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STUDIES ON THE BIOSYNTHESIS OF ABH AND LEWIS EPITOPES ON O-GLYCANS

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Ce qui a été cru par tous,
et toujours et partout,
a toutes les chances d'être faux

Paul Valéry

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. Absorption of anti-blood group A antibodies on P-selectin glycoprotein ligand-1/immunoglobulin chimeras carrying blood group A determinants: core saccharide chain specificity of the Se and H gene encoded alpha1,2 fucosyltransferases in different host cells. **Lofling, J.**, Hauzenberger, E. and Holgersson, J. 2002 *Glycobiology 12, 3; 173-82*

II. Glycosyltransferases involved in type 1 chain and Lewis antigen biosynthesis exhibit glycan and core chain specificity. Holgersson, J. and **Lofling, J.,** 2006. *Accepted for publication in Glycobiology*

III. An engineered cell line for Lewis B-mediated *Helicobacter pylori* adhesion. **Lofling, J.**, Diswall, M., Eriksson, S., Borén, T., Breimer, M. and Holgersson, J., *submitted to Infection and Immunity*

IV. Core saccharide dependence of SLe^x biosynthesis. **Lofling**, J. and Holgersson, J., *submitted to Glycobiology*

ABSTRACT

ABH and Lewis antigens are carbohydrate epitopes (glycans) found on a wide variety of cell surfaces. They are involved in many biological processes, e.g transplantation rejection, bacterial and viral adhesion, cell-cell communication and metastasis. The *in vivo* synthesis of glycans is mediated by glycosyltransferases (GTs). Common GTs for the formation of ABH and Lewis antigen is fucosyltransferases (FucTs), either α2- or α3/4-FucTs. Glycans on proteins are usually O- or N- linked. On O-glycans, ABH and Lewis epitopes are carried by different core structures. In this thesis we report on the glycan- and core chain-dependence *in vivo* of FucTs and β3galactosyltransferases (β3GalTs) involved in ABH and type 1 chain Lewis antigen biosynthesis. To this end, P-selectin glycoprotein ligand-1 (PSGL-1) or CD43 (both O-linked type), or α1-acid glycoprotein (AGP; N-linked type) IgG fusion proteins were expressed in cells co-transfected with FucTs and the blood group A gene or β3GalTs and different core enzymes. The knowledge gained was used to engineer various tools of diagnostic or therapeutic value. These include absorbers of anti blood group A-antibodies, and cells that can successfully be used in adhesion of *Helicobacter pylori*, which is a bacterium that causes gastric cancer and ulcers.

Blood group A epitopes were synthesized on PSGL-1/mIgG in 293T, COS and CHO-K1 cells and the epitope density was found to be highest in CHO co-expressing FUT2 and the A synthase. This PSGL-1/mIgG was used for absorption of anti-blood group A antibodies in human blood group O serum. At least 80 times less A trisaccharides on PSGL-1/mIgG in comparison to a synthetically made absorbent were needed for the same level of antibody absorption.

FUT1 and FUT2 were both found to direct α2-fucosylation on type 1 chains on both N- and O-glycans. When comparisons were made for Galβ4GlcNAc (type 2) and Galβ3GalNAc (type 3 or core 1) preference on O-glycans, FUT1 and FUT2 preferentially fucosylated type 2 and type 3, respectively.

β3Gal-T1, -T2 and -T5 could synthesize type 1 chains on N-glycans, but only β3Gal-T5 worked on O-glycans. The latter enzyme acted on both core 2 and core 3 O-glycans. Furthermore, the specificity of FUT3 and FUT5 in Le^a and Le^b synthesis was different, with FUT5 fucosylating H type 1 on core 2, but FUT3 fucosylating H type 1 almost only on core 3. We also found that the Le^a-specific antibody T174 did not react with Le^a on core 3.

On CD43/IgG, FUT3, FUT5, FUT6 and FUT7 gave rise to SLe^x on core 2 O-glycans in CHO, whereas only FUT5 and FUT6 were effective in producing SLe^x on core 3, as detected with the monoclonal antibody CSLEX. PSGL-1 did also support SLe^x production by FUT3, FUT5, FUT6 and FUT7 on core 2. In contrast, no SLe^x at all was detected on PSGL-1 carrying core 3 modifications. KM93 reacted well with SLe^x on core 2, but did not at all stain the same epitope on core 3 O-glycans, regardless of the fusion protein used. FUT3, FUT5, FUT6 and FUT7 were all able to produce SLe^x on AGP in CHO but not in COS and 293T.

Taken together, our findings point to the importance of protein sequence and core saccharide structure for FucT activity and Lewis epitope biosynthesis, as well as for the binding of antibodies. Our results further provide a good framework for future investigations on the role of carbohydrates in metastasis, bacterial and cellular adhesion and the role of multivalency for inhibition of protein-carbohydrate interactions.

CONTENTS

LIST OF ABBREVIATIONS

AGP	α_1-acid glycoprotein
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FucT	fucosyltransferase
GalNAc-T	N-acetylgalactosaminyltransferase
Gal-T	galactosyltransferase
GI	gastrointestinal
GSL	glycosphingolipid
GT	glycosyltransferase
IgG	immunoglobulin G
IgM	immunoglobulin M
Le	Lewis
LN	lactosamine
LPS	lipopolysaccharide
mAb	monoclonal antibody
PBS(T)	phosphate-buffered saline (with 0.05% Tween-20)
PEI	polyethylenimine
PMN	polymorphonuclear cell
PNGaseF	peptide:N-glycosidase F
pp-GalNAc-Ts	UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase
PSGL-1	P-selectin glycoprotein ligand-1
RBC	red blood cell
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sia	sialic acid
SiaT	sialyltransferase
SLe^x	sialyl Lewis X
TBS(T)	tris-buffered saline (with 0.05% Tween-20)
TGN	trans-Golgi network
Tx	transplantation

PREFACE

Meier *et al.* reported in 1993 about expression of SLe^x on a recombinant LFA/IgG protein (1). The idea was that the protein could be used as a method to produce and purify large quantities of specific carbohydrate structures. In 1997, Liu *et al.* published an article on the absorption of xenoreactive antibodies from serum (2). This was done with a recombinant P-selectin glycoprotein ligand-1/mouse IgG (PSGL-1/IgG), which was produced in a cell-line that co-expressed the porcine α3GalT. The authors found that the PSGL-1/IgG was much more effective than any other product available at that time for absorption of anti-Galα3Gal antibodies. Therefore, my supervisor Jan Holgersson also came up with other potential applications based on PSGL-1/IgG, as well as other IgG tagged fusion proteins, as absorbers of anti-blood group ABO antibodies, vaccine adjuvants, and inhibitors of microbial attachment. This is about the time I joined the lab, which was in June 1999. My main task has been to dissect the synthesis of various carbohydrate epitopes, more specifically the Lewis and ABH determinants, using a more relevant, physiological experimental set-up than enzymatic assays *in vitro*.

For my own sake, and to orient the reader, I have given an introduction to glycosylation, the field of glycobiology and to the evolutionary aspects of glycans, because I wanted to put my contribution to science into a bigger context, rather than merely present it narrowly in a biochemical sense. Newcomers to the field (and all others for that matter) with further interest in any topic presented in this thesis are encouraged to begin with the book "Essentials of Glycobiology" (3), the review "Biological roles of oligosaccharides: all theories are correct" by Varki (4) and the many reviews that are cited in this thesis, especially the one by Winifred Watkins from 1980 (5), despite its flaws in the discussion on the Secretor gene. Of course, a review on mucins must be recommended, and a short one with a nice historical background is the article by Hang and Bertozzi (6). The last recommendation goes to the Minireview "Evolutionary considerations in relating oligosaccharide diversity to biological function" by Gagneux and Varki (7). It is a paper with lots of food for thought.

Jonas Löfling
Stockholm, 2006

INTRODUCTION

The sequencing of the entire human genome was recently published and the number of genes turned out to be surprisingly small, not exceeding 30,000 (8,9). Estimations points to the involvement of about 200-500 genes in the process of generating or modifying glycoconjugates in vertebrates (10). Thus, at least 1% of the genes are involved in the production of the human glycome. By the way, the word glycome was probably first mentioned in the literature in 2000 (11), but the explanation that it means the total glycan composition produced by an organism was first provided in 2001 by Hirabayashi (12).

Except for the involvement of sugars in metabolism, the part glycans play varies from crucial to not necessary (4). Glycans are normally found on glycoconjugates and these are found in almost every organism, from bacteria, fungi and plants to mammals. The usual glycoconjugates found in nature are glycoproteins, proteoglycans (which strictly speaking are glycoproteins, but so special that they normally are considered an own class), GPI anchored glycans and glycolipids. It is estimated that over 50% of all proteins are modified by glycans (13). The emphasis of my thesis is on the two main modifications of protein glycosylation, namely N- and O-linked, and they will be described in detail later, but first follows a general introduction to glycobiology.

GLYCOBIOLOGY

'Defined in the broadest sense, glycobiology is then the study of the structure, biosynthesis, and biology of saccharides (sugar chains or glycans) that are widely distributed in nature' (3). It has become more and more urgent for researchers to turn to the field of glycobiology due to the simple fact that sugars are involved, directly or indirectly, in many biological processes. The prime reason for this is that their main location is at the cell surface. This in turn is likely because oligosaccharides are well suited as carriers of information, due to the extremely high combinatorial possibility involved when joining carbohydrates together (see below). Also, since the affinity of sugar-protein interactions are not high and that the interaction often depends on multivalency, there is an opportunity to create a more fine tuned system, often only in certain tissues [e.g (14-18)], rather than a mere "on-off" system or a strict (almost irreversible) binding, which occurs in e.g. biotin-streptavidin interactions (19).

Venter *et al.* write "The modest number of human genes means that we must look elsewhere for the mechanisms that generate the complexities inherent in human development and the sophisticated signaling systems that maintain homeostasis" (8). No matter the reason, I found it odd that they in this context do not mention glycosylation at all.

A SHORT HISTORICAL INTRODUCTION TO THE STUDIES ON CARBOHYDRATES

Historically, the study of carbohydrates started in a sense long time ago, with the experiments on fermentation. The beginning of modern carbohydrate chemistry is probably the structural determination of D-Glucose in 1891 by Emil Fischer, reviewed in (20). Investigations by Leloir's group suggested that the formation of the disaccharide trehalose phosphate was mediated by an enzyme, which was also the first glycosyltransferase (GT) to be purified with certainty (21). Later, it was discovered that the localization of GTs is usually in the Golgi, and thus the localization of the biosynthesis of a major part of the glycans was defined (22).

CARBOHYDRATE STRUCTURES

Structures and nomenclature

The term carbohydrates generally refers to monosaccharides linked together as oligomers or polymers, called oligosaccharides or polysaccharides, and these are commonly referred to as saccharides, sugar chains or glycans. The monosaccharides are in nature mostly five- or six-carbon compounds. Sialic acids (Sias) are one of the exceptions, since they are built from nine carbons. All sugars in living organisms are normally in D-configuration, except fucose, which has the L-configuration (3).

The implications of the structure for biology

Since the monosaccharides have five or six carbons and that in addition the sugars can be linked in two different ways, α or β, it is possible to combine just a few of them to many different combinations. In fact, three hexoses put together could result in anywhere from 1,056 to 27,648 unique trisaccharides (3). This should be compared to the merely six variations that is possible for three nucleotide bases combined to a trimer. The combinatorial possibility for assembling a hexasaccharide is calculated to about 10^{12} (23). This inherent complexity of carbohydrates makes them excellent tools in nature as carriers of information, as already mentioned. It should be emphasized that, out of the many possible combinations, only very few are actually used *in vivo* (24).

PROTEIN-GLYCAN INTERACTIONS

The main characteristic for a protein-glycan interaction is that it is quite weak, often with a K_d value in the mM range (3,25). Therefore, multivalency is the common way to obtain high avidity in a biological system, working much like a Velcro® or IgM compared to IgG. The role of multivalency and the glycan carrier has been proven in many cases, e.g. in bacterial adhesion (26-28) and in the interaction of DC-SIGN or the mannose receptor with Lewis antigens (29). Our

interpretation of the superior activity of recombinant mucins in absorbing anti-carbohydrate antibodies in serum (2,30) is that this is principally because of multivalency. On a molar basis, the efficacy of PSGL-1/IgG carrying Galα3Gal was about 5000-fold better than the trisaccharide absorbent that was synthetically made. The absorption of anti blood group A antibodies was also much better than with a synthetic absorbent, but only with about an 80-fold difference.

EVOLUTIONARY ASPECTS OF GLYCOBIOLOGY

All eukaryotic cells are covered with a dense layer, the glycocalyx, which is made up by carbohydrates. As mentioned before, this gives glycans a role in cell-cell communication and also a protective function as part of the mucus layer in the body. However, the glycocalyx also makes the cells susceptible to attack from carbohydrate-binding microorganisms. A question glycobiologists for a long time have tried to answer is why there is such a great structural diversity of glycans in biology. To find an answer to this question it is necessary to examine the roles glycans play (4).

How much do we owe to the Red Queen effect?

