-binding proteins and we believe that a reporter protein like our PSGL-1/mIgG2b expressing a repertoire of target glycan structures carrying different linkages and substitutions will be beneficial in that respect.

5 CONCLUSIONS

- ❖ PSGL-1/mIgG2b carrying the multivalent *O*-linked carbohydrate receptors of Shiga-like toxin and *C. difficile* TcdA was successfully produced in the glyco-engineered CHO-K1 cells, C-PP1 and C-PGC2, respectively (Paper I and Paper 1V)

    - PSGL-1/mIgG2b carrying the blood group P1 bound strongly to Stx1 in Western blot and Biacore assays, while Stx2 bound with lower affinity

    - PSGL-1/mIgG2b carrying the Galα1,3Gal determinant was proved to be a high efficiency inhibitor of the hemagglutination property of TcdA and displayed moderate neutralization capability of TcdA cytotoxicity

- ❖ CHO cells were successfully glyco-engineered transiently and stably in order to produce PSGL-1/mIgG2b that carried *O*-glycans with different *O*-glycan core saccharides (Paper II and Paper III)

    - the recombinant mucin-type fusion protein with its *O*-glycans was used as probe to understand the biosynthetic pathways of *O*-glycans and the potential competition between glycosyltransferases involved in *O*-glycan core chain biosynthesis

    - The core chain specificity of human ST6GALI and CHST4 was assessed by means of transient expression

    - A repertoire of sialylated (α2,3- or α2,6) *O*-glycans based on different core saccharides with either type 1 (Galβ3GlcNAc) or type 2 (Galβ4GlcNAc) outer chains was stably generated in CHO-K1 cells for determining the fine *O*-glycan binding specificity of sialic acid-specific microbial adhesins and lectins

6 FUTURE PROJECTS

6.1 Paper I

The high avidity binding of P1-decorated PSGL-1/mIgG2b to Shiga-like toxin 1 and 2 (Stx1 and 2) makes it a potential therapeutic inhibitor of these toxins. The next step would be to evaluate its inhibitory effect on Shiga-like toxin binding by using a cellular model for hemolytic uremic syndrome (HUS) with relevant target cells. For this purpose, microvascular kidney endothelial cells will be used as target cells and the toxins will be FITC-conjugated or biotinylated in order to quantify the binding. The inhibitory effect of the recombinant mucins will be assessed by pre-incubating the toxins with C-PP1 produced recombinant mucin type fusion protein. The role of endothelial cell activation on Shiga-like toxin binding will be assessed using TNF-α and IL-1β. If differences in binding activity of Shiga-like toxins to activated and non-activated endothelial cells are detected, it would be beneficial to use glycomics techniques to identify the difference in global glycan phenotypes between cells of the two activation states. Another direction to this study would be to employ a mouse model of HUS (Keepers et al. 2006) and assess the effect of intravenous administration of recombinant mucin carrying P1 determinants or control mucins in this model.

6.2 Paper II and Paper III

The repertoire of recombinant mucins carrying sialylated *O*-glycan core structures generated in the study will be used to define the fine *O*-glycan binding specificity of sialic acid-specific microbial adhesins and lectins. The preferential binding of avian and human influenza virus to α2,3- and α2,6-linked sialic acids, respectively, has been shown in many studies (Gagneux et al. 2003; Viswanathan et al. 2010; Stevens et al. 2006; Olofsson et al. 2005). Using our panel of recombinant mucins carrying core 1-4 and extended core 1 *O*-glycans presenting terminal α2,3- and α2,6-linked sialic acid on type 1 or type 2 outer chains, the binding specificities of many different virus strains, for example the oculotropic viruses EV70 and adenovirus 37 (Ad37), can be characterized (Nokhbeh et al. 2005).

6.3 Paper IV

Even though, the neutralization ability of C-PGC2-produced fusion protein against TcdA cytotoxicity has been shown in this study, further investigations are required to provide a novel therapeutic approach for preventing the toxic effects seen on the large intestine during a *C. difficile* infection. In a recently established mouse model of *C. difficile*-associated disease (CDAD) (Chen et al. 2008), we would evaluate the therapeutic effect of the recombinant mucins administered locally. In Paper I, we have successfully used SPR to assess the binding strength between the C-PP1 mucin and the Stx-1 toxin. In a similar way, it would be beneficial to get an estimate of the binding strength of the C-PGC2 mucin using SPR. It would also be interesting to assess the TcdA inhibitory activity of the mucin using cells other than CHO-K1 such as colon epithelial cells that express the native human receptor of the toxin. This would be useful in establishing a more physiological cell-based model.

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