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Optimisation of the cellular metabolism of glycosylation for recombinant proteins produced by mammalian cell systems

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Abstract

Many biopharmaceuticals are now produced as secreted glycoproteins from mammalian cell culture. The glycosylation profile of these proteins is essential to ensure structural stability and biological and clinical activity. However, the ability to control the glycosylation is limited by our understanding of the parameters that affect the heterogeneity of added glycan structures. It is clear that the glycosylation process is affected by a number of factors including the 3-dimensional structure of the protein, the enzyme repertoire of the host cell, the transit time in the Golgi and the availability of intracellular sugar-nucleotide donors. From a process development perspective there are many culture parameters that can be controlled to enable a consistent glycosylation profile to emerge from each batch culture. A further, but more difficult goal is to control the culture conditions to enable the enrichment of specific glycoforms identified with desirable biological activities. The purpose of this paper is to discuss the cellular metabolism associated with protein glycosylation and review the attempts to manipulate, control or engineer this metabolism to allow the expression of human glycosylation profiles in producer lines such as genetically engineered Chinese hamster ovary (CHO) cells.

Abbreviations: ADCC – antibody-mediated cytotoxicity; BHK – baby hamster kidney (cells); C2GnT – core 2 GlcNAC transferase (UDP-GlcNAc: Galβ1,3GalNAc-R β1,6-N-acetyl glucosaminyl transferase); CHO – Chinese hamster ovary (cells); CMP – cytidine monophosphate; DO – dissolved oxygen; EPO – erythropoietin; ER – endoplasmic reticulum; FT – fucosyl transferase; G0 – agalactosylated glycans; G1 – monogalactosylated glycans; G2 – digalactosylated glycans; GalNAc – N-acetyl galactosamine; GDM – GDP mannose 4,6 dehydratase; GDP – guanosine diphosphate; GFAT – glutamine: fructose 6-phosphate amidotransferase; GlcNAc – N-acetyl glucosamine; GnT – N-acetyl glucosaminyl transferase; HIV – human immunodeficiency virus; IFN – interferon; IgG – immunoglobulin; LAMP – lysosomal membrane glycoprotein; ManNAc – N-acetyl mannosamine; mPL-I – mouse placental lactogen I; NANA – N-acetyl-neuraminic acid; NGNA – N-glycolyl-neuraminic acid; OST – oligosaccharyltransferase; ST – sialyl transferase; ST3Gal1 – sialyl transferase 3 (CMP-sialic acid: Galβ1,3GalNAc2,3 sialyl transferase); TIMP – tissue inhibitors of metalloproteinases; t-PA – tissue plasminogen activator; UDP – uridine diphosphate; UTP – uridine triphosphate

Introduction

Approximately 50% of proteins produced from eukaryotic cells are glycosylated (Apweiler et al. 1999). This post-translational modification of proteins distinguishes the synthetic apparatus of eukaryotic cells from prokaryotes, which lack the intracellular structures and enzymes necessary for glycosylation. Glycoproteins are produced as pools of different glycoforms with varying glycan structures attached to a single invariant peptide backbone. The characteristic glycoform profile is dependent upon the glycan structures added during co-translation and modified post-translationally. Variations may be found in the site-occupancy (macroheterogeneity) or in the structure of attached glycans (microheterogeneity).

The glycan structures are important because they can affect many of the biological properties of the glycoprotein including pharmacokinetics, bioactivity, secretion, *in vivo* clearance, solubility, receptor recognition and antigenicity (Takeuchi et al. 1988; Narhi et al. 1991; Wasley et al. 1991; Storring 1992).

Animal cell cultures are used in bioprocesses for the production of biopharmaceuticals because of their capabilities of post-translational modification of proteins including glycosylation (Butler 2004). The basic protein structures can be controlled and directed by the expression of appropriate genetic sequences. However, controlling the pool of glycan structures (glycomics) that occupy a recombinant protein is still difficult. The glycoform profile of a recombinant glycoprotein expressed by a cell line in culture is affected by various parameters including the host cell line (Goto et al. 1988; Kagawa et al. 1988; Gooche 1992; Sheeley et al. 1997), the method of culture (Jenkins and Curling 1994; Gawlitzek et al. 1995; Schweikart et al. 1999), extracellular environment or the protein structure (Jenkins et al. 1996; Reuter and Gabius 1999).

For the production of a specific recombinant protein as a biotherapeutic it is essential to ensure that a consistent glycosylation profile is maintained between batches (Restelli and Butler 2002). Therefore, it is important to understand the culture parameters that can be controlled to enable strategies in the production bioprocess to ensure the consistency of biopharmaceutical production. The need for culture control can be taken one step further if there is a requirement to express a certain range of glycoforms or even a specific glycoform. This is desirable if enhanced therapeutic efficacy of a specific glycoform is shown.

The purpose of this chapter is to review the cellular metabolism associated with protein glycosylation and review the attempts to manipulate, control or engineer this metabolism to allow the expression of human glycosylation profiles in producer lines such as genetically engineered Chinese hamster ovary (CHO) cells.

Glycan structures attached to proteins

N-glycans

This is the most widely studied structural form of glycosylation and has the greatest effect on overall protein structure and function (Kornfeld and Kornfeld 1985; Spellman 1990). The glycan consists of a core pentasaccharide ($Man_3GlcNAc_2$) attached via an N-glycosidic bond to an Asn residue of a consensus amino acid sequence (sequon) Asn-*X*-Ser/Thr, where *X* can be any amino acid except proline (Figure 1).

The occupation of a sequon occurs co-translationally in the endoplasmic reticulum and involves the transfer of a consensus oligosaccharide structure ($Glc_3Man_9GlcNAc_2$) from a dolichol-linked pyrophosphate donor to the Asn side chain of an available sequon (Figure 2). The enzyme involved in this transfer is oligosaccharyl transferase (OST) and is a complex of several subunits bound to the endoplasmic reticulum (ER) membrane (Yan and Lennarz 2005). Only around 65% of all appropriate glycoprotein sequons are occupied and the oligosaccharyl transfer reaction appears to depend upon various cellular factors including the protein translation rate, the availability of the oligosaccharyl donor and the activity of the enzyme. Petrescu et al. (2004) surveyed the occupation of sequons on several thousand proteins and found a significantly large number of occupied glycans that have low accessibility in the folded protein. Although this may seem surprising, it must be realised that protein glycosylation precedes folding and so the inaccessibility of a sequon in the fully folded protein does not mean that it is necessarily inaccessible to the OST enzyme. The glycans may play a part in maintaining the folded structure by

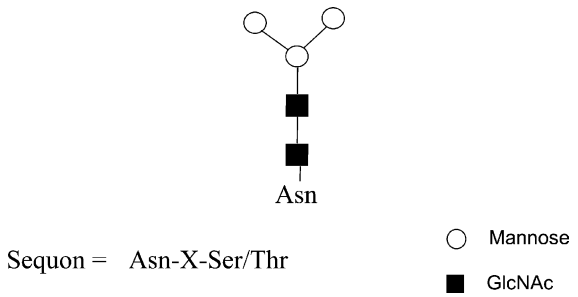

Figure 1. Core N-glycan structure.