Gagneux and Varki quoted Dobzhansky with "nothing in biology makes sense, except in the light of evolution" (7) and that is really important to remember when trying to put the picture together. Another way to state this is "glycobiology is evolution" (Varki, Gagneux, 2006). In other words, to elucidate the complete role a particular glycan play in nature, consideration of both exogenous (parasites and commensals) and endogenous (the need for cellular interactions) pressure is needed. One problem is that the time frame that is possible to study practically is very limited from an evolutionary perspective. Therefore, many of the hypotheses will be hard to test. Anyway, it can be assumed that exogenous pressure is a stronger driving force, because low fitness of an individual must be better than no fitness (an extinct species). Thus, a major driving force in evolution of glycans probably has been the need to escape from adhesion of, and infection by, lethal pathogens. The same principles apply to e.g. sickled-cell erythrocytes, where selection has been exerted by the malarial parasite on the host to stay heterozygote for the sickle-cell anemia trait due to the higher fitness of the carriers of one allele (31). A cost for this human adaptation is that homozygotes are instead suffering from sickle-cell anemia (32).

There should also be an endogenous pressure for a particular glycan if it is to remain expressed in the end, otherwise genetic drift would cause the genes responsible for its synthesis to disappear from the genome. However, a possible scenario is that even if the gene is inactivated, the presence of a pseudogene can function as an advantage for the organism, who can use it for recombination with related genes, thereby creating new, improved versions of the original gene before complete deterioration of it (33).

One argument for the endogenous pressure has been that the glycosylation in development is regulated in a highly specific manner, see e.g. (3,10,34). Yet, many GT null organisms seem to be healthy and completely viable. However, it might

be hard to know where to look for a phenotype due to the counteracting evolutionary forces, also known as the Red Queen effect (7), the glycan may only be needed in some tissues it is expressed in. Further, a null phenotype can be explained by the presence of compensating homologs, strain-specific differences that affect the phenotype differently (35) or that the glycan actually has no endogenous function (4).

Herd immunity

At this point, the phenomenon 'herd immunity' needs to be clarified. Gagneux and Varki discussed this matter (7) and I will here give my opinion on the subject and clarify some points needed to put my work in this thesis in an evolutionary context. "Herd immunity" (or perhaps more correct would be herd effect, but I will not discuss that matter (36)) refers to a phenomenon where some individuals are resistant to a potential epidemic disease and therefore they limit the spread by conferring "immunity" to the "herd" (37).

Gagneux and Varki speculate if "herd immunity" is possible even within an organism (7). This is surely the case, and the location of the carbohydrates most likely explain the tissue tropism of many microbes (38). Envision two different organisms, one with the same glycan on all cells and another with the glycan on only some of its cells. It is not hard to realize that if a toxin binds to this glycan, the organism with the glycan on all cells will then probably suffer from systemic disease whereas the other organism only will have local problems. However, this tropism must also arise because the organism in itself needs to have certain "zip codes", as argued by Renkonen *et al.* (39). Certainly, one example of the need for specific tissue distribution is the selectins and their ligands, which instructs circulating leukocytes to migrate from the circulation into the tissue in different parts of the body (40,41). This is also related to the importance for a multicellular organism to distinguish between "self" and "non-self", which is a very suitable role for glycans. Interestingly, King *et al.* reported that the evolution of key cell signalling and adhesion protein families predates the origin of animals (42). One of the protein families found was C-type lectins, which is a family of carbohydrate binding proteins, suggesting that protein-carbohydrate interactions was needed for the formation of multicellular organisms.

The evolution of glycosyltransferases

There are many fascinating details in the evolution of GTs and the creation of the glycome in organisms. The existence of Galβ4GlcNAc (lactosamine, LN) is widespread in mammals, whereas for example in nematodes and insects, a similar structure, GalNAcβ4GlcNAc (LacdiNAc or LDN), is present. The occurrence of this structure is in mammals restricted to some glycoprotein hormones. One enzyme involved in the synthesis of LDN in *C. elegans* is a member of the β4GalT family. When expressed in CHO cells, it produces poly-LDN structures (43), analogous to poly-LN sequences. Another enzyme with β4GalNAc-T activity was later cloned from man (44). A third enzyme was cloned from insects (45), and has a different acceptor specificity, but is still a member of the β4GalT family.

The Gal-T families

The β3GalT family contains several β3GalNAcTs (46,47), β3GlcNAcTs as well as β3GalTs, and the α3GalT family encompasses also α3GalNAcTs, the blood group A and Forsmann synthases. In the α4GalT family there are both α4GalTs and α4GlcNAcTs (48). Therefore, Hennet suggested that a more appropriate name for the families would β3GT, α3GT, etc.

Other points on the evolution of GTs

Among anciently related organisms, there is functional and structural conservation of GTs (49) and usually also of transporters of nucleotide sugars (50).

In mammals, a common structure is lactosylceramide (Galβ4Glc-Cer). The counterpart in insects is mactosylceramide (Manβ4Glc-Cer). A study on the evolution of elongation of this structures was recently published by Wandall *et al.* (51). Interestingly, the gene Egghead, which elongates mactosylceramide, can use both lactosyl- and mactosylceramide. This is in contrast to the paralogous mammalian genes, which require lactosylceramide (51).

The localization of FUT3, FUT5, FUT6 as well as FUT1 and FUT2 and DC-SIGN and also CD33 (52) is to chromosome 19. The products of these genes directly affect each other and it is interesting that they have all evolved quite rapidly, possibly because of an endogenous pressure.

All mentioned examples point to the need for organisms to create new functions of GTs, and this has usually happened through one or a few point mutations (53). It still remains to be seen how much of the diversification that is caused by the Red Queen effect. No matter the cause of the diversity, knowing more in detail how different GTs produce terminal glycans will help us in our understanding of the role glycans play and it may also help us in developing more specific therapeutics.

Microbe-host interactions

Microbes have evidently played a big role in the evolution of multicellular organisms. *Vibrio fischeri* has been shown to live in symbiosis with a squid, *Euprymna scolopes*. Surprisingly, the bacterium signals to the host via a tracheal cytotoxin (TCT) (54) that is a fragment of peptidoglycan, which is the factor responsible for the extensive tissue damage characteristic of whooping cough and gonorrhea infections.

It has recently been shown that the human intestinal microbial flora is very diverse (55). Not much is known about how these microbes find their specific niche in the gastrointestinal (GI) tract, but specific glycans may be important. There is emerging evidence for a highly specific glycosylation in the GI tract, both in rodents (56), pigs (57) and humans (58,59) and that this is more subject to inter- than intra-species variation. Gordon and Hooper points to this fact, and suggest that it is due to the need to keep commensals at different sites in the GI tract (60,61). The production of specific glycosidases is one way commensal bacteria may utilize the varying glycosylation differently (62-64).

The group of Jeffrey Gordon has for a long time studied the interaction between commensals and their hosts. A remarkable finding was that *Bacteroides thetaiotamicron* induces the enzymatic activity that leads to the addition of terminal α2Fuc in mice. Moreover, this is only seen when the bacteria can utilize fucose as an energy source (60,61,65,66). Another study on *Bacteroides* showed that the bacteria are defective in colonizing the host under competing conditions if they cannot decorate themselves with L-fucose on the cell surface (67).

Microbial adhesion

In order for a pathogen to cause infection, it is often a necessity for the pathogen to adhere to a host cell. Many of the receptors on the cells are glycans (38,60,68,69). Examples include terminal β3-linked galactose (70), ABH and Lewis antigens (71-75), SLe^x (76-78), GAGs (79,80) and Sia (81). It has also been found that commensal bacteria utilize the same receptors as pathogens (82), which could make it difficult for humans to mutate away some of the glycans used by pathogens. The proteins responsible for attachment by bacteria are called fimbriae or, adhesins. For viral proteins, the term hemagglutinin is commonly used. The importance of Lewis antigens expression in the host and of the BabA and SabA adhesins in *Helicobacter pylori* colonization has been demonstrated in both mice (83) and humans (84). Because of the role of adhesion in the colonization process, it is interesting to correlate the expression of glycans in milk to host protection from disease. Indeed, Newburg and co-workers have found this to be the case. This is likely because the glycans act as decoys (85,86), and thus highlights the necessity for microbial attachment to glycans on the host cells in the infectious process.

Natural antibodies

Karl Landsteiner's pioneering studies on the ABO blood group system are from as early as the late 19th century (87,88). He found that an individual with a blood group type different from another individual's blood group has antibodies directed against this person's red blood cells. This is a major obstacle for blood transfusions and is also a problem in allo-transplantation (Tx), when transplanting across the blood group ABO barrier. Work by Bensinger *et al.* using columns with synthetically made A epitopes showed that absorption of the antibodies is a good method to prevent rejection (89). Lately, Glycorex Transplant AB has exploited this technology with a column, Glucosorb®, which is used in the clinic for incompatible blood group AB0 Tx (90).

A big problem in Tx is the shortage of organs (91). Glucosorb® is one way to make more organs available for Tx to a wider range of recipients. Another possibility could be to use organs from another species, such as pig. However, pigs and other mammals express at least one, in this context, very important carbohydrate that Old world monkeys and humans do not. This is due to a recent mutation in an α3GalT that generates the glycan Galα3Gal, and since this was described by Galili *et al.* the epitope is also referred to as the Galili epitope. Old world monkeys do not express this epitope, instead they produce high amounts of antibodies directed against it (92).

Blood group ABH and Galα3Gal are not the only carbohydrate epitopes against which some or all humans produce antibodies. The Lewis blood group system and the P blood group system are other examples with a clinical relevance (5). The rare human blood group Pk is characterized by the glycan Galα4Gal, which is also found in several, but not all, orders of birds (93). This structure is a receptor for Shiga-like toxins, as well as for *Streptococcus suis* (28,94). Moreover, Blixt *et al.* found evidence for the existence of antibodies directed against terminal Galβ3 structures (95). Another group of carbohydrate-reactive antibodies are the Hanganutziu-Daicher (HD) antibodies. They were first found in patients who had been injected with serum from horses and later developed so-called serum sickness. It turned out that the antibodies are directed against a Sia, N-glycolyl neuraminic acid, NeuGc [see e.g. (96)].

Why do we have natural antibodies and why is this important to know?

Although natural antibodies against various carbohydrates occur in many species, no good explanation has yet been provided for the triggering of the development of these antibodies (87). It has been suggested that they originate from interactions of the immune system with bacteria. T. Springer made some work on this in the 1960s, and showed that bacteria are likely inducing natural antibodies (97,98).

The origin of the natural antibodies is important for their specificity, and because of that, it is relevant if one wants to make an absorber of these antibodies. For example, anti-H or anti-A mediated agglutination of erythrocytes is not inhibited by monosaccharides, because the underlying structure and multivalency is critical for the inhibition (99) and similar results exist for the inhibition of anti-Galα3Gal antibody mediated cytotoxicity (100).

A plausible explanation for the existence of many natural antibodies is herd immunity, as given by studies on the blood group ABH system and pathogens (101,102). It has been seen that transmission of virus particles is hindered by the existence of pre-formed antibodies against ABH determinant incorporated in the envelope of the virus (103).

Mouse models

The use of mouse models for elucidation of the role of glycans was recently reviewed by John Lowe and Jamey Marth (104) and a comparison between null mice and CDG patients have been done, e.g. by Eklund and Freeze (105).

It is clear that many studies on mouse models has given and will give us many clues on the role of glycans. It has been seen that some of the reported knockout mice are totally fertile, viable and seem to have no obvious phenotype except a minor one (see also the discussion in Evolutionary Aspects of Glycobiology, p. 5). This is in contrast to what is seen in humans where many of the same enzymes are naturally knocked, which leads to phenotypes that are either severe or normal (106,107). But examples also exist for the reverse situation, i.e. a more severe phenotype in mouse than in humans. One of the explanations for this might be that the systems are not completely comparable. Another reason for the difference in phenotypes certainly lies in intraspecies variations in the genetic background

(107,108). A third one is that the mutations in themselves may not be equal, because in mouse the targeted mutation studies have used null alleles. By contrast, the CDGs are typically caused by alleles with missense mutations, and this will likely leave the corresponding protein with partial function (109). A further probable explanation may also be that the lethal mutations corresponding to the mouse knockouts are not seen, for obvious reasons. In most cases, however, the mouse mutation studies are largely consistent with the results from the studies on CDG patients (105,109).

GLYCANS AND CANCER

Alterations of glycans in cancer are well-known (110-112) and have attracted the attention of many researchers. The suggestion by Marionneau *et al.* was that the correlation between certain blood group antigens and cancer "is merely incidental and devoid of evolutionary impact" (113). On the other hand, Hakomori states that the early events in tumor formation may be related to glycosylation (110) and there is evidence for the actual involvement of glycans, not only SLe^x, in the behavior of tumor cells (114). Because of the possibility to use glycans as targets for cancer specific therapy and for diagnosis, it is still very important to study the glycosylation in relation to cancer. In fact, some carbohydrates have been used for cancer vaccination in clinical trials. The antibodies that have been formed in the patients show selectivity in complement lysis of cells depending on which glycoconjugate the antibodies target. A hypothesis is that this is because of a difference in proximity to the cell surface of glycolipids versus glycoproteins (115). Therefore, studies on the synthesis of the tumor-associated glycans on specific glycoconjugates are needed as well.

Cancer associated epitopes

The T and Tn antigens (Galβ3GalNAcα and GalNAcα, respectively), as well as their sialylated counterparts, have for a long time been known as tumor associated carbohydrate epitopes (110,112,116). They are thought to be the result of incomplete glycosylation, but why this happens is not really clear. A mechanism proposed by Hull and Carraway was that the intracellular routing of the protein carrying the epitopes is abnormal (117), and data from Egea *et al.* also supports this theory (118). Another reason can be a change in the expression of one or more GTs (119,120).

Other epitopes whose expression is often changed in tumor tissues are Lewis determinants, both type 1 and type 2 based, sometimes in discordance with the RBC phenotype (121-125).

Yet another mysterious glycosylation phenomenon in cancer is the occurrence of blood group A structures in O individuals, called incompatible A (126). There is not yet any totally satisfactory explanation available. Because enzymatic activity has been detected in some cases, a proposed mechanism has been a conversion of the O-gene to a gene that codes for a functional enzyme. An intriguing hypothesis is that this phenomenon is caused by an enzyme of exogenous origin, e.g. from *H. pylori* colonizing the stomach mucosa (127).

GLYCANS AND DISEASE

Aberrant glycosylation in humans can lead to various diseases. Many of them are classified as Congenital Disorders of Glycosylation, CDG (106,107). Others are caused by a defect in GAG (128) or mucin biosynthesis, as in cases of familial tumor calcinocis caused by mutations in UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 3 (pp-GalNAc-T3) (129). A newly discovered, unexpectedly large, group of glycosylation defects in humans are gain of glycosylation mutations (130).

CONCLUDING REMARKS TO THE INTRODUCTION

The many examples given in this chapter illustrate the importance of glycans. That a carbohydrate is important may be inferred from various other approaches. One way could be to search for important microorganisms that express a certain carbohydrate, i.e. through molecular mimicry studies. There are in fact many bacteria and parasites that have been found to express Sia (131) on their cell surface and this assists their survival in their hosts. Because most successful vaccines are directed against the LPS of bacteria (132), the LPS is likely important for proper colonization, since otherwise the bacteria that had mutations in LPS synthesis would survive and cause disease. Actually, the importance of LPS has been known for a long time, through e.g. the early observations by Fred Griffith and later Avery *et al.* (133). That symbionts sometimes decorate themselves with fucose as a factor for higher fitness (67) is further evidence for the importance of carbohydrates in host-microorganism interactions. Finally, *H. pylori* is known to express Lewis antigens in its LPS, but the consequence of this is not known. It is suggested to assist in host colonization, but also in the induction of auto-antibodies (134).

MAJOR CLASSES OF OLIGOSACCHARIDES AND GLYCOPROTEINS

GLYCOLIPIDS

In higher animals, the monosaccharide attached directly to ceramide is normally glucose or sometimes galactose, which gives glucosyl- or galactosylceramide, respectively. Glucosylceramide is typically elongated, starting with a galactose, which results in lactosylceramide. Lactosylceramide is further extended in many possible ways, leading to several defined core structures. Since the principal focus of this thesis is on glycoproteins, the glycolipids will not be dealt with in further detail.

Instead, the two main classes of glycoproteins, i.e. those with N- and those with O-linked glycans will be described in the next sections. The other known modifications, C-mannosylation and the recently found N-mannosylation of tryptophan, will not be discussed.

N-GLYCANS

N-glycans are produced in a different manner than are O-glycans. The creation of N-glycans starts with the assembly of an oligosaccharide using dolichol phosphate. The oligosaccharide is then transferred *en bloc* to the protein in the ER via a multienzyme complex called the oligosaccharyltransferase. The oligosaccharide is attached to an asparagine, in the motif X-Asn-Ser/Thr, where X is any amino acid except proline and aspartic acid (135). Many studies have been devoted to the role of N-glycans. For examples on this, as well as on the role of O-glycans, see (4).

AGP

AGP or orosomucoid is a glycoprotein normally synthesized by the liver. The carbohydrate content represents 45% of the molecular weight, attached to five or six N-glycosylation sites. It is one of the major acute phase proteins in humans, rats, mice and other species (136). We used AGP as a protein probe in order to compare the synthesis of N- to that of O-linked sites (for which other reporter proteins were used, see below).

MUCIN TYPE O-GLYCANS

The O-glycosylation that will be described and discussed in this thesis is only the mucin type O-glycosylation. For short, I will refer to this as O-glycosylation and the resulting glycans as O-glycans. O-fucosylation, O-GlcNAc and other types of O-glycosylation will not be dealt with further.

The synthesis of O-glycans starts with the addition of one or several GalNAc residues being linked to serine or threonine in alpha-configuration. Contrary to what is found for N-glycosylation, there are no good consensus sites for O-

glycosylation, even though there have been many attempts to find one, e.g. (6,137). Enzymes of the pp-GalNAc-T family add the GalNAc (see more below) and some of them will only use naked peptides, whereas others needs an already glycosylated peptide (138). After the addition of GalNAc, other enzymes elongate the glycan chain, usually starting with a core-forming enzyme followed by a SiaT or a FucT.

The role of O-glycans is not clear. Generally, O-glycans on mucin domains make the protein more extended and protease stable (139,140).

MUCINS

Mucins function as multivalent carriers of glycans and are abundant on epithelial surfaces, which means they probably serve as both endogenous and microbial receptors, and potentially as decoys for microorganisms.

Mucin was originally the term for a highly glycosylated protein in mucus. The mucins have more than 50% of its weight made up by carbohydrates. These are attached to a mucin domain, which consists of a variable number of tandem repeats (TRs) of amino acids, commonly known as VNTRs (Variable Number of TRs). The VNTRs are serine (Ser), threonine (Thr) and proline rich, and the Ser and Thr residues are often glycosylated, which gives the protein an extended structure, see Fig. 1 and (139,140). The mucins have traditionally been named from MUC1 and up, but they are not homologous. Rather, the classification is based on the properties given above (141). Proteins rich in O-glycosylation, but without TRs have from time to time been called mucins as well. Another term is mucin-like protein, which may be better. However, there are examples of "real" mucins that have splice variants without VNTRs, like MUC1/Y, reviewed in (142). Unlike proteoglycans, mucins do not carry uronic acid or xylose. The gene localization and organization is well conserved between species, but the tandem repeats are not (143). This is probably a major determinant for species and cell-specific glycosylation of mucins, because both the sequence and number of the VNTRs are important for the glycosylation of a mucin (144).

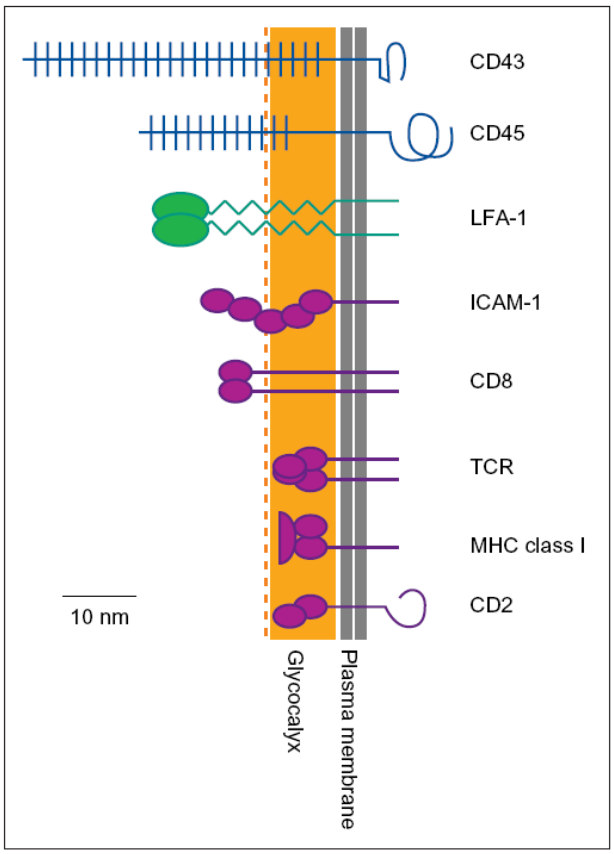

Fig. 1. Scale model of common T-cell surface molecules. The estimated lengths of the extracellular and cytoplasmic domains of CD43 are compared with immunoglobulin superfamily receptors, CD2, MHC class I, TCR, CD8, ICAM-1; the integrin LFA-1; and another cell surface mucin, the leukocyte common antigen CD45 (Ref. 11). The lipid bilayer and glycocalyx (extending to the dashed line) are drawn to scale as 4–5 nm and 10 nm thick, respectively. Abbreviations: ICAM-1, intercellular adhesion molecule 1; LFA-1, leukocyte function-associated molecule 1; MHC, major histocompatibility complex; TCR, T-cell receptor. Taken from (145) with permission from the publisher Elsevier Science.

CD43 and PSGL-1

In this thesis, the extracellular parts from CD43 and PSGL-1, two mucin-like proteins were used. I will shortly describe these two proteins:

Sialophorin, or leukosialin, CD43, is a highly glycosylated protein with one N-linked glycosylation site, and most importantly, 93 extracellular Ser and Thr (146). CD43 has been shown to be a ligand for E-selectin, both on various tumor cells (147,148) and on T cells (149,150). The ability of recombinant CD43 and lamp-1 produced in CHO to inhibit E-selectin adhesion of metastatic tumor cells was compared by Fukuda's group (151). The two molecules are approximately equally potent inhibitors, even though there is much more SLe^x on lamp-1 (152). Other functions ascribed to CD43 are principally in immunological contexts (145).

PSGL-1 was cloned in 1993. The sequence predicts a protein that has four N- and 53 O-linked glycosylation sites in the extracellular domain (153). PSGL-1 is a ligand for all selectins and also the best characterized one (40,154,155).

STRUCTURES COMMON TO DIFFERENT TYPES OF GLYCANS

The common biosynthetic pathways of the structures studied in this thesis are presented in Fig. 2-4. The symbols are adopted from (3).

Cores

Eight O-glycan core structures have been found in mammals (156). The most prominent in humans are core 1 and core 2. Core 3 and 4 are found mainly in the gastrointestinal tract and in the airway epithelium and the rest have only been detected in a few instances in nature. The core structures enable the creation of highly diverse glycans, for example in the stomach (56-59) and salivary gland (157,158).

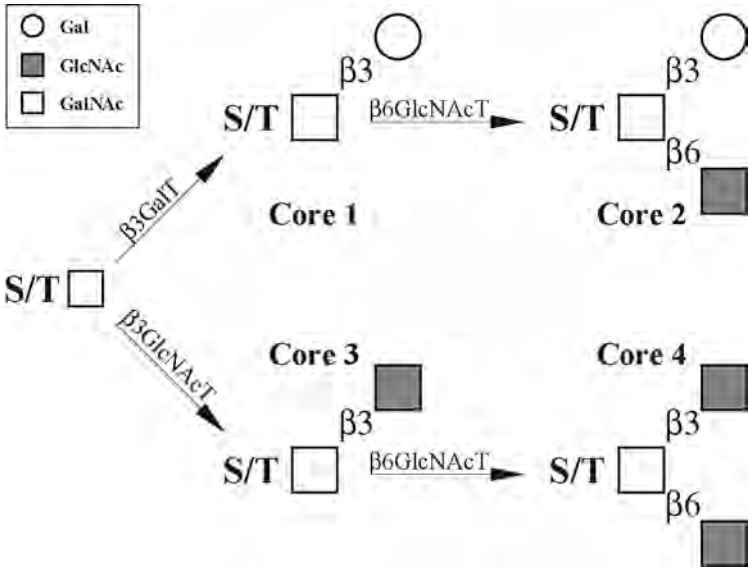

Fig. 2. Schematic representation of the formation of the core 1-4 O-glycan structures.

Fucose

Fucose is a deoxyhexose, and is present in most organisms as a common substituent both in terminal and core structures of many glycans in mammalian cells [reviewed in (34)]. Many roles have been ascribed to fucosylation, sometimes via indirect evidence. As an example, the involvement in the compaction phase of the developing blastocyst was implicated because antibodies directed against the epitope fucose-containing epitope SSEA-1 (later found to be Le^x) blocks the compaction. However, the development of FUT9-/- mice is normal, despite the lack of SSEA-1 (159).

The core fucosylation of N-glycans is carried out by FUT8, and mice with null mutations in FUT8 show severe growth retardation and 60% dies within 3 days of birth (160). The effect of knocking out FUT8 is on multiple proteins, and leads to dysregulation of both the EGF- and TGF-β1 receptors (160,161).

Clinical studies on patients with a form of LAD II, known as CDG type IIc, show that these patients have severe alterations in multiple systems, which is caused by a defect in fucose metabolism (162,163). The patients are further suffering from recurrent infections because of defective leukocyte extravasation due to impaired selectin ligand synthesis. Two patients were also mentally retarded and had skeletal abnormalities (163). The phenotype of mice deficient in FX, a crucial enzyme in GDP-fucose synthesis, is also pleiotropic but the phenotype is modulated in different strains by unknown factors (35) and it differs from the

human phenotype, probably because the Notch-Fringe signaling pathway seems to be affected in FX null mice but not in CDGIIc. Not surprisingly, FX has also been suggested to participate in the control of metastasis of colorectal cancer cells via the synthesis of SLe^a (164). The existence of a de novo synthesis and a salvage pathway for GDP-fucose further indicate the need of fucose for whole organisms (34). However, fucose is not necessary for individual cells, because cell lines lacking fucose are viable with no gross abnormalities (165).

Furthermore, many reports exist on investigations on associations between blood group (166) or fucosylated antigens and disease (76,167-171). This may be a direct causual relationship, e.g. the expression of Le^b and binding of H. pylori, or SLe^x and metastasis, but could also be indirect, e.g. that expression of one epitope competes with the expression of another.

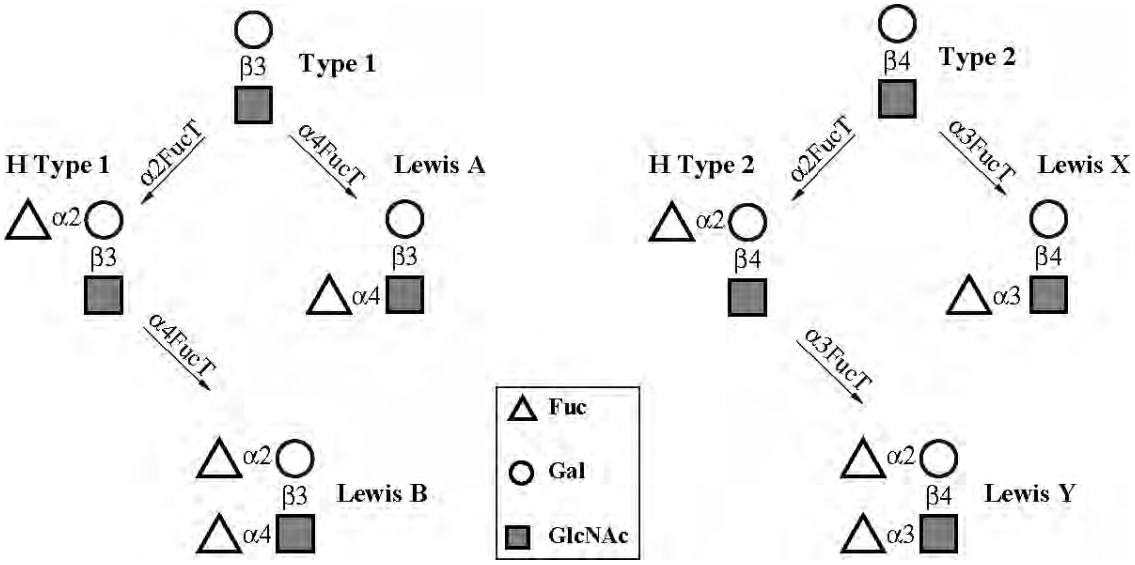

Fig. 3. The formation of fucosylated type 1 and type 2 epitopes.

Type 1

The type 1 disaccharide (Galβ3GlcNAc) has in humans mainly been detected in body fluids and excretions and mesodermally derived tissues such as lining and glandular epithelia (126). In other animals, structural evidence exist for its occurrence in various other tissues, e.g. in rat brain (172), on bovine prothrombin (173) and interphotoreceptors of the eye (174), calf thymocytes (175) and rat AGP (176).

The functional role of type 1 is not clear. The production of type 1 may be a way to regulate sialidation. Since ST6GalTs do not work well on type 1, only Siaα3 will be produced on type 1. Sometimes there will also be a discrimination of the location of the epitopes, usually type 1 on the GlcNAcβ3 and type 2 on the GlcNAcβ6-branch in humans, see e.g. (177,178). This is not the case in the rat small intestine where the GlcNAcβ6-linked branch is extended by type 1 or type 2 chains (179).

The difference in location of the epitopes could be important, because it can potentially affect the binding of endogenous lectins, such as galectins and Siglecs.

Le^a and Le^b

The structure for the Le^a determinant was deduced in 1957 by Watkins and Morgan and for Le^b in 1962 by serological inhibition tests with oligosaccharides isolated from human milk by Kuhn [as reviewed in (5,99)].

The next step was to elucidate the pathway for the synthesis of Le^a and Le^b. The consensus is that the production of Le^b goes via H type 1 and not Le^a (Fig. 3). *In vitro* data on purified H and Se enzymes showed that the addition of α4Fuc to type 1 results in a substrate that is not accessible for α2FucTs (180,181). There are, however, a few reports on expression of an α2FucT in cancerous tissues or Kato III cells that could produce Le^b from Le^a, though the identity of the enzyme(s) in these reports were not clear (182,183). Prohaska *et al.* found that the Le^a activity appears ahead of Le^b activity, and they therefore suggest that Le^a formation occurs before Le^b (184). Other, yet unexplored, options are that the Sec1 is actually functional in some instances (maybe due to recombination with FUT2?), since the rabbit RFT-II has a substantial activity with lacto-N-fucopentaose II (185), or that the fucosyltransferase is of exogenous origin, e.g. from a virus or bacterium .

The role of Le^a and Le^b is not clear, but the epitopes possibly play a part in cell-cell interactions, such as those mediated by DC-SIGN (186,187), They may also be involved in egg-sperm contacts (188,189).

SLe^a

The only biological connection noted for SLe^a so far has been in the form of the epitope CA19-9 and its association with malignant cancers, reviewed in (190). A connection to metastasis was later proposed, because SLe^a was found to be an E-selectin ligand.

The existence of SLe^a and Le^a has for a long time been considered to be confined to plants and some primates due to convergent evolution (191). However, it is intriguing to speculate that the ability to produce α4-fuc existed in the ancestor of vertebrates and that it was subsequently lost in some lineages. That is based on the evidence that some, but not all, pig strains have Le^b in milk (192) and that Le^a was found in one frog species but not in another (193). Interestingly, in frogs Le^a is situated on core 6 (GlcNAcβ6GalNAc), which is more similar to core 2 than to core 3 (see Fig. 2). A poster from Yamnikova *et al.* reported that some avian influenza viruses bind to SLe^a (194). This means that there could be an α4FucT in at least some birds, even though there are no reports on this. The only known bird FucTs have a motif characteristic of α3FucTs and not α4FucTs (195,196).

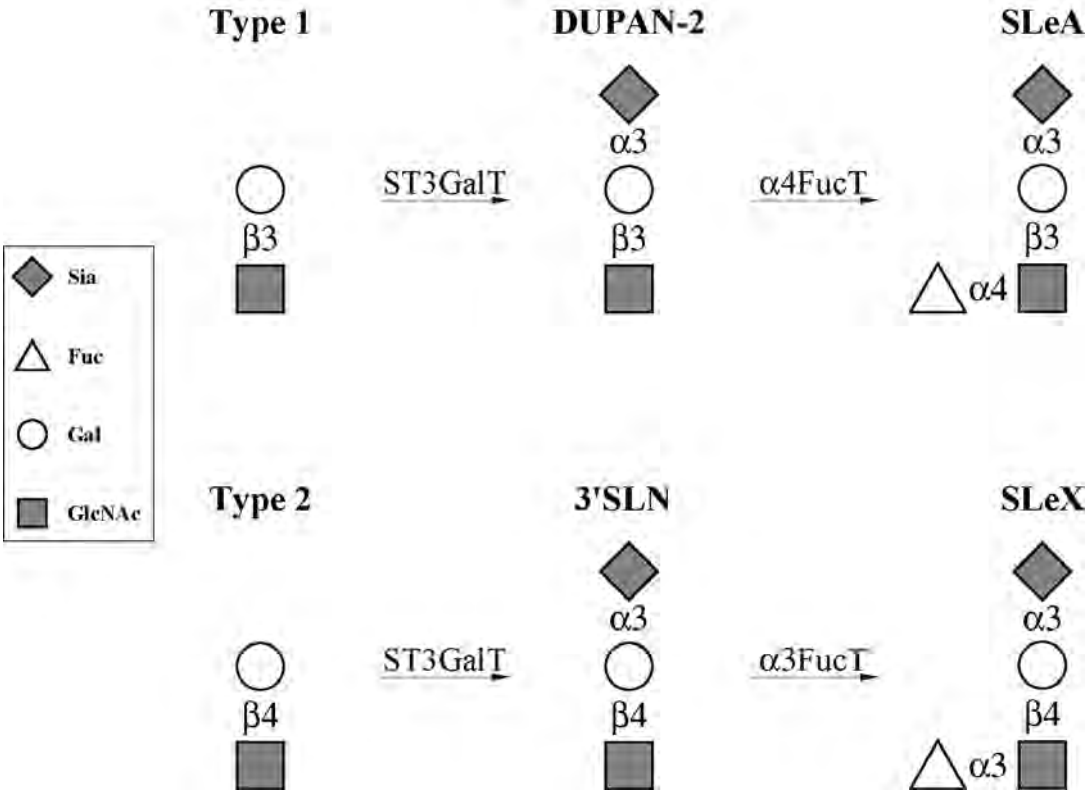

Fig. 4. The formation of the SLe^a and SLe^x epitopes.

Type 2

SLe^x

The major ligand for E-, P- and L-selectin is the tetrasaccharide SLe^x (40,41,197). Furthermore, the similar epitope VIM-2, Siaα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAc, has also been shown to modulate E-selectin binding (40,197,198). Because of its importance for selectin binding, the correlation between SLe^x and metastasis formation has been extensively studied. However, this is outside the scope of this thesis. It is reviewed elsewhere in detail (190).

H. pylori binds to SLe^x via SabA (76). The connection between efficient binding of dimeric-SLe^x by H. pylori and an earlier observation that FH6 (which recognize this epitope) correlated with poor prognosis in gastric carcinomas (199) suggest a link between gastric cancer and the bacterium.

In addition, Fukuda's group has proposed that SLe^x in high concentration on tumor cells might result in killing of the cells by NK cells (200). It was reported that CD94 might function as a receptor for SLe^x, because blocking with anti-CD94 antibodies alone inhibited the killing and no additional blocking effect was seen in combination with SLe^x oligosaccharides.

Le^x and Le^y

Le^x and Le^y were originally termed X and Y, because they were not really connected to the serological Lewis system, but since they were later discovered to be isomers to Le^a and Le^b they have been renamed.

Le^y has been reported to be a potential mediator of angiogenesis, but the article is rather confusing in terminology and the specificity of the antibody used in the study is not completely defined (201). Le^x is found in many organisms and its principal role has been suggested to be the involvement in compaction of the morula-stage embryo (see the Fucose section, p. 15 for further details).

Blood group epitopes

The ABO (ABH) blood group

The major blood group system of carbohydrate nature is the ABO system and was defined serologically in 1900 by Landsteiner (88). The elucidation of the chemical structures was performed mainly by Morgan and Watkins and Kabat and has been reviewed in (99). In brief, the blood group O is determined by the lack of A and B determinants, because the O-gene codes for a non-functional enzyme. Instead, the precursor epitope for A and B is present, the H determinant.

A first report in 1976 by Akira Kobata's group suggested that H antigens only existed on type 2 on erythrocytes (202), but when the matter was further investigated, fucose in α2-linkage on core 1 (which is the same as H type 3) was detected (203). This structure was originally described by Donald on ovarian cyst glycoproteins (204) but has later been found on glycolipids as well (205). The ABH epitopes have also been found on type 4, which only occurs on glycolipids (126). Because ABH determinants exist in many tissues and not only on RBCs, a more appropriate description is that they constitute a histo-blood group system.

The Bombay phenotype (O_h) was described in 1952 (206), in three individuals from Bombay, hence the name of the phenotype. The molecular basis for the O_h phenotype was later described by the group of John Lowe (207). It has been stated that these individuals are normal, healthy and fertile and show no distinct phenotype except the absence of blood group H structures. However, new findings show that the von Willebrand factor of these individuals is more susceptible to ADAMTS13 cleavage (208).

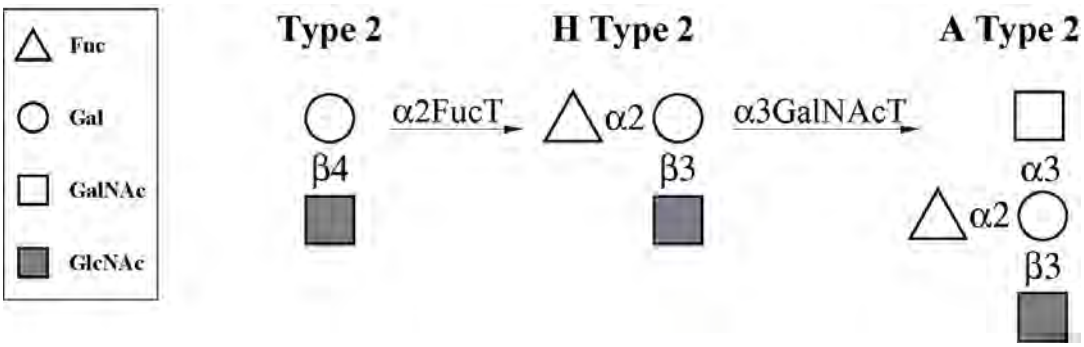

Fig. 5. The synthesis of a blood group A determinant, A type 2.

The Lewis blood group

The Lewis antigens, together with the blood group ABO epitopes, were chemically studied primarily by Watkins and Morgan as well as Kabat. As reviewed in (99), many enzymes that could degrade blood group substances were found in the 1940s and 50s. In addition, inhibition studies with simple sugars implied that fucose, galactose and galactosamine residues were important parts of the ABO and Lewis substances. However, it was also realized that this was not enough, since e.g. fucose was involved in both Le^a and H determinants. All this, together with a major breakthrough (the isolation of oligosaccharides from milk by Kuhn, published in 1957), subsequently led to the conclusive elucidation of the chemical structure of Le^a later that year (209).

The importance of the structural conformation of type 1 and type 2 chains

The difference in conformation between type 1 and type 2 is significant for the enzymes that will use the structures as substrates, or for antibodies and microorganisms that will bind to them. This is clearly seen in Fig. 6. See FucTs on p. 26 for further details.

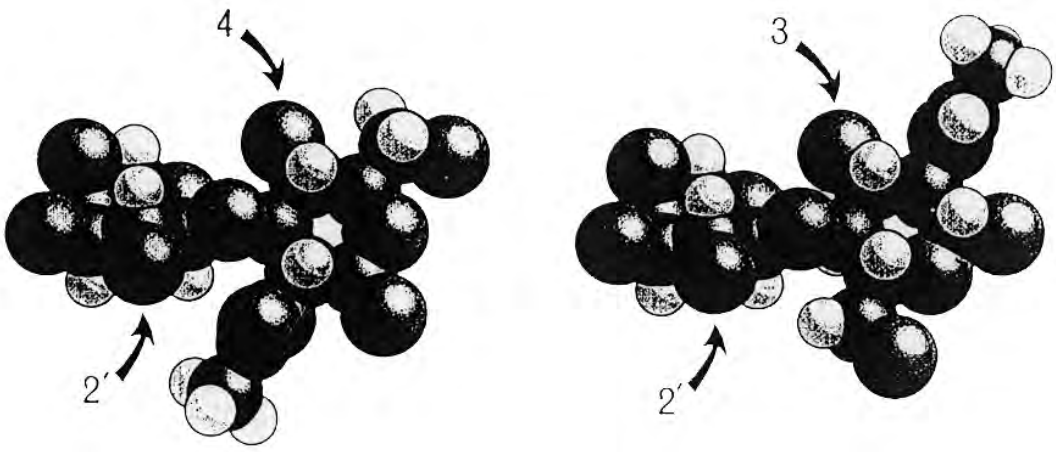

Fig. 6. Comparison of the accessibility to the 2' hydroxyl group (arrows) of the terminal galactose in type1 (left) and type 2 (right), respectively. The acetyl group of the subterminal βGlcNAc restricts access to the 2' hydroxyl group in type 1, whereas it leaves a different and freer access to the same hydroxyl group in type 2. Also shown are the 3 and 4 hydroxyl groups on the GlcNAc where FUT3 and FUT5 can add fucose. It should be noted that hydroxyl hydrogen atoms are not shown. The 3D model, produced by P-G Nyholm, was obtained by hard-sphere exo-anomeric calculations. Taken from Henry *et al.* (210) and reprinted with permission from Blackwell publishing.

GLYCOSYLTRANSFERASES

GTs are type II membrane proteins with an N-terminal, short cytoplasmic tail, a 16-20 amino acid transmembrane anchoring domain and a long lumenal region. This one can be divided into an extended stem region and the catalytic domain, which is located in the C-terminus (211). Most of the GTs reside in the ER or Golgi (including the trans-Golgi network; TGN), where the principal part of glycosylation takes place. There are several reviews on the roles of the Golgi for terminal glycosylation of glycoconjugates, e.g. (212-214).

THE GLYCOSYLATION MACHINERY OF THE CELL

Many factors control the glycosylation potential of the Golgi apparatus. It is not only the repertoire of existing GTs, but also their relative distribution, sub-compartmentalization and availability of activated sugars (214). In a review by Saul Roseman, he also mentions the possibility that Mn^{2+} and other ions can limit the glycosylation reactions as well (20). However, overall the role of divalent cations in regulating GT reactions in the Golgi is poorly understood (214).

The first studies on *in vitro* synthesis by GTs that were done suggested that the glycosylation would take place in a sequential order (215). It was thought that there is a continuous movement of the protein through the Golgi apparatus (216) and that there would be competition between different GTs. This was also the interpretation of results from structural studies of protein glycosylation, which indicates that the glycosylation is heterogeneous and incomplete. Furthermore, studies on mucins from cancer cells has pointed to a competition in the early stages of core structure synthesis (120,156,217,218). This theory of competition is based on the observation that core 2 formation is inversely correlated to the activity of ST3GalT1 in pancreatic and breast cancer cells, mainly T74D (either core 2 or the Siaα3Galβ3GalNAc epitope is formed). Also, ST3GalT1 null mice show an increase in core 2 structures, which is presumed to be due to less sialylation of core 1. However, flag-tagged MUC1 produced in other breast cancer cell lines carries the glycan Siaα3Galβ4GlcNAc(Siaα3Galβ3)GalNAc in substantial amounts. This structure is also reported to be a major one on CD43 from several types of cells (146).

Compartmentalization of the glycosylation reaction

All the mentioned examples below are to illustrate a system in which the *in vivo* synthesis of glycans is not only a result of mere competition based on the Km values for the individual GTs, but instead is highly controlled on many different levels. Possible explanations on how the control is carried out are also discussed. First of all, some words of caution I think are appropriate:

Because the compartmentalization varies depending on the origin of the cells (211,219), extrapolating results from one type of cell to another can be dangerous and is most likely a factor for some of the confusion and contradictory results in the literature.

Reports from the group of Assou El-Battari have given evidence both for a direct competition between glycosyltransferases (220), but recently also against it (221). Results from Yano *et al.* suggest that the glycosylation machinery is likely divided into several sub-compartments of the Golgi (in addition to the traditional *cis*-, *medial*-, and *trans*-division), and each part is not interacting with the other (222). Looking into the literature, there is more indirect evidence for this, e.g.:

- The study of Goelz *et al.*, who (223) found that the synthesis of SLe^x but not of Le^x in DHFR-ELFT cells is affected by swainsonine. Their results could be explained by different cellular locations for the formation of the epitopes.
- De Graffenreid and Bertozzi noted that although the *in vitro* specificity of two sulfotransferases, GlcNAc6ST-1 and 2, is similar, there is a marked preference *in vivo*, with GlcNAc6ST-1 glycosylating N-glycans, and GlcNAc6ST-2 mostly O-glycans. This is related to a difference in the stem region between the enzymes, which leads to distinct Golgi localization (224).
- Marcos *et al.* examined the role of the human ST6GalNAc-I and ST6GalNAc-II in the synthesis of the cancer-associated sialyl-Tn antigen. *In vitro*, both enzymes were very similar, whereas the in vivo specificity was different. ST6GalNAc-I strongly preferred to use the Tn epitope, whereas ST6GalNAc-II instead used the T epitope (225).
- A paper on β3Gal-Ts (enzymes involved in type 1 chain biosynthesis, see β3GalTs, p. 25-26) from Salvini *et al.* showed that β3Gal-T5, but not β3Gal-T1 or T2, competes with the synthesis of type 2 on N-linked glycans (226). These data can also be interpreted in another way, namely that the enzymes have different Golgi localization, thereby affecting the synthesis of type 1 differently.
- Fernandez-Rodriguez *et al.* found that MUC1 and CD43 purified from Colo 205 cells are not carrying the same set of carbohydrates (227). Even though no direct proof was provided for separate pathways through the cell for the mucins, the difference in the kinetics of glycosylation led the authors to suggest that this is the case. Anyhow, cellular sorting of MUC1 has been reported to influence its glycosylation (228).
- The expression of FUT1 in CHO cells and in mouse mammary glands gave a surprising result. Even though many proteins carry LN sequences, only a few are fucosylated (229,230). Further, the number of transgene copies does not seem to influence the production of fucosylated glycans. This also points to a sorting mechanism in the cell, whereby there will be a sub-cellular control of the glycosylation, far greater than predicted from *in vitro* data alone. Zöllner and Vestweber also provide evidence for this theory, when studying the synthesis of SLe^x by α3FucTs (231). Their data also show that there is a strictly controlled fucosylation pattern, with ESL-1 being one of the few proteins modified in CHO cells by FUT4, FUT5, FUT6 and FUT7, but surprisingly not by FUT3. They further found that the mRNA levels of FUT4 do not influence this selectivity. An objection to these results could be that only fucosylation has been studied, but results from our lab on the formation of Galα3Gal in endothelial cells show a similar selectivity in glycosylation of proteins (232).

- The localization of pp-GalNAc-T1-3 is strangely enough throughout the Golgi (233). It is not clear why this is the case. If the simple model with sequential glycosylation of the maturing protein through the Golgi were assumed to be correct, pp-GalNAc-Ts would be situated in the *cis*-Golgi compartment only. Still, in normal cells, there are no or very little free GalNAc-residues on their mucins. Perhaps there is some quality control between pp-GalNAc-Ts and core 1 β3GalT and/or COSMC. This control could be disturbed in cancer cells, as seen in a redistribution of proteins and subsequent emergence of the T and Tn epitopes in cancer cells (117,118). A second explanation is that the pp-GalNAc-Ts are excluded from COPI vesicles, which are involved in Golgi transport (212).

The conserved oligomeric Golgi (COG) complex

The COG complex is an eight-subunit (Cog1–8) peripheral Golgi protein involved in Golgi-associated membrane trafficking and glycoconjugate synthesis (234,235). The COG can probably be a determining factor for the localization of many GTs, and thus father explain the discrepancies in the literature discussed so far.

Furthermore, a well known fact is that the pH optimum differs for GTs, see e.g. (236). This can be yet another mechanism for the localization of GTs, as Axelsson *et al.* found that a change in intracellular pH leads to a reorganization of the Golgi (237).

Enzyme complex formation

I would like to add the little explored field of GT complex formation to the concept of sub-compartmentalization. The subject is also discussed in (224). Enzyme complex formation of GTs must be a major factor for a cell to control the glycosylation procedure. It will ensure a quick and proper synthesis. It is reported that enzymes involved in glycolipid biosynthesis have been shown to associate in distinct complexes, both physically and functionally, and the same is seen for several enzymes responsible for heparan sulfate synthesis (212). Data from Holmes *et al.* indicate that synthesis of type 1 and type 2 on glycolipids is separated in Colo 205 cells, because the different susceptibility to brefeldin A of the two pathways (238). The same is likely true for the enzymes involved in elongation in N- and O-glycosylation, but there are few reports on this. Viitala *et al.* found that the peptides from digested erythrocyte proteins from an blood group AB individual have either A or B determinants, and not both (239), despite the fact that each peptide carries an average of 3 or 4 determinants. The proposed explanation for this was the formation of enzyme-acceptor complexes.

Recycling of the substrate

The recycling of the substrate may influence its glycosylation, at least for O-glycosylated proteins. This was reported for a mucin, ASGP-1, produced in 13762 rat ascites cells (240,241). Similar results were seen in studies on two transmembrane and one secreted form of MUC1; the two forms of MUC1 are glycosylated differently due to recycling of only the transmembrane forms (228). Recycling of a protein should lead to a higher density of glycans on this protein (241), and that is in fact what has been seen (228,240). It is possible that the recycling may also only involve parts of the glycosylation machinery, e.g. the TGN.

OTHER FACTORS THAT INFLUENCE GLYCOSYLATION

Modifying factors

The synthesis of lactose in the mammary glands has been examined thoroughly. β4Gal-T1 is normally involved in LN synthesis. However, when the enzyme is complexed with α-lactalbumin, it instead forms lactose (242). β4Gal-T1 and α-lactalbumin has since been used as a general model to explain differences in glycan synthesis, e.g. incompatible A (see Glycans and cancer, p. 10). It has been said that it cannot be ruled out that there is a factor, which modifies the enzymatic specificity. Yet, to my knowledge, there are no other proven examples of GTs that change their specificity when they are associated with any co-factor (243).

Epigenetic factors

The role of epigenetic factors in regulating glycan synthesis has not been studied as extensively as other parts of glycobiology. But it is clear that chromatin structure (244) should play a big part in how the glycosyltransferases are expressed and thus ought to regulate also glycosylation. Therefore, it might be equally important to study transient and stable transfectants. You get an average of gene expression in transient transfectants, whereas in stable transfectants, the site of integration and the formation of concatamers can influence the level of expression.

Furthermore, methylation of promoters and CpG islands is yet another way for regulation of GT expression that has been observed (245).

INHIBITORS OF GLYCOSYLATION

Traditionally, inhibitors of glycosylation have been used for the determination of the glycan type. However, the results on inhibition of N-glycosylation with tunicamycin vary depending on the protein and cell type used (246). The drug may also have other effects such as inhibition of mRNA synthesis or cytotoxicity (6). Similar effects are also seen with e.g. benzyl-α-GalNAc, and in this case, the accumulation of modified benzyl-αGalNAc may cause unwanted competition with endogenous substrates and other side effects (6). It is also known that the effect of α-benzyl GalNAc is not equal from cell to cell and also not on all proteins in one cell (247,248).

GLYCOSYLTRANSFERASE FAMILIES

PP-GALNAC-TS

There are about 20 different pp-GalNAc-Ts in man. In addition, there are around 30 pseudo-genes (138). This is in sharp contrast to the enzymes elongating the Tn-antigen. For example, there is from in man only one core 1 β3GalT, one core 3 β3GlcNAcT and three core 2 β6GlcNAcTs cloned. From *D. melanogaster* and *C. elegans*, several pp-GalNAc-Ts were cloned in 2002 and it was found that the sub-families are well conserved, both at protein and substrate levels (249). This is in clear contrast to e.g. the CD33-related Siglec family (52), which is rapidly evolving.

Some pp-GalNAc-Ts are only expressed in certain tissues, e.g. pp-GalNAc-T13, which is specifically expressed in brain (250), whereas others like pp-GalNAc-T1 is found in most tissues (138). The pp-GalNAc-Ts have different preferences for various substrates, though these are not mutually exclusive because each enzyme appears to have a rather broad range of substrates *in vitro* (6,251). In sharp contrast, the cellular synthesis is highly controlled, as seen in several studies (252,253). In a cell-free system, using microsome fractions of the cell-line LS174T, MUC2 peptide glycosylation was tested. Out of about 13,000 possible pathways for the initial GalNAc addition to the peptides, (based on random initiation and *in vitro* data), only two were used. It might be that the Golgi localization is the key factor in this respect as well (6), as discussed above.

GLCNACTS

The β3GlcNAcT that was studied directly in this thesis is β3GlcNAc-T6, or the core 3 synthase gene (254). In man, there seem to be only one enzyme with high activity for core 3 synthesis, whereas there are several of the members in the β3GlcNAcT family that can form poly-LN (48,255).

There are currently three known enzymes in humans that can produce core 2 on O-glycans (256-258). We focused on the β6GlcNAc-T1 because it is ubiquitously expressed (258).

GALTS

The main families of galactosyltransferases are β3GalTs and β4GalTs, but they are perhaps best described as β3GT and β4GT families (48,259). Other GalTs are e.g. the blood group B enzyme and the α3GalTs responsible for the biosynthesis of the major xenogeneic antigen (48).

β3GalTs

Enzymatic activity

The formation of type 1 in humans is performed by three β3GalTs, β3Gal-T1, T2 and T5 (226,259-265). However, it is not clear what type of glycoconjugates they modify, because *in vitro* specificity assays with these enzymes have given somewhat contradictory results. There are only a few publications on these enzymes and its proposed mammalian orthologs. Most of them are cited in this section:

Don Carlson's group found in the early 80's evidence for enzymatic β3GalT activity in pig trachea and rat intestine (266,267). The enzyme was much better in utilizing core 3 than core 2 structures when assayed for type 1 production *in vitro* (267). This enzyme is probably β3Gal-T5, which also is the enzyme that regulates the levels of the tumor marker CA19-9 in human serum (268).

Evolutionary aspects

At least two enzymes in *C. elegans*, Bre-2 and Bre-5, belongs to the family of β3GTs and they have been demonstrated to be involved in glycolipid synthesis in the worm (70,269). Bre-5 was initially thought to be a β3GalT but is instead a β3GlcNAcT. Bre-2 is likely a β3GalT, but it has not been proven directly. Bre mutants were shown to be resistant to *Bacillus thuringiensis* toxin, and no other phenotypes were found. Therefore, it seemed as if the genes were dispensable for the worm. However, a screen for suppressors of Lin-12 (a receptor in the Notch signaling pathway) revealed that Bre-5 was one of them. Bre-5 is sometimes claimed to be an ortholog of Brainiac in Drosophila and β3Gal-T5 in humans, but without a proper phylogenetic analysis, this is impossible to say.

What is fascinating about the β3GalTs is that β3GalT1 and T2 are highly evolutionary conserved (Lofling, unpublished). β3Gal-T1 is remarkably 100% identical on the amino acid level between mouse and man and 95% between chicken and man. This fact has not been pointed out, probably because the only comparisons made have been between all β3GTs and because the mouse genes were cloned based on their similarity to β3Gal-T1 (the only enzyme available in the databases at that time). β3Gal-T2 from human and mouse are 95% percent identical, which is also rather high. It can be speculated that the reason for the high similarity is that the β3Gal-T2 gene is located inside another gene, HPRT2, which is highly conserved and is associated with hyperparathyroidism (270).

No individuals carrying mutations in β3Gal-T1, T2 or T5 have been found. Whether this suggests that the enzymes are vital, or that mutations in these genes would go without phenotypes, remains to be seen. The absence of SNPs in the coding regions also indicates that more time should be spent on investigating this enzyme family. Anyway, a knock-out of β3Gal-T5 will probably not be lethal, since some of its exons were found to be rearranged in Wolley monkeys (271). What will be of high importance is to see whether knockout mice for any of the genes will have any phenotype. In this respect, it should be pointed out that even though the coding sequences are highly similar, the tissue expression pattern is different for these genes (260).

β4GalTs

The β4GalTs have not been studied directly in this thesis. Therefore, I instead refer to the reviews by Hennet (48) and Amado *et al.* (259). Worth mentioning is that deletion of the β4Gal-T1 in mice led to compensatory biosynthesis of type 1 instead of type 2 on plasma proteins (272). This was unexpected, because *in vitro* assays on liver extracts from these mice revealed very low galactosyltransferase activity.

SIATS

In nature, Sia is always linked from its second carbon to the growing glycan chain. The SiaT family is big and consists of α3, α6 and α8 SiaTs (273). The SiaTs responsible for selectin ligands are mainly SiaTIV (274) and SiaTVI (275). These have not been investigated directly but only in the role as a competitor to other enzymes. This has already been discussed in this thesis and therefore, the SiaTs are not going to be discussed further.

FUCTS

α2FucTs

In humans there are two functional α2FucTs. FUT1 was cloned in the early 1990s and FUT2 a bit later by J Lowe's group (276). There is also a pseudo-gene, called Sec1, which is expressed in some primates and mammals but not in humans (277-279).

FUT1 and FUT2

It has often been said that FUT1 controls production of H type 2, whereas FUT2 is responsible for H type 1 and 3 synthesis, mainly due to serological observations, the co-existence of the structures and enzymes, and some *in vitro* data, see e.g. the Introductions to (280,281). Data from enzymatic assays indicate that FUT1 compared to FUT2 shows a preference for type 2, and that FUT2 has a somewhat lower Km for type 1 than FUT1 has (180,181,276,282), but there results varies. This is likely due to differences in the source of the enzymes, purification methods and experimental set-up. *In vivo*, FUT1 can fucosylate type 1 as shown by us and others (283-285).

A longstanding question has been the role of the Secretor gene in the synthesis of H antigens. In 1955, observations by Levine *et al.* on a family with three individuals of the Bombay phenotype were published. It was found that in this family, there were two children, one A1B, se, and the other O, Se, whose parents were A1, se and Oh, se, respectively (286). Since the authors did not consider this a case of non-paternity or non-maternity, the problem was how to explain the apparent non-mendelian discrepancy. The title says it all "Gene interaction resulting in the suppression of blood group substance B". The Secretor gene was accordingly assumed to be a regulatory gene to the H gene. This is also the theory favored by Watkins in her much-cited review from 1980 (5). Data from the group

of Lemieux showed that the stereochemical effects of the different linkages in type 1 and type 2 was markedly different (see Fig. 6, p. 20), which suggested that there would instead be two different enzymes for making H antigens (287). This was also the hypothesis put forward by Oriol *et al.* in 1981 (288) and later biochemical evidence was provided by the same group (289) and others (282). The debate was finally settled in 1995, when the Se gene was cloned (276).

The regulation of α2Fuc, and FUT2 - the enzyme thought to be responsible for its synthesis - in the GI tract of mammals is complex, with several factors, such as cell types, age, suckling or weaning, insulin concentration and bacterial colonization, affecting the degree of fucosylation (66,290,291). However, mice deficient in FUT2 develop normally without any observed abnormal development of the GI tract (292). Staining with *Aleuria aurantia* lectin is abolished, indicating absence of terminal fucose. It remains to be seen if there is some α2Fuc left resulting from compensation by either FUT1 or Sec1 (279,293) that was not stained by the lectin. Fucα2Gal glycans have further been implicated in mechanisms such as long-term memory, and a recent investigation on this topic found that expression of synapsin Ia/Ib is regulated by fucosylation (294). The *in vivo* relevance of these findings is not known.

α2FucTs in other organisms

In rabbits the gene corresponding to FUT1, RFT-I, is only expressed in brain. This enzyme has an approximately equal Km value for both type 1 and 2, whereas the RFT-II has a Km that is about five- and three-fold higher for type 2 and type 3 than type 1, respectively. (295). Transfection experiments in COS-7 cells indicated a substrate difference between the two enzymes *in vivo* as well.

From rats, three α2FucTs have been cloned, but only two were found to be active. The two functional ones, FTA and FTB, were shown to have the ability to induce strong staining with *Ulex europeus* lectin on CHO cells, indicating that they use type 2 well, whereas only FTB can make H type 3. Surprisingly, this was in opposite to the results from enzymatic data, which suggested that FTB barely use type 2 as an acceptor (296).

In cow, there are probably four distinct enzymes (297). Their specificity is mainly directed towards core 1, rather than type 1 and type 2.

The C. elegans CE2FT-1 is a α2FucT with low (5-10%) overall sequence identity to the rabbit, human and mouse α2FucTs. It has an unusual specificity, because it has a specificity towards some Gal-terminated structures, e.g. Galβ4Xyl-R and Galβ6GlcNAc-R, but not toward type 2 (298).

α3/4FucTs

The α3/4FucT family in humans consists of FUT3, FUT4, FUT5, FUT6, FUT7 and FUT9 (299). In this thesis, FUT9 was not examined because it does not produces SLe^x (299).

FUT3, FUT5 and FUT6

FUT3 is the enzyme responsible for the Lewis blood group and has therefore been subjected to many investigations (299-301). FUT6 was cloned in 1992 and shares 85 % and 83% amino acid sequence identity with FUT3 and FUT5, respectively (302). The remarkable thing is that despite the close similarity of FUT3, 5 and 6, they have distinct specificities for different substrates.

Mutagenesis and domain swapping experiments combined with phylogenetic studies have led to the mapping of the amino acids that are responsible for the difference in preference for type 1 and type 2 for FUT3 and FUT5 (299,303-305). Actually, the main determinant for the selectivity for type 1 or type 2 is just one amino acid.

The in vivo relevance of FUT5

An early study found little or no expression of the FUT5 gene in any tissue (306), and because of that the *in vivo* relevance of this enzyme is considered questionable. A subsequent investigation with the same primer design has come to the same conclusion (307) and in other investigations (308-310) no FUT5 expression was seen. Actually, in the literature, there are papers that mention expression of FUT5 but not FUT3 in some cell lines (247,281,311) or both FUT3 and FUT5 (312). Furthermore, a recent report by Nystrom *et al.* provides evidence for up-regulation of FUT5 in cells infected with herpes virus (313). Also, data from FUT3 negative individual have suggested that there is another functional α4FucT present, at least in gastric mucosa (121,125,314,315). Björk *et al.* reported on small amounts of Le^b glycolipids in blood group OLe(a+b-) non-secretor and OLe(a-b-) secretor individuals (316), and results from Hakomori and Andrews also support the fact that glycolipids from some adenocarcinomas carry both Le^a and Le^b, regardless of the Lewis blood type of the donor (317). In addition, a study by Lucas *et al.* found that the human zona pellucida protein contains Le^b, irrespective of the blood phenotype (188).

Even if Le^b cannot be detected on an individual's RBCs, an α4FucT can be present and active in small amount in other tissues (301). This was also seen in a study by Mollicone *et al.* (318). They reported that even if the enzyme is active *in vitro*, it is found in substantially lower amounts in transfected COS cells. It is unclear if all cases with expression of Le^a and Le^b in RBC Lewis negative individuals can be explained by residual activity of FUT3 in other tissues. Several lines of evidence indicate that this is not the only explanation, e.g.:

One gene from a Lewis negative individual that was expressed in COS cells was unable to produce Le^a, in contrast to the wild-type allele (319), and similar results were also reported for the *le1* allele (320). Therefore, it seems more probable that there is another functional α4FucT present in many of these cases, likely FUT5.

α4FucTs in other organisms

The existence of at least one functional α4FucT in plants has been demonstrated by the presence of Le^a on plant N-glycans (321), by the detection of enzymatic activity (322), and by the cloning of transferases from various plants (323,324). However, the acceptor specificity of the tomato FucT differed from that

in white campion in the transfer of fucose to type 2. This hints on the existence of several distinct α4FucTs in plants, or that the enzymes have evolved differently to meet the specific requirements of the respective species. Furthermore, one study showed that expression of human FUT3 in tobacco plants leads to alterations in the gibberellin response, thus suggesting that α4Fuc may have a role in plants (325).

A putative ancestor gene of the human FUT3-5-6 was cloned from cow in 1997 by Oulmouden *et al.* (326). The enzyme is solely working on type 2 and not type 1. The putative gene in mouse and rat is a pseudogene, and the syntenic location is poorly conserved compared to the human one.

FUT4 and FUT7

FUT4 and FUT7 are the only enzymes known to be expressed in a significant degree in normal leukocytes or most leukocytic cell lines. They are therefore the α3FucTs responsible for the generation of selectin ligand under normal conditions *in vivo* and that is the reason for the many investigations on the *in vitro* specificity of these enzymes in SLe^x synthesis (40,41).

The ability of FUT4 to synthesize SLe^x has been debated. Some groups could not detect any production of SLe^x by FUT4, whereas others did. This discrepancy was later shown to be due to the use of two subclones of CHO (327). In BHK cells, a reporter protein with N-glycans was shown to carry SLe^x when co-expressed with FUT4 (328). Another group found that FUT4 could produce SLe^x on glycolipids but not on glycoproteins in CHO cells (329). The substrate specificity of FUT7 *in vitro* is restricted to only sialylated type 2, thus yielding only SLe^x and not Le^x (330).

Due to the differing results from the enzymatic assays, and because there are no studies in cells on the specificity of FUT4 and FUT7 for O-glycan cores, we examined this issue in Paper IV.

BLOOD GROUP ABO ENZYMES

The blood group A and B enzymes differ only by four amino acids. Actually, out of these four amino acids, only two are critical for the discrimination between Gal and GalNAc (331). Furthermore, the blood group A can be sub-divided into several subgroups, with A1 and A2 as the predominant ones (5). It has been debated whether the A1 and A2 enzymes differ in specificity for the H epitopes (126,205,332-335), but no clear consensus exist. A proposed difference was the ability to use H type 3 (207,336), but since it has not been the aim of this study to investigate this issue, I instead refer to the cited papers in this section. The same goes for the sub-types of the O and B genes, as well as for FUT1 and FUT2 (5).

AIMS

The general aim of this thesis was to investigate the specificity of the GTs involved in the biosynthesis of the ABH and Lewis epitopes. The knowledge gained was further to be used for biopharmaceutical purposes, such as absorption of anti-blood group A antibodies, as well as to create a cellular model for *H. pylori* adhesion.

MATERIAL AND METHODS

For a more detailed description, see Materials and methods in the separate papers. Here will mainly issues concerning the thesis in general be described and discussed.

CONSTRUCTS

All constructs for transient transfections were in the CDM8 vector, and the gene expression was driven by the CMV-promoter.

All fusion protein constructs were made by fusing the extracellular parts of the proteins CD43, AGP or PSGL-1 to a mIgG2$_b$-Fc tag on the cDNA level.

For stable transfections, the EF1α-promoter was used, due to the higher risk of silencing of the CMV-promoter (Brian Seed, personal communication).

CELL LINES

CHO-K1

The use of principally CHO-K1 for our studies was based on the fact that they have a limited repertoire of GTs. For example, they do not express any α6SiaTs (337), the O-glycan structures are core 1 based (338,339) and the N-glycans are predominantly complex-type with only type 2 (340). Furthermore, the cells are easy to culture, grow quickly and are readily transfectable. Also, the existence of mutant CHO cell lines, Lec cells (341), makes it possible in the future to try to specialize the synthesis even more.

293T

293T cells are of human origin, and therefore suitable for biotechnological applications. The main problem with 293T cells is that their glycans are not well characterized. We even suspect that the cells express the Galili epitope (Gustafsson *et al.*, unpublished). They grow rather quickly, but a problem is that they detach easily from the surface when they get confluent. However, an advantage is that 293T cells have higher transfection efficiency than COS and CHO cells, especially with cheap reagents such as polyethylenimine (PEI).

COS-7m6

The use of COS-7 cells for controlled production of fucosylated antigens could be troublesome, because there are reports on FucTs in these cells (342). In our hands, there is no detectable activity of α2-or α3-FucTs in COS-7m6 cells, in contrast to what has been found by Clarke and Watkins in the parental COS-7 cells. The type 1 chain is known to exist in COS-1 (319,343) and COS-7 cells (300,315), but we have not found any evidence for this in our COS-7m6 cells. We believe the difference is likely due to the use of different subclones.

WESTERN BLOTTING AND IMMUNOSTAINING

SDS-PAGE was run either according to Laemmli (344) or using the Invitrogen NUPAGE gel system. Transfer of proteins was done in a BioRad Mini Protean II system as described before (30), because the transfer efficiency was better using this system than the corresponding Invitrogen system.

Antibody specificity

The results in this thesis are largely based on immunological detection of the glycan epitopes. Therefore I will go through the antibodies used and their specificity and the controls we had.

The controls

We tried to use two different ways to control for non-specific binding of the antibodies. First of all, BSA-conjugates were used whenever possible. Unfortunately, we did not find any type 1- or Le^a-conjugates. Secondly, controls for the recombinant proteins were produced by leaving out one or more of the enzymes. For example, when testing for production of Le^b, only an α2- or an α4-FucT, or both but no β3Gal-T were co-transfected with the fusion protein constructs.

SLe^x

CSLEX and KM93 are two mAb, which have been used in many investigations, e.g. (345-348). They both react with SLe^x, however, KM93 may even recognize non-fucosylated sialylated structures (349). Our finding that KM93 does not react at all with SLe^x on core 3 is interesting (Paper IV), because of previous reports that show that KM93 has a wider range of reactivity than CSLEX (346,350). Another factor that influence the binding of different SLe^x-reactive mAb is sulfation (345), but this has not been studied for KM93. KM93 is known to bind to both human and rat polymorphonuclear cells (PMNs) whereas CSLEX only binds to human PMNs (350). This could be due to a difference in sulfation or in the type of glycoconjugate carrying the SLe^x epitope.

SLe^a

The 1116-NS-19-9 antibody reacts with the epitope CA19-9, also known as SLe^a, which is present on both glycolipids and glycoproteins (190). The antibody is quite specific for SLe^a-neoglycoconjugates and does not cross-react with other tested structures (351).

H type 1

The specificity of the H type 1 mAb 17-206 (also known as BG-4 from Signet Laboratories) has to my knowledge not been published. It is claimed to react only with H type 1. In Paper I, we saw that the antibody stains H type 1 conjugated to BSA in Western blotting. However, no reactivity with this antibody was detected toward any recombinant protein produced in this paper or in Paper II. This is also substantiated by studies on glycoproteins isolated from mice (352).

H type 2

The H type 2 antibody 92FR-A2 was produced by immunization of mice with synthetic type 2. It has been tested against H type 1, 3 and 4, and does not cross-react with these structures, but with Le^y (353).

H type 3

The specificity of the H type 3 antibody HH-14 is only described in a patent by Henrik Clausen. The reactivity is to H type 3 only, and not to A type 3.

Le^a and Le^b

The Le^a specific mAb 78FR2.3 was found to cross-react with Le^b and type 1 precursors conjugated to BSA in an ELISA assay (354). The characterization of the reactivity of the mAbs T174 and T218 is described in (122). Their specificity is claimed to be towards Le^a and Le^b, respectively, but there are minor reactions with a few other structures (122). T174 and T218 have further been used in the Lewis-typing of LPS from H. pylori (355). Monterio *et al.* found that T218 in ELISA assays only recognizes parts of Le^b and not Le^b in itself in the LPS (356). However, these authors also show that this cross-reactivity does not occur on immunoblots. Other investigators have tested the reactivity of T218 in ELISA towards various synthetic conjugates, and they did not detect any cross-reactivity to Le^a (357). Because the potential cross-reactivity of these three antibodies, we made sure that appropriate controls were included through our experimental set-up, as discussed above (Paper II). No reaction was seen with T218 when one of the enzymes necessary for Le^b synthesis was left out in the transfections (Paper II and III). In addition, no reactivity could be seen with the mAbs T174, T218 and 78FR2.3 when a β3Gal-T was not used (Paper II). What is impossible for us to say is if T174 and 78FR2.3 cross-reacts with Le^b. However, no staining of Le^b-BSA was observed.

The mAb 98FR2.10 was not used extensively for the Western blotting, because of its known cross-reactivity to H type 1 (354); a cross-reactivity was also seen in our results. However, the mAb was used in TLC immunostaining, because in that

case it was possible to distinguish Le^b from H type 1 because of the difference in mobility of the glycolipids and the inclusion of proper controls.

Le^y

The mAb F-3 (358) has also been seen to cross-react with other structures, mostly H-type 2. This antibody was used for TLC staining of glycolipids isolated from wt CHO-K1, and the stable transfectants of CHO-K1, which expressed Le^b, 1C5 and 2C2.

TRANSFECTIONS

For paper I, PEI was used as a convenient way to transfect the cells. In the course of the work on other projects, we found that Lipofectamine 2000 was a much better reagent to use for CHO cells, thanks to a higher transfection rate. That Lipofectamine 2000 was used instead of PEI should not affect the results, because proper controls were used in all experiments.

Stable transfectants were produced in Paper III, and this was done by transfecting the cells with all constructs at the same time. Selection with drugs was then performed, usually for about two weeks, and clones were subsequently selected based on the cellular staining for the desired epitope.

The interpretation of our results is dependent on some critical issues, mainly related to the antibody specificities (see above) and the transfections. One is the use of transient expression of the constructs. However, it was assured that equal amounts of the different vectors were added to each flask of cells in the following ways:

All flasks used for transfection were seeded at the same time with the same number of cells per flask.

All constructs contained the same promoter and were in the same vector backbone, which should not lead to a biased expression other than what might be caused by the gene sequence itself.

A master-mix was prepared in DMEM from the constructs used for several flasks, and aliquots from this mix were then used for transfections.

We checked all blots for the amount of fusion protein produced. In all cases it was about the same. Since fusion protein gene transcription was also driven by the CMV promoter, equal amounts of fusion protein suggest that the total concentration of CMV promoter-containing plasmids in the cells was the same.

The experiments were repeated at least once with plasmids from a second plasmid-preparation to make sure that the DNA quality was stable.

Also, it should be mentioned, that results from transient transfections by definition is an average of several transfectants, opposite to what is the case for single-cell cloned stable transfectants. Integration site, concatamer formation and other factors influence the expression of a gene in stable transfectants, making interpretation of such results in no way easier. This is for example seen in the results from Burdick *et al.* (359) and Kanoh *et al.* (360).

Furthermore, the number of transcripts were not quantified, since mRNA levels do not necessarily correlate to protein levels (361-363), or enzymatic activity for that matter (229,231). Another worry with transient expression is that the protein is over-expressed leading to a misplaced subcellular localization. If transient transfections cause an over-expression of glycosyltransferases in the Golgi, more likely it should have been found that FUT3 and FUT5 both worked on core 2 and core 3, and not on only one of them, as is the case in Paper IV. Supporting the results are also several findings by other groups, such as those by *e.g.* Liu *et al.* and Pykari *et al.* (283,364).

ABSORPTION OF ANTI-BLOOD GROUP A ANTIBODIES

The absorption of anti-blood group A antibodies (anti-A antibodies) in pooled serum from blood group O individuals was evaluated with hemagglutination (as performed at the Blood Bank, KUS, Huddinge) and with an ELISA assay. The ELISA plates were coated with A-PAA-biotin. The antibody concentration before and after absorption was measured. Also, total protein content was assessed by the BCA protein Assay Reagent (Pierce).

RESULTS AND DISCUSSION

ABSORPTION OF ANTI-A ANTIBODIES

We tested the absorption of anti-A antibodies in pooled O serum from blood donors. This was done with PSGL-1/mIgG produced in CHO, 293T and COS cells together with FUT2 and the blood group A enzyme and compared to chemically synthesized PAA-conjugates. Evaluation of the experiment was first done by hemagglutination. Data from such experiments are not possible to quantify, but they gave us an indication of the relative absorption potential of the PSGL-1/IgG from each of the cell-lines. Since the relative blood group A density on PSGL-1/IgG from CHO cells was the highest, we used this protein for a direct comparison to the A-PAA conjugate. This time, the absorption of the antibodies was evaluated with ELISA (see Materials and Methods, the previous page).

The absorption of anti-A antibodies was about 80 times better with PSGL-1/IgG carrying blood group a epitopes than the A-PAA conjugated glass beads. In contrast, a much higher efficiency was seen for absorption of anti-Galα3Gal antibodies (2).

Because we evaluated the absorption of anti-A antibodies with ELISA using a PAA-conjugate (much the same as the one used for the absorption), whereas in Liu *et al.* 1997, a cytotoxic test was used instead (2), the difference could be due to that the antibodies have structural requirements other than just the terminal epitope. Another possibility is that the actual amount and clustering of the A determinants on the PSGL-1 differs in these two cases.

THE COMPLEXITY OF LEWIS EPITOPE BIOSYNTHESIS

FUT1 and FUT2 specificity in O-glycan fucosylation in cells

We confirmed the specificity of FUT1 and FUT2 in the biosynthesis of ABO structures. FUT1 and FUT2 seemed to prefer type 2 and type 3, respectively, for the biosynthesis of H antigens, in keeping with most studies *in vitro* (Paper I). However, when making Le^b on CD43 and PSGL-1, we noted that FUT1 was even better than FUT2, contrary to the prediction from *in vitro* data and glycolipid analysis (Paper II).

Recombinant proteins as probes for glycosylation studies

The concept of using tagged proteins as glycosylation probes *in vivo* has been used in other studies (1,144,339,359,365). We believe this is a better idea to test the specificity of GT than the use of glycosylation inhibitors, due to the reasons discussed in the section Inhibitors of glycosylation, p. 23. The recombinant IgG tagged proteins were very helpful in determining the specificity of glycosyltransferases in cells on both N- and O-glycans. This was evident with the enzymes FUT3 and FUT5, as well as with β3Gal-T1, 2 and 5 (paper II).

The *in vivo* specificity of β3Gal-T1, T2 and T5

The involvement of β3Gal-T1, T2 and T5 in the biosynthesis of type 1 structures was characterized and our results indicate that only β3Gal-T5 works on O-glycans, whereas β3Gal-T1, T2 and T5 all work on N-glycans, albeit with different efficacy (Paper II). The data clarify some unclear results from the past (259,261,265).

Different sorting of CD43/IgG compared to PSGL-1/IgG?

As discussed earlier, mucin glycosylation is quite elaborate *in vivo*. The polypeptide chain is important for the glycosylation *in vivo* (144,227).

The lack of SLe^a reactivity on core 3 on CD43/IgG co-expressed with FUT3 and β3GalT5, compared to the lack of Le^x and SLe^x on core 3 on PSGL-1 suggest that the proteins take different routes in the cell (Paper II and IV). This was proposed to be the case for MUC1 and CD43 isolated from Colo-205 cells, even though the authors did not present clear data supporting this. Recent data from Mathieu *et al.* showed that expression of FUT1 competed with the formation of SLe^x, but not with that of SLe^a (221). This is in agreement with our data presented in Paper II and IV.

Taken together, these results support a recent study which showed that glycosylation in cells is divided not only into *cis*-, *medial*- and *trans*-Golgi, but also into more specific sub-structures (222). However, the mechanism for this is not clear. It could very well be that using other protein backbones might give additional clues. Data from de Graffenreid and Bertozzi indicate that the specificity correlated with the localization of the glycosyltransferases, which is often determined by the stem region (212,224), and this will be interesting to test in relationship to our results

The order of synthesis of Le^b

We cannot in our data (Paper II) distinguish if the production of Le^b goes via Le^a or H type 1. In any case, we (unpublished) have not seen staining of H type 1 on cells or any fusion protein with the antibody 17-206. On the other hand, when FUT3 was also expressed, this resulted in expression of Le^b. It could be that FUT3 is needed because the synthesis *in vivo* goes via Le^a. However, it is not clear if the antibody binds to H type 1 on O-glycans, even if it binds to H-type 1-BSA. The situation of blood group A and Le^y synthesis in COS cells as reported by Clarke and Watkins is reminiscent of this, because they did not find any α2FucT-activity, no H type 2, and yet there is mRNA for FUT1 and even formation of Le^y and blood group A on the cell surface (342).

The O-glycan core and protein sequence is important for the formation of SLe^x by different FucTs

The glycosylation of PSGL-1 from HL-60 cell were determined in 1996 by Wilkins *et al.* (366) and reported to differ from that of CD43 (367). However, PSGL-1 was purified over a column of P-selectin, which was not done with CD43. This has not been pointed out, but it is probably the basis for some discrepancies in the discussion in papers on SLe^x and PSGL-1 versus CD43. Anyway, it is clear that only a fraction of the sialyl-LN epitopes present gets fucosylated, and this is in contrary to what is expected from the enzymatic assays on FUT7, which efficiently fucosylates sialyl-LN *in vitro* (368,369). We therefore wanted to study the production of SLe^x on O- and N-glycans on different proteins and in various cell-lines. Paper IV shows that FUT3, 5, 6 and 7 all have distinct abilities to produce SLe^x on O-glycans on core 2 and core 3, and also that this differs between CD43 and PSGL-1, whereas FUT4 did not make any SLe^x. A difference was also seen between the cell lines COS and 293T for FUT3. Another peculiar finding was that AGP only carried CSLEX reactive epitopes when produced in CHO.

Physiological relevance?

Even though PSGL-1 and CD43 primarily have been found on cells of hematopoietic origin, this may be because focus has been on cells that should express selectin ligands. Anyhow, these proteins have been found in various types of cancer tissue and cell lines of nonhematopoietic origin (370-373). Therefore, studies on SLe^x biosynthesis on core 2 and core 3 on these proteins might be important for an understanding of bacterial adhesion in the airway epithelium and GI tract, and metastasis formation *in vivo*. Experiments with e.g. CD24 and CD44 isoforms in relation to the potential of producing SLe^x on different core structures could also be worthwhile doing (374,375).

Le^b expressing cells

The establishment and characterization of transfectants of CHO cells stably expressing β3Gal-T5, β3GlcNAc-T6 together with FUT2 and FUT3 (Paper III) gave some more information on the complexity of glycan biosynthesis. We found that one clone, 1C5 expressed Le^b only on O-glycans, whereas another clone, 2C2, expressed the same epitope on both glycolipids, N- and O-glycans. Le^y was detected on glycolipids and N-glycans, but not O-glycans isolated from 2C2 and no Le^y staining at all was seen for 1C5.

Another explanation for the difference in the expression of the glycan epitopes, which merits investigation, is that there is a variation in expression levels of some of the enzymes, as seen in the expression of FH6 in FUT6 transfected KM12-LX subclones (376). This could lead to the observed difference between 1C5 and 2C2. Our unpublished observations on other stable CHO transfectants suggest that they have a defect in the N-glycosylation pathway, speaking against the explanation of varying expression levels of GTs. A yet unexplored possibility is that there could be a defect or reorganization of COGs (for more details, see chapter COG, p. 22) in these subclones. At this point, it should be emphasized that the expression of Le^b

was tested only by staining of secreted proteins. It would be interesting to see if cell lysates would give a different result, and also to see if the location of Le^b is on many or just a few proteins.

It will also be interesting to see whether the difference in Le^b expression affects the adhesion of *H. pylori*. Our preliminary data on *H. pylori* adhesion to TLC show no binding of the bacteria to glycolipids isolated from the two clones (see Fig. 7), but we believe that this is due to insufficient amount of loaded material.

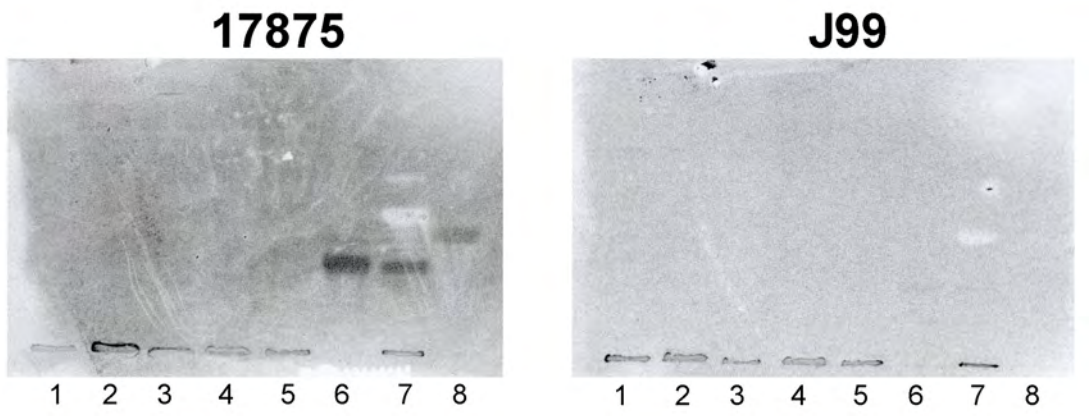

Fig. 7. *H. pylori* TLC overlay.
The samples were loaded (40 µg / lane) in the following order: Lane 1. CHO, lane 2. CHO-Le^b (1C5), lane 3. CHO-Le^b (2C2), lane 4. CHO-H type 1, lane 5. CHO-Type 1, lane 6. Le^b-6 (0,5 µg), lane 7. Neutral glycolipids from RBCs OLe(a-b+) (25 µg) and lane 8. H-5-1 (0,5 µg). The plates were overlayed with bacteria, either the CCUG 17875 or the J99 strain. This was performed by Susann Teneberg and Mette Diswall according to (377).

SUMMARY

I believe that the findings presented in this thesis illustrate that the field needs to change its approach somewhat (and in fact perhaps has done so to some extent). Instead of using small glycans, which has been the traditional way, other inhibitors and tools need to be used if we are going to get a deeper understanding of what really is happening in biology. Not only the terminal, but also the underlying glycan structure is surely playing part in biological happenings.

CONCLUSIONS

- Recombinant PSGL-1/IgG carrying blood group A was much better in absorbing anti-A antibodies in serum than was a synthetically made substrate. We believe this is due to the multivalent presentation of the A epitope on the mucin. However, this was still quite different from the results of another study on antibody absorption from serum (2), and this shows that extrapolating results from a similar study to another can be misleading.
- The glycan and core chain specificity of glycosyltransferases are not redundant, but rather complementary, as seen for FUT3 and FUT5.
- The glycoprotein substrate specificity in CHO cells of β3Gal-T1 and T2 was found to be N-glycans, and for β3Gal-T5 both N- and O-glycans.
- The α3fucosylation in vivo on PSGL-1/IgG and CD43/IgG differs between core 2 and core 3, possibly due to a sorting mechanism in Golgi
- The biosynthesis of fucosylated glycans on core 2 and 3 was peptide dependent for FUT5.
- We have confirmed some *in vitro* data, while also providing evidence for a more complex synthesis *in vivo* than could be predicted from enzymatic synthesis.
- FUT1 and FUT2 can both produce Le^b in cells.
- Stable transfectants of CHO-K1 can be made to express Le^b. Remarkably, two clones, 1C5 and 2C2, differed in their N-, O-glycan, glycolipid and cellular expression of Le^a, Le^b and Le^y. Furthermore, these clones will likely be suitable for studies on BabA-mediated *H. pylori* adhesion.

FUTURE PLANS

It will be interesting to expand the studies in this thesis in some of the following ways by doing:

- Tests on the absorption of anti-A antibodies with mucin from different cell-lines to see if the antibody repertoire is mainly directed against the terminal tri-saccharide of if the underlying structure is influencing the binding of the antibody.
- Bioinformatics on the β3GT family.
- Studies on the evolution of α4FucTs. It will be interesting to see the specificities of the α4FucT(s) responsible for Le^a synthesis in frog.
- Mapping of transcription factors to define the evolution of the expression pattern of β3Gal-Ts, as well as of α4FucTs.
- Tests for the relevance of core structures as well as protein backbone for glycan presentation in biological phenomena, such as DC-SIGN or H. pylori interactions with Le^b.
- *In vitro* studies on FUT3 and FUT5 specificity
- Determination of the factors for the difference in sorting for mucins, especially CD43 and PSGL-1, in relation to the core 2 and 3 synthases.
- Experiments with other mucins and mucin-like proteins (Seagal's law- "A man with one watch always knows what time it is, a man with two watches is never sure").
- Adhesion studies of H. pylori and other bacteria to test the relevance of attachment to a specific receptor, in this case Le^b. For example, will the host cell and bacterial signaling depend on attachment to Le^b? Also, it would be possible to see if the type of glycoconjugate expressing Le^b will influence the attachment and signaling.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kolhydrater, glykaner, finns överallt i naturen. De är inte bara en energikälla, utan framförallt ett sätt för celler att kommunicera med varandra och signalera vad för typ av cell den är. Alla celler är täckta med ett lager av kolhydrater, som är fästa vid proteiner och lipider. Dessa kolhydrater sticker ut som små träd och buskar, och är därför det första en annan cell kommer att stöta på. Tyvärr utnyttjar också bakterier och andra mikroorganismer dessa små strukturer för att fastna och ta sig in i celler. Det kanske mest kända exemplet är influensaviruset som behöver en viss kolhydrat, sialinsyra, för att kunna infektera celler.

Det unika med glykaner är att de kan sätta ihop tre byggstenar (så att de bildar en s.k. trisackarid) på mer än 1000 olika sätt. Att göra en trisackarid på kemisk väg kan vara otroligt krångligt och tidsödande. Kroppen har löst detta problem genom att använda vissa enzymer, glykosyltransferaser (GTs) till att sätta ihop olika glykaner. Av alla glykaner som finns i naturen, är glukos (Glc), galaktos (Gal), fukos (Fuc), sialinsyra (Sia), glukosamin (GlcNAc) och galaktosamin (GalNAc) de vanligaste. Motsvarande enzymer blir då GlcT, GalT, FucT osv.

Eftersom kroppens egna celler har behov av dessa olika glykaner, men även farliga mikroorganismer utnyttjar dessa, har under tidens gång antalet GTs utökats enormt, så att en relativt stor del av vår arvsmassa används till att koda för GTs. Dessutom så skiljer sig ibland egenskaperna åt för motsvarande GTs från olika organismer.

Traditionellt inom glykobiologin (vårt forskningsområde) har ofta studier på GTs endast gjorts i provrör (*in vitro*) med renade enzymer. Resultatet från sådana studier har pekat på att syntes av glykaner sker stegvis, och beror på en tävling mellan de olika GTs som finns i cellen. Problemet är att det i naturen är mer komplext än så. I de flesta celler ordnas nästan alla GTs i en organell kallad Golgi, för att cellen ska kunna styra tillverkningen av glykaner på ett väldigt kontrollerat sätt. Dessutom så verkar det som att Golgi är uppdelat i massa olika subenheter, som var och en kanske har specifika GTs.

För att studera bildandet av glykaner i celler har vi utnyttjat gener som kodar för olika GTs för att undersöka hur dessa fungerar i celler vid syntesen av blodgruppsdeterminanter. Framförallt har vi använt FucTs och GalTs, samt s.k. förgreningsenzymer.

Vad vi har funnit är att bildandet av olika blodgruppsdeterminanter är ännu mer komplext än vad som kunnat förutses av försök *in vitro*. På en biokemisk nivå var det intressant att trots att två enzymer, FUT3 och FUT5, har 91 % identiska aminosyrasekvenser, så skiljer de sig åt markant. Det har förut varit känt att dessa enzymer vid syntes av Lewisepitoper *in vitro* använder de s.k. typ 1 och typ 2 glykanerna olika bra. Däremot har det inte undersökts hur vilka strukturer FUT3 och FUT5 kan använda i celler, speciellt inte de s.k. core 2 och core 3 strukturerna.

Därför var ett huvudmål för mig att studera hur denna syntes sker i celler. Ett nytt, oväntat fynd, var att enzymerna även använder core 2 och core 3 strukturerna olika, trots att enzymerna alltså är nästan identiska i uppbyggnad. Detta har också intressanta kopplingar till hur evolutionen av dessa enzymer har gått till och kan också leda till en djupare förståelse för hur samspelet mellan bakterier och glykaner har påverkat evolutionen av FucTs.

Kunskapen har också använts för olika applikationer. Bl.a. har ett preparat utvecklats för att kunna ta bort antikroppar ur blodet innan transplantation hos mottagaren av ett organ av annan blodgrupp än givaren. Detta är oerhört viktigt, för om de antikropparna inte tas ur blodet stöts organet bort. Vi har sett att preparatet är bättre än syntetiskt tillverkade preparat, vilket vi tror beror på s.k. multivalens (samma princip som ett kardborreband fungerar – en tagg är inte speciellt stark, men många tillsammans är det). En annan tillämpning som har varit möjlig är att göra cellinjer som kan användas vid studier av adhesion av bakterier, framförallt den bakterie som orsakar magsår och magcancer, *Helicobacter pylori*.

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