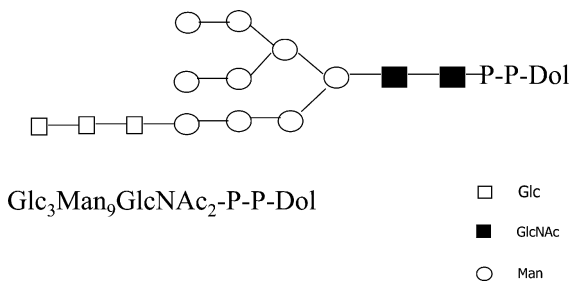

Figure 2. N-glycan precursor attached to dolichol carrier.

reducing the conformational freedom of the local peptide backbone (Petrescu et al. 2004). However, disulfide bridge formation is also co-translational in the ER and may interfere with the accessibility of some sequons leaving them unoccupied. For some proteins such as tissue plasminogen activator (t-PA) this may lead to variable occupancy of a specific site and lead to macroheterogeneity of glycoforms (Allen et al. 1995).

A further consideration is the position of the sequon in relation to the primary structure of the protein. Statistical analysis of a large number of glycoproteins has indicated that the frequency of non-glycosylated sequons increases toward the C-terminus (Gavel and von Heijne 1990). The critical distance appears to be 60 amino acid residues from the C-terminus when reduced glycan occupation occurs. This distance corresponds to the distance between the ribosome P-site and the active site of the OST and it has been hypothesised that the protein chain is not available for N-glycan attachment once it is released from the ribosome. However, this phenomenon of poor glycosylation efficiency toward the C-terminal does not appear to be universal for all proteins (Walmsley and Hooper 2003).

The assembly and processing of N-glycans on proteins

The precursor for glycosylation is the oligosaccharide ($Glc_3Man_9GlcNAc_2$) which is formed by the step wise addition of monosaccharides from nucleotide sugars (UDP-GlcNAc and GDP-Man) or lipid intermediates (Dol-P-Man and Dol-P-Glc) to a dolichol-linked pyrophosphate acceptor to form the complex: $Glc_3Man_9GlcNAc_2$-P-P-dolichol. The 14-mer oligosaccharide is transferred to the consensus sequence (Asn-*X*-Ser/Thr) on the growing peptide via the complex reaction of the OST enzyme, which is thought to be approximately 65 amino acid residues from the ribosome P-site (Yan and Lennarz 2005). Further processing by trimming of the attached glycan occurs by glucosidases and mannosidases that are located within the membrane of the ER. Trimming is initiated by α-1,2 glucosidase I (Gluc I) which removes the terminal glucose. The next two glucose residues are then removed sequentially by a single α-1,3 glucosidase II (Gluc II). At least one mannose residue may then be removed by mannosidase I (Man I) before the newly synthesised glycoprotein is transported to the Golgi cisternae by means of vesicles. This sequential process of trimming in the ER will only occur efficiently if the protein assumes an appropriate folding pattern and so the process has been thought of as a quality control mechanism required for the formation of the protein tertiary structure (Ellgaard and Helenius 2003).

The core structure may be processed further to a complex-type glycan with the sequential addition of monosaccharides such as GlcNAc, galactose, fucose and sialic acid through a series of transferases that are present in the Golgi. The full sequence of reactions in the N-glycan pathway is shown in Figure 3. The transferase reactions in the Golgi do not always go to completion and so give rise to heterogeneity of the final glycan structure (microheterogeneity). This heterogeneity is evident as variable antennarity, with the number of branches from the central mannose of the core structure ranging typically between 2 (biantennary) and

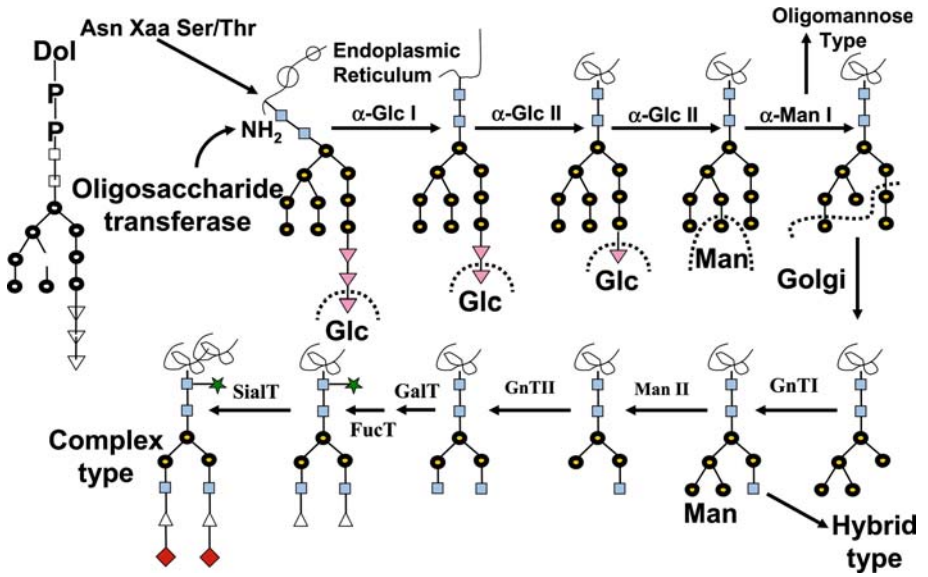

Figure 3. Glycosylation pathway for N-glycans.

4 (tetraantennary). Terminal sialylation of the antenna, fucosylation of the innermost core GlcNAc (proximal) or the outer arm GlcNAc (peripheral) and addition of a 'bisecting' GlcNAc to the central core Man residue are also examples of glycan processing that is variable and may be incomplete in the Golgi. These factors that affect glycan heterogeneity are explained further in Section 'Glycan heterogeneity arising from processing in the Golgi'.

In mammalian cells high-mannose glycan structures are produced as intermediates in the formation of complex glycans. These may have two to six additional mannose structures attached to the core as a result of processing in the endoplasmic reticulum prior to translocation to the Golgi. However, in lower eukaryotes such as fungi and yeast the high-mannose structures are released from the cell as end-products of glycosylation with multiple mannose residues and also in some cases, hybrid structures have been found (Figure 4). These consist of branches from the mannose core of both complex and high-mannose type structures (Meynial-Salles and Combes 1996).

A diverse range of glycan forms may be produced in some recombinant proteins. For example the human immunodeficiency virus (HIV) envelope glycoprotein (gp120) produced from CHO

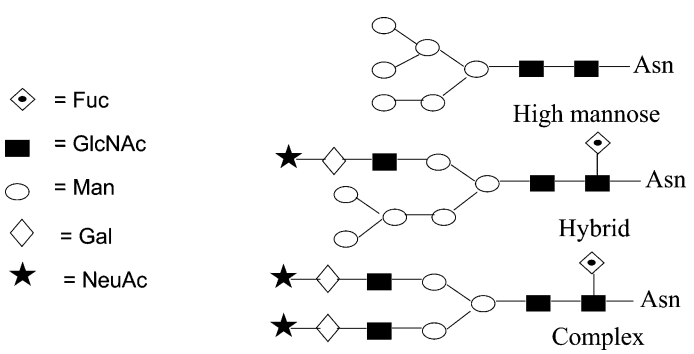

Figure 4. N-glycan structures of glycoproteins.

cells contains 24 occupied sequons of which 13 are complex glycans and 11 are high mannose or hybrid structural forms (Leonard et al. 1990).

O-linked glycans

Although most attention has been placed on N-linked glycosylation of proteins, O-linked glycans are smaller structurally but equally ubiquitous amongst eukaryotic glycoproteins. The most common form of O-glycan is the mucin-type which involves the addition of N-acetylgalactosamine to a serine or threonine residue in a protein. The O-glycan is added post-translationally to the fully folded protein. No consensus sequence has been identified, although glycosylation often occurs in a region of the protein that contains a high proportion of serine, threonine and proline (van den Steen et al. 1998). These residues probably enable the region of the protein to assume a conformation that is accessible to the GalNAc transferase enzyme responsible for the addition of the GalNAc. The first step for the assembly of the mucin type O-glycans is the addition of N-acetylgalactosamine (GalNAc) residue to a Ser/Thr by a GalNAc transferase (Gal NacT) from UDP-GalNAc (Van den Steen et al. 1998). Further elongation leads to a large number of structures, synthesised by various glycosyltransferases, producing eight different core structures (Figure 5). Each of the core structures can give rise to a series of modified structures as illustrated in the pathways for core 1 and core 2 glycans in Figure 6. These core structures can be further modified by sialylation, fucosylation, sulfation, methylation or acetylation.

The most common is the core 1 structure (Galβ1-3GalNAc) that may be monosialylated or disialylated. These are the most prominent O-glycan structures found in glycoproteins produced from CHO cells (Backstrom et al. 2003). Prati et al. (2000) enabled some metabolic engineering of O-glycan synthesis in CHO cells by simultaneously up-regulating and down-regulating selected enzymes to alter the metabolic pathway. They co-expressed the core 2 GlcNAc transferase (C2GnT) and an antisense fragment of the sialyltransferase (ST3Gal I) enzyme in a CHO cell line already transfected with a fucosyltransferase. The effect of this change diverted the O-glycosylation pathway from the formation of core 1 glycans to the formation of core 2 glycans, which are the well studied sialyl-Lewis *X* glycan structures that mediate cell–cell adhesion (Figure 7).

Culture parameters that may affect glycosylation

It is important to control culture parameters in order to ensure consistency of glycosylation of a

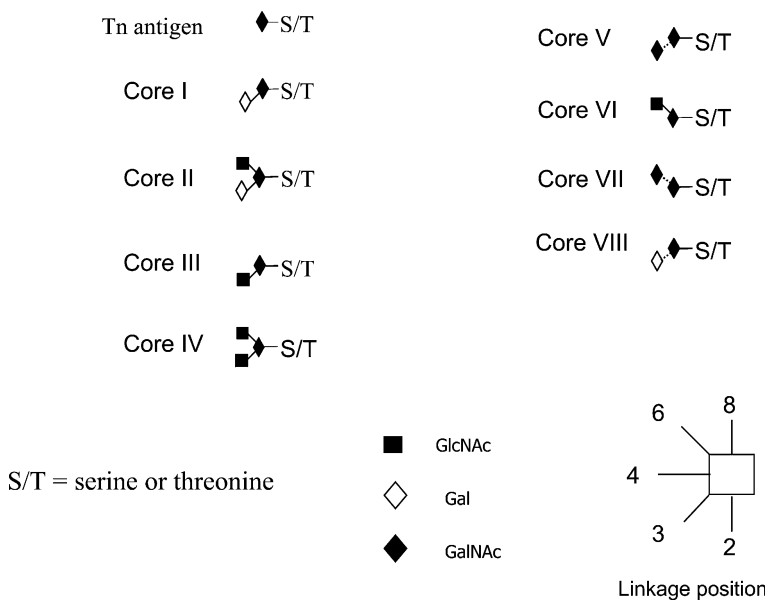

Figure 5. Core structures of mucin-type O-linked glycans.

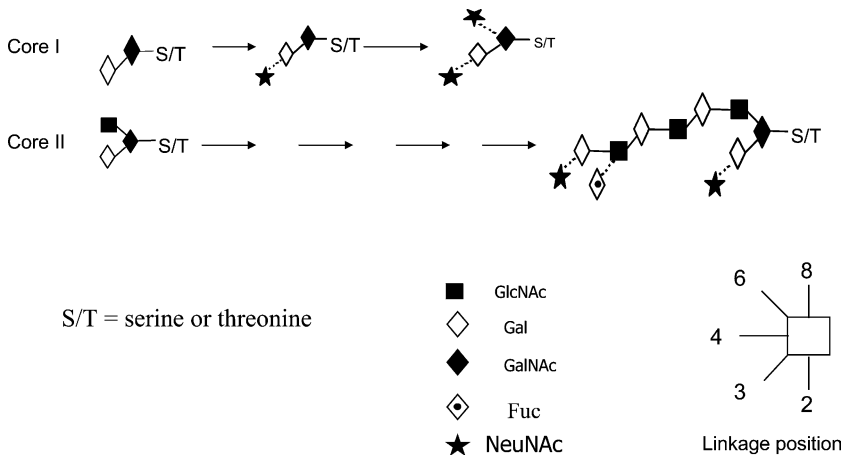

Figure 6. Processing mucin-type O-linked glycans in CHO cells.

recombinant protein in a culture bioprocess. However, this may not be so easy given that the extent of glycosylation may decrease over time in a batch culture (Curling et al. 1990). This is likely to be due to the continuous depletion of nutrients, particularly glucose or glutamine and accumulation of metabolic by-products, which have been shown to limit the glycosylation process (Hayter et al. 1992; Jenkins and Curling 1994; Nyberg et al. 1999). Culture conditions such as nutrient content, pH, temperature, oxygen or ammonia, may have a significant impact on the distribution of glycan structures (microheterogeneity) found on the resulting recombinant protein. This of course is of major concern in trying to produce consistent biopharmaceuticals. It can lead to enhanced glycoform heterogeneity, significant batch to batch variation in the production process and diminished

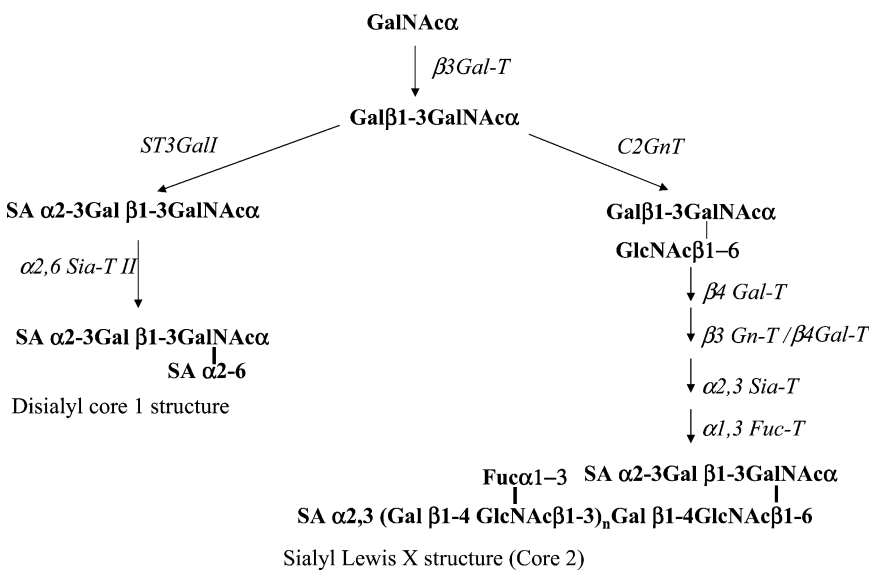

Figure 7. O-linked glycosylation pathways showing the branching point for the synthesis of core 1 or core 2 structures. Prati et al. (2000) showed that inhibition of the enzyme, *ST3Gal I* and the over-expression of the enzyme, *C2GnT* resulted in enhanced production of core 2 structures at the expense of core 1 structures.

therapeutic efficacy. In order to maintain product quality it is important to understand the parameters that cause the variation in glycosylation.

Supplements

Glucose and glutamine

Glucose starvation may result in an intracellular depleted state or a shortage of glucose-derived precursors of glycans (Rearick et al. 1981). Hayter et al. (1992) showed that in a glucose-limited chemostat culture CHO cells produced an increase in the proportion of non-glycosylated gamma-interferon, although pulsed additions restored normal levels of glycosylation rapidly. Fed-batch strategies may be designed to ensure that the concentrations of these key nutrients do not decrease below a critical level that could compromise protein glycosylation (Xie and Wang 1997). From a series of fed-batch culture studies, the lower levels of nutrients for the production of gamma-interferon from CHO cells were found to be 0.1 mM glutamine and 0.7 mM glucose (Chee et al. 2005). Nutrient levels below these critical concentrations led to decreased sialylation and an increase in hybrid and high mannose-type glycans. Reduced site-occupancy of N-glycans was also shown in immunoglobulin synthesis from mouse myeloma cells at low glucose concentration (<0.5 mM; Stark and Heath 1979). Davidson and Hunt (1985) showed under-glycosylation and the presence of abnormal truncated glycans in viral proteins derived from CHO cells deprived of glucose.

A plausible mechanism for reduced site-occupancy of a recombinant protein produced by glucose-depleted or glutamine-depleted CHO cell cultures was offered by Nyberg et al. (1999). They showed that in both cases the low level of glycosylation was related to a decreased intracellular concentration of UDP-GlcNAc but from different metabolic causes. Metabolic flux analysis showed that glutamine depletion was likely to decrease significantly the formation of glucosamine phosphate via the glutamine: fructose 6-phosphate amidotransferase (GFAT) enzymic reaction. On the other hand, glucose-depletion affected the synthesis of UTP, which was found at a low intracellular concentration. Both UTP and glucosamine-phosphate are the key precursors of UDP-GlcNAc, which in turn is required for glycosylation of proteins.

Tachibana et al. (1997) showed that by replacing glucose with GlcNAc in the media of a human hybridoma, they were able to change the glycosylation profile of the hypervariable region of the light chain of the antibody, with a 10× increase in affinity binding to its antigen. This change was associated with a lower level of sialylation of the light chain glycans. The role of UDP-GlcNAc in the metabolic network for glycosylation is shown in Figure 8.

Glucosamine

Glucosamine is a precursor for UDP-GlcNAc, which is an important intracellular nucleotide-sugar that is the substrate for a range of GlcNAc transferases present in the Golgi. Supplementation of cultures with glucosamine leads to an elevated level of intracellular UDP-GlcNAc, particularly if added in conjunction with uridine (Baker et al. 2001). The UDP-GlcNAc requires transport into the Golgi lumen before it can be acted upon by the GlcNAc transferases (GlcNAc T). The elevated level of UDP-GlcNAc has been shown to enhance the antennarity of glycan structures produced in baby hamster kidney (BHK) cells, probably through a stimulation of the specific GlcNAc TIV and TV (Gawlitzek et al. 1998; Grammatikos et al. 1998; Valley et al. 1999). However, the phenomenon is not universal for all cell lines. Baker et al. (2001) found enhanced antennarity following glucosamine supplementation in CHO cells but not NS0 cells that produced the same recombinant glycoprotein. However, in all cases an elevated UDP-GlcNAc appears to cause a decrease in sialylation, which may be explained metabolically by the inhibition of CMP-sialic acid transport (Pels Rijcken et al. 1995).

Galactose

Terminal galactosylation of glycans of recombinant antibodies exhibits significant variability dependent upon the state of the medium. Andersen (2004) has shown that feeding cultures with galactose up to a concentration of 36 mM can ensure high levels of terminal galactosylation as shown in the production of a number of antibodies. Galactose feeding was shown to increase the UDP-galactose pool in the cell up to 20× of control levels and corresponded to a concentration

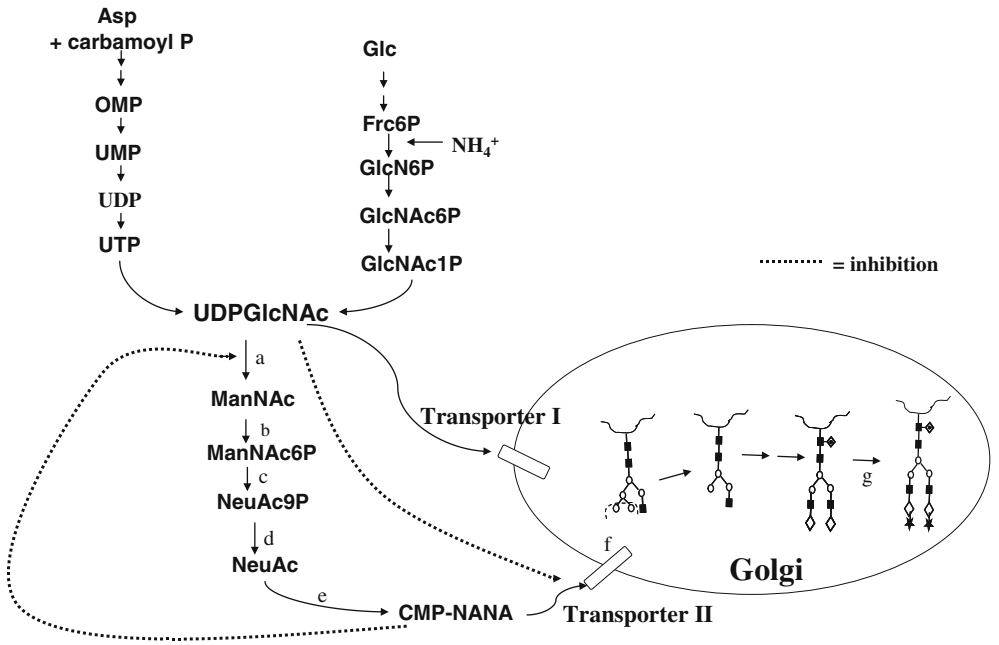

Figure 8. The central role of intracellular UDP-GNAc in the control of sialylation. The enzymes identified in the figure as follows: a: UDP GlcNAc 2-epimerase, b: ManNAc kinase c: Neu 5Ac-9-phosphate synthetase, d: Neu 5Ac-9-phosphate phosphatase, e: CMP-Neu 5Ac synthetase, f: CMP-Neu 5Ac transporter, g: CMP sialyl transferase. The broken lines indicate inhibition by UDP-GlcNAc and CMP-NANA.

of 7 fmol/10^5 viable cells. However, in a separate study Clark et al. (2005) showed that the sialic acid content of a glycoprotein was not increased by galactose feeding. They attributed this to an enhanced intracellular sialidase activity in the galactose-fed cultures that increased the potential for desialylation. Figure 9 shows the metabolic network in which UDP-Gal is a precursor transported into the Golgi by a specific transporter prior to addition to the N-glycan on the protein.

Ammonia

Ammonia (NH_3) or the ammonium ion (NH_4^+) accumulates in culture as a by-product from cellular glutamine metabolism (glutaminolysis) or from the non-enzymatic decomposition of glutamine in the medium. It has been known for some time that the accumulated ammonia is inhibitory to cell growth (Butler and Spier 1984), an effect that is greater at high pH (Doyle and Butler 1990).

The major affect that ammonia exerts on glycosylation is to decrease terminal sialylation, a phenomenon observed in the production of a variety of recombinant proteins (Andersen and Goochee 1994; Zanghi et al. 1998; Yang and Butler 2000). Andersen and Goochee (1995) reported that even a low level of ammonia (2 mM) could affect the sialylation of O-glycans. There are two possible mechanisms to explain this effect. The first of these is the observed increase in the UDP-GlcNAc/UTP ratio that is brought about by the enhanced incorporation of ammonia into glucosamine, a precursor for UDP-GlcNAc. This nucleotide-sugar competes with the transport of CMP-NANA into the Golgi and would therefore decrease the available substrate concentration for sialylation. The second plausible mechanism for the effect of ammonia is that it raises the pH of the Golgi thereby shifting from the optimal pH of the sialyl transferase enzymes (Valley et al. 1999).

pH

Under adverse external pH conditions the internal pH of the Golgi is likely to change resulting in a reduction of the activities of key glycosylating enzymes. The pH of the medium was shown to have some effect on the distribution of glycoforms of IgG secreted by a murine hybridoma (Rothman

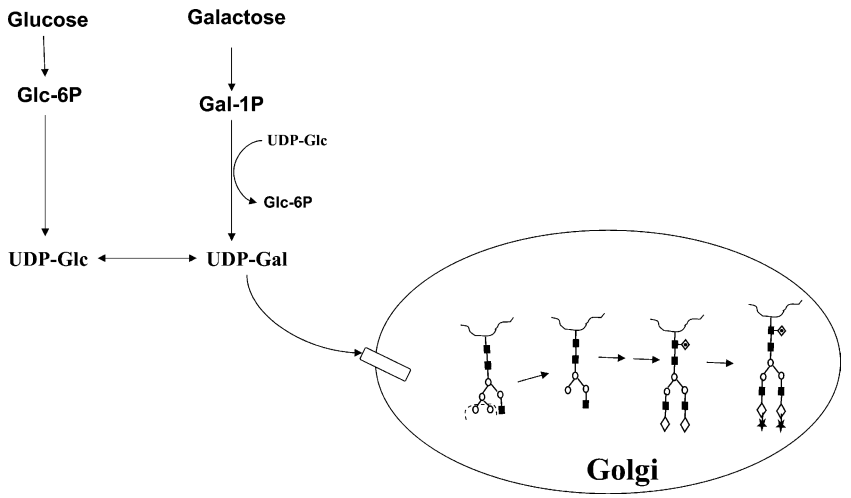

Figure 9. Pathway for galactosylation from galactose feeding.

et al. 1989). Borys et al. (1993) related the extracellular pH to the specific expression rate and glycosylation pattern of recombinant mouse placental lactogen-I (mPL-I) by CHO cells. They observed that the maximum specific mPL-I expression rates occurred between pH 7.6 and 8.0. The level of site occupancy was maximum within this pH range, decreasing at lower (<6.9) and higher (>8.2) pH values.

Oxygen

The control of the dissolved oxygen (DO) level is an important parameter to maintain optimal metabolism and growth of producer cells in bioprocesses (Jan et al. 1997; Heidemann et al. 1998). The effect of DO on the glycosylation of a recombinant protein from CHO cells was observed by a changing glycoform profile (Chotigeat et al. 1994). In particular, an increase in sialyltransferase was observed at high oxygen levels that translated into increased sialylation of recombinant follicle stimulating hormone (FSH).

By controlling DO set-points between a range of 1–100% air saturation the terminal galactosylation of an immunoglobulin (IgG) was changed significantly with a gradual decrease in the digalactosylated glycans (G2) from 30% at the higher oxygen level to around 12% under low oxygen conditions (Figure 10; Kunkel et al. 1998). The mechanism for the effect of DO is unclear but it is unlikely to be due to a change in the activity of the transferase enzyme (Kunkel et al. 2003). One explanation is that reduced DO causes a decline in the availability of UDP-Gal, which might occur through reduced synthesis or reduced transport into the Golgi lumen. A second explanation is that galactosylation might be sterically impeded by the early formation of an inter-heavy chain disulfide. It has been proposed previously that the timing and rate of formation of the disulfide bond in the hinge region of IgG is critical to the extent of galactosylation (Rademacher et al. 1996). The redox environment of the ER or the Golgi may perturb the pathway of inter-chain disulfide bond formation.

The effect of the producer cell line on protein glycosylation

The pattern of protein glycosylation is dependent upon the expression of various glycosyltransferase enzymes that are present in the Golgi of the cell. Differences in the relative activity of these enzymes among species can account for significant variations in structure. In one systematic study of glycan structures of IgG produced from cells of 13 different species significant variation was found in the proportion of terminal galactose, core fucose and bisecting GlcNAc (Raju et al. 2000).

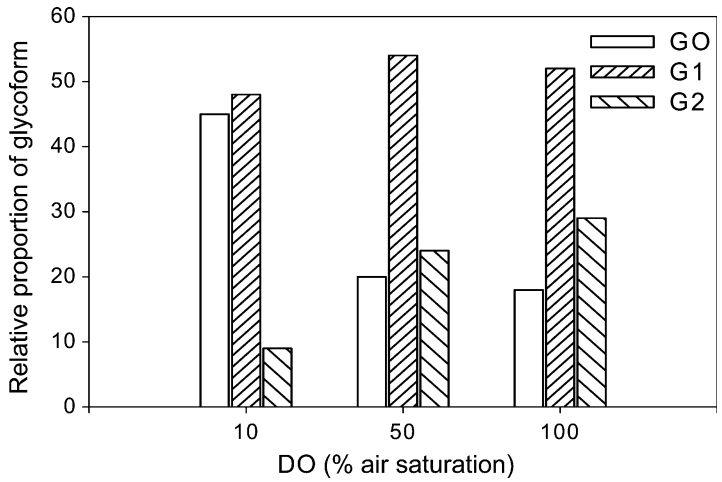

Figure 10. Effect of dissolved oxygen on the glycoform profile of IgG. The proportions of agalactosylated (G0), monogalactosylated (G1) and digalactosylated (G2) glycans are shown following the growth of cells three different dissolved oxygen conditions.

The fact that glycoproteins normally exist as mixtures of glycoforms suggests that the protein structure is not the primary determining factor in glycosylation. The glycoforms that emerge from the Golgi are end products of a series of incomplete enzymic reactions. Thus, the choice of the host cell line is particularly important factor in the glycoform profile of a recombinant protein (Rudd and Dwek 1997). Sialylation patterns of the secreted protein are particularly affected by the host cell (see Section 'Sialylation').

The effect of growth rate and protein production on glycosylation

N-glycosylation occurs co-translationally in the ER over a limited time period in which the N-glycan sequon is exposed to the active site of the oligosaccharyl transferase (OST) enzyme. This means that the glycan site occupancy may be dependent upon the rate of the elongation step of protein synthesis. There are several studies that support this. Shelikoff et al. (1994) showed that C127 cells that expressed recombinant prolactin enhanced glycosylation of the secreted protein from 20% to 80% in the presence of cycloheximide. Cycloheximide is highly specific in its inhibition of protein elongation and so could extend the time available for glycosylation by reducing the elongation rate.

Human tissue-type plasminogen activator has 3 N-glycan sites one of which is variably occupied (Asn-184). This gives rise to the production of a mixture of type 1 (3 glycans) and type 2 (2 glycans) from the culture of transfected CHO cells. The relative levels of each of these two forms of t-PA is dependent upon various factors including the cellular growth rate and it is suggested that site-occupancy could vary with the fraction of cells in the G0/G1 phase of the cell cycle (Andersen et al. 2000).

This suggests a mechanism by which glycosylation efficiency improves at a reduced rate of protein translation. A decrease in growth rate produced by supplementation of butyrate or lowering the culture temperature resulted in an increased site occupancy and higher levels of type 2 t-PA (Andersen et al. 2000). This also supports the hypothesis that the lowering of the rate of protein elongation would increase the exposure time of the glycan site to the oligosaccharide transferase enzyme in the ER.

The residence time of proteins in the Golgi can also be important in the determination of the extent of exposure to glycosyltransferase enzymes. Wang et al. (1991) incubated cells at 21 °C to decrease the flow of glycoproteins through the Golgi. They found that the glycans produced under these conditions had 100% more N-acetyllactosamine repeats than the controls, suggesting that glycan processing increases with increasing residence time in the Golgi.

Nabi and Dennis (1998) found similar results that the extent of polylactosamine formation in lysosomal membrane glycoprotein (LAMP-2) was dependent upon the Golgi residence time. Polylactosamine consists of repeated Gal β1–4GlcNAc disaccharide units that are produced by the repeated action of two transferases, β1–3GlcNAc transferase and β1–4Gal transferase. Low temperature culture was used to increase the residence time of the synthesised protein in the Golgi and enhance its glycosylation by increased exposure to the limiting transferase enzymes.

Glycan heterogeneity arising from processing in the Golgi

N-acetyl glucosaminyltransferases

Significant heterogeneity of glycan structures is a result of variable activity of a series of N-acetyl glucosaminyltransferase (GnT) enzymes that are found within the Golgi. These enzymes are important in converting a high mannose glycan structure to a complex structure. They introduce variable antennarity onto the core mannose structure and also may allow a bisecting GlcNAc to occur between two antennae. N-acetyl glucosaminyltransferase1 (GnT1) is the first of these enzymes and is responsible for the transfer of GlcNAc from the nucleotide-sugar donor (UDP-GlcNAc) to the α1,3 mannose arm of the high mannose structure, M_5. This creates a hybrid structure M_5Gn, which is converted to M_3Gn by the sequential action of a mannosidase. The free α1,6 mannose arm of the core structure is then extended by GnTII to produce M_3Gn_2 which is a complex biantennary glycan. Both arms may be extended by the sequential action of galactosyl transferase and sialyl transferase enzymes to produce fully sialylated biantennary structures. However, the other GnTs can allow further branching. GnTIV may add GlcNAc in a β1,4 linkage to the α1,3 mannose arm of the core structure and leads to M_3Gn_3, which is a triantennary complex glycan. GnTV has equivalent activity for addition of a GlcNAc in a β1,6 linkage on the α1,6 mannose arm of the core structure. Thus, the combined activity of GnTIV and GnTV leads to a tetra-antennary structure, M_3Gn_4 (Figure 11).

A bisecting N-acetyl glucosamine ($GlcNAc^b$) may also be added via a β1,4 linkage to the core mannose through the activity of GnTIII. This $GlcNAc^b$ cannot be extended further because of steric interference that does not allow access of the galactosyl transferase. However, GnTIII is not expressed in CHO or BHK cells and therefore the bisecting GlcNAc is normally absent in glycoproteins produced from these cell lines.

The importance of each of the GnT enzymes is emphasised by the determination of a set of abnormal glycan structures found in a human patient with a genetic mutation in the GnTII enzyme that leads to a severe pathological condition characterised as a Congenital Disorder of Glycosylation type II (Butler et al. 2003). This led to the biosynthesis of a series of hybrid glycan structures that appeared to be present in all serum proteins.

This complex of reactions that involve the GnTs has been modelled mathematically and described as the central reaction network of glycosylation (Umana and Bailey 1997). They based the model on kinetic constants and mass balances associated with the formation of 33 different glycan structures and used the model to predict the effect of GnTIII on the glycosylation metabolism of CHO cells, in which the enzyme is normally absent (Figure 12). The ability of CHO cells transfected with the GnTIII gene to produce bisected glycans was shown for beta-interferon and an immunoglobulin (Sburlati et al. 1998; Umana et al. 1999). In the case of immunoglobulin it was shown that the presence of a bisected $GlcNAc^b$ resulted in a significant enhancement of effector function through antibody-mediated cytotoxicity (ADCC; Umana et al. 1999).

Fucosylation

The core-fucosylation pathway includes the synthesis of the fucose donor GDP-fucose, the transport of the nucleotide-sugar from the cytosol into the Golgi, and the transfer of fucose to the nascent glycoprotein by the enzyme α1,6 fucosyltransferase (α1,6FT) (Hirschberg 2001; Freeze 2002). Alpha 1,6 fucosyltransferase is a glycosyltransferase enzyme that catalyses the transfer of fucose from GDP-fucose to the Asn-linked GlcNAc residue (Voynow et al. 1991; Noda et al. 1998). The enzyme is a type II transmembrane protein, con-

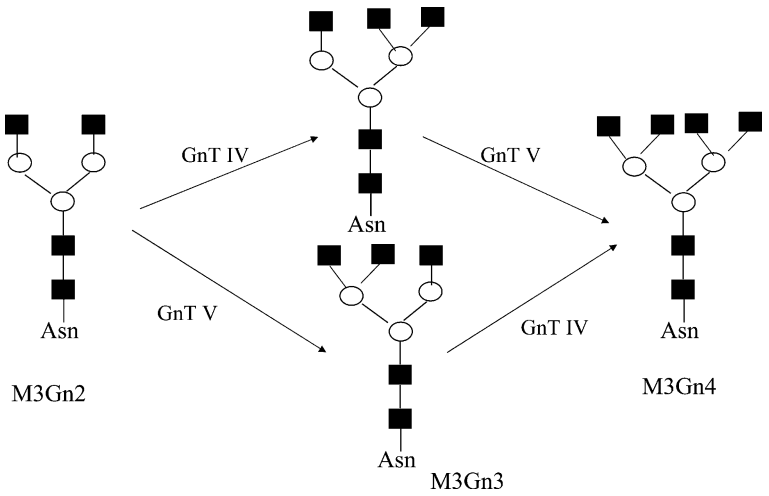

Figure 11. Production of tri- and tetra-antennary structures.

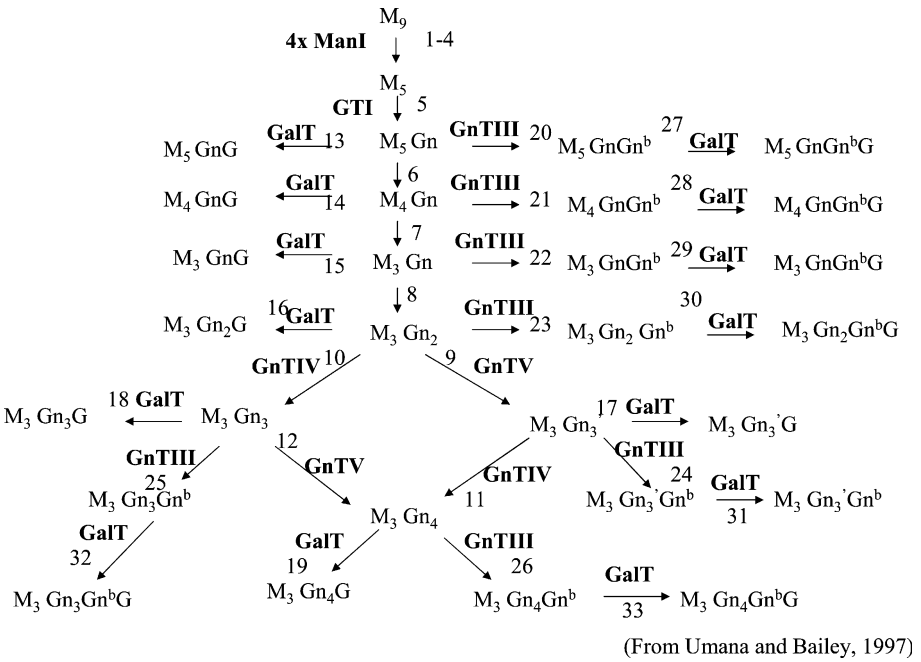

Figure 12. Reaction network for N-linked glycosylation.

sisting of a short amino-terminal cytoplasmic tail, a transmembrane domain and a large intraluminal carboxyl-terminal catalytic region (Breton et al. 1998).

Although the α1,6 fucose residue is frequently found in the N-glycans of a variety of glycoproteins and it has been shown that this modification is widespread in a variety of tissues (Srikrishna et al. 1997), little is known about the function of the core fucose residue. Fucose-containing glycoproteins are removed from the blood into the liver and fucose/mannose receptors exist on the surface of macrophages and mediate phagocytosis (Miyoshi et al. 1999). Some studies have found that core fucose residues play an important role in defining oligosaccharide conformation needed for

specific carbohydrate–protein interaction (Stubbs et al. 1996) and they can regulate the activity of some glycoproteins (Wang et al. 2001).

A congenital defect in the activity of the enzymes involved in the de novo synthesis of GDP-fucose from GDP-mannose has been reported in humans (Sturla et al. 1998). This involves the enzymes GDP-mannose 4–6 dehydratase (GDM) and FX protein, a NADP(H)-binding protein that apparently catalyses a combined epimerase and NADPH-dependent reductase reaction (Tonetti et al. 1996; Ohyama et al. 1998). Altered core-fucosylation has been found to occur in many glycoproteins of tumor cells (Yamashita et al. 1989; Miyoshi et al. 1999). A high α1,6 fucosyltransferase activity has been associated with hepatocellular carcinoma (Uozumi et al. 1996; Saitoh et al. 1993), ovarian adenocarcinoma (Takahashi et al. 2000) and several other human cell lines including myeloma, pancreatic cancer, lung cancer and gastric cancer (Yanagidani et al. 1997). This increase in the α1,6 fucosyltransferase activity in tumor cells was shown to be at the level of transcription (Noda et al. 1998).

There are reports of altered fucosylation of glycoproteins in patients with cystic fibrosis; attributed to a change in the intravacuolar pH of the Golgi apparatus (Barasch et al. 1991). Therefore, a change in the glycosylation of a glycoprotein could occur because of an altered activity of the glycosyltransferases as a result of a perturbation in the pH or due to a change in the proper localisation of glycosyltransferases (Sears and Wong 1998). Furthermore, because of the high specificity of the enzyme α1,6 fucosyltransferase (Longmore and Schachter 1982), a modification in the glycan substrate might cause a significant change in the resulting fucosylation.

Fucosylation may be particularly important to the biological activity of some biopharmaceutical proteins, such as IgG, in which the fucose plays an important role in the determination of the binding capacity of the protein to Fc receptors (see Section 'Functional glycomics').

Sialylation

A high level of terminal sialylation of glycans is important in therapeutic glycoproteins in order to avoid the effects of asialoglycoprotein receptors present in the liver and macrophages that cause the removal of the glycoprotein from the circulatory system (Weiss and Ashwell 1989). This interaction with the receptors reduces therapeutic efficacy by decreasing the effective half life of the glycoprotein in the circulatory system.

Sialylation is the last intracellular stage of the glycosylation process that takes place in the trans-Golgi. This involves the enzymatic transfer of sialic acid from the nucleotide sugar precursor, CMP-sialic acid to an available galactose on the emerging glycan structure that is attached to the newly synthesised protein. Possible limitations to the process that might cause incomplete sialylation include the availability of CMP-sialic acid and the activity of the sialyl transferase enzyme.

The pool of CMP-sialic acid (CMP-NANA) in the trans-Golgi is generated from a well-defined biosynthetic pathway in which glucose is the original precursor but the unique portion of the pathway derives from UDP-GlcNac (Figure 8). The metabolic conversion from UDP-GlcNac to CMP-NANA proceeds via 5 enzymic steps and one transport step into the Golgi that includes the formation of a 9-carbon sialic acid from the fusion of a hexose and a 3 carbon pyruvate structure. There is a metabolic control network in this pathway governed through two key steps – the UDP-GlcNAc 2-epimerase enzyme and the transporter II. The activity of the epimerase enzyme is regulated allosterically through the cytoplasmic concentration of CMP-NANA (Rijcken et al. 1995). This feedback mechanism is important to prevent the excessive accumulation of CMP-NANA. It has been shown that the human hereditary disease sialuria, characterised by the accumulation of excessive sialic acid, arises through a mutation in the allosteric site of this enzyme (Seppala et al. 1999). A further regulatory mechanism associated with the transporter (II in Figure 8) involves competitive inhibition of CMP-NANA transport into the Golgi if the cytoplasmic level of UDP-GlcNAc is too high. This may occur in the presence of excess ammonia which is required for the formation of glucosamine 6-phosphate, a precursor of UDP-GlcNAc (Valley et al. 1999).

One of the strategies that can be used to enhance the intracellular concentration of CMP-NANA is to supply one of its metabolic precursors. Because of its location in the metabolic

pathway and high cell membrane permeability, N-acetyl mannosamine (ManNAc) is the best candidate for this, as sialic acid or CMP-sialic acid has poor membrane permeability. Gu and Wang (1998) showed that the supplementation of 20 mM ManNAc to CHO cell culture increased the intracellular pool of CMP-sialic acid 30× and enhanced the sialylation of gamma-interferon. However, the enhancement of sialylation was selective and limited. One N-glycan site (Asn^{25}) was unaltered at a high level of sialylation (90%) whereas in the second site (Asn^{97}) the proportion of incompletely sialylated biantennary structures was reduced from 35% to 20%. This suggests that in this system there were other limiting factors, which were probably related to the rate of the transport system or the relative accessibility of the glycan sites to the transferase enzymes. The results from the use of this strategy in other systems have been variable. Baker et al. (2001) reported an increase of intracellular CMP-NeuAc as a result of ManNAc feeding to either CHO or NS0 cells but no increase in overall sialylation for a recombinant protein (TIMP 1) containing two N-glycan sites. However, this strategy did change the ratio of N-glycolylneuraminic acid/N-acetylneuraminic acid from 1:1 to 1:2 in the NS0 cells. In a further study using this type of feeding strategy, Follstad (2004) showed a 36% increase in protein sialylation from the addition of 4 mM each of ManNAc, fructose, galactose and mannose. The metabolic network used to rationalise this feeding strategy is shown in Figure 13.

Analogues of ManNAc may also be incorporated into the glycosylation pathway leading to non-natural sialylated glycans that may be incorporated into secreted proteins or cell surface proteins (Jones et al. 2004). This strategy has potential in sialic acid engineering that may lead to novel glycoproteins with unique properties.

Sialylation can be maximised by enhancing the activity of specific glycosyltransferase enzymes. Weikert et al. (1999) used this approach with CHO cells that had already been selected for the secretion of different recombinant glycoproteins and transfected them with human genes to allow the over expression of both β1,4-galactosyltransferase and α2,3-sialyl transferase. The predominant structures (>90%) secreted by these re-engineered cells were fully sialylated bi-, tri- and tetra-antennary glycans. This decreased the heterogeneity of glycoforms produced by the cells and the more highly sialylated structures were shown to have improved pharmacokinetics as tested in animals.

The structure of sialic acid varies between species with N-glycolyl-neuraminic acid (NGNA) found in goat, sheep and cows rather than the N-acetyl-neuraminic acid (NANA) found in humans. NGNA is the predominant sialic acid in

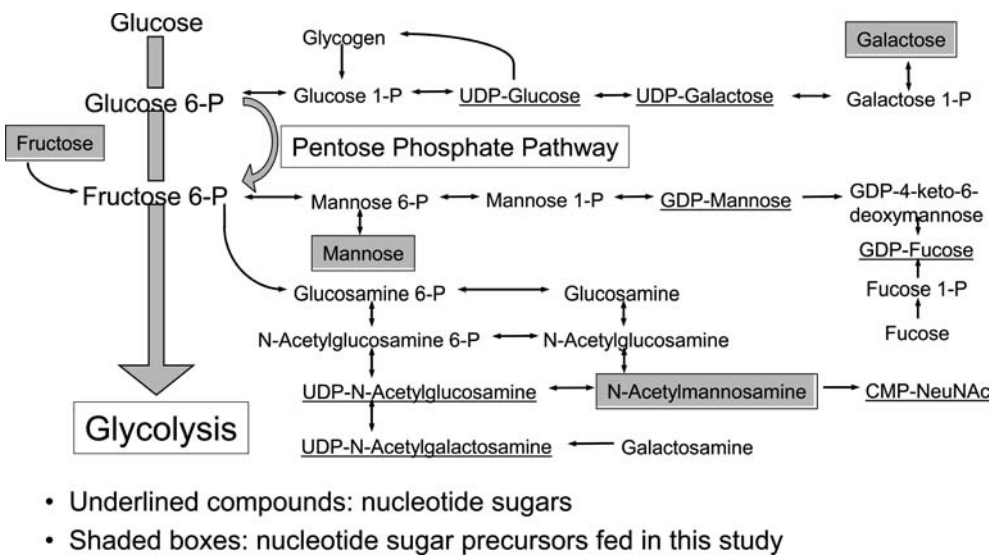

Figure 13. Nucleotide sugar biosynthesis.

mice but CHO-produced glycoproteins have predominantly NANA, although a small proportion (up to 15%) of NGNA can occur (Baker et al. 2001). These differences in glycan structure are important for the effectiveness of biotherapeutics because of the potential immunogenicity of these structures in humans.

There are 18 different types of sialyltransferases that have been cloned from various animal species and they give rise to various terminal linkages (Angata and Varki 2002). The two that are most important for human glycoproteins are the α2,6 and α2,3 sialyl linkages to galactose, each of which is catalysed by a specific enzyme – the α2.3 or α2,6 sialyl transferase (ST). In humans both enzymes compete for the same substrate and this results in glycoproteins with either linkage type, depending upon the cells. The linkage and expression levels of terminal sialic acids do have important effects in human cells that include interactions with the extracellular matrix and susceptibility to apoptosis (Dawson et al. 2004). The sialyl transferase enzyme, (α2,6 ST) is absent in the hamster and so CHO and BHK cells produce glycoproteins with exclusively α2,3 terminal sialic acid residues. However, these differences in sialylation profiles between CHO and human cells do not appear to result in glycoproteins that are immunogenic. Natural human erythropoietin (EPO) consists of a mixture of sialylated forms – 60% are 2,3 linked and 40% are 2,6 linked. Because of the restricted sialylation capacity of CHO cells, the commercially available EPO is sialylated entirely via the α2,3 linkages (Takeuchi et al. 1988). Nevertheless, recombinant EPO produced from CHO cells has proven to be a highly effective therapeutic agent with no evidence of an adverse physiological effect due to the structural differences in terminal sialylation.

The sialic acid pattern of glycoproteins from producer cell lines can be humanized by transfecting with appropriate glycosyltransferase enzymes. Attempts have been made to enable α2,6 sialylation in hamster cells by transfection of the α2,6 sialyl transferase gene. BHK cells produced proteins containing a mixture of α2,3 and α2,6 sialyl structures following transfection with an α2,6 ST gene (Grabenhorst et al. 1995). CHO cells that were co-transfected with genes for gamma-interferon (IFNγ) and α2,6 ST produced sialylated IFNγ, 40% of which was in the α2,6 form. This form of IFNγ was shown to have improved pharmokinetics in clearance studies compared to IFNγ produced from a normal CHO host (Bragonzi et al. 2000).

Immunogenicity of non-human structures

Mouse cells express the enzyme α1,3 galactosyltransferase that generates Gal α1,3-Galβ1,4-GlcNAc residues that are highly immunogenic in humans (Jenkins et al. 1996).

In one study of NS0 murine cells, 30% of glycan antennae of a recombinant protein terminated with the Gal α1,3-Gal motif (Baker et al. 2001). The NSO cells and their derived hybridomas are used extensively for the production of IgG monoclonal antibodies. However, in the immunoglobulin structure terminal galactosylation of the conserved glycan site at Asn_{297} of the Fc region is restricted by steric hindrance (Wormald et al. 1997) and so the extent of terminal α1,3-Gal is likely to be much lower (Sheeley et al. 1997).

Fortunately, the α1,3 galactosyltransferase enzyme appears to be inactive in CHO and BHK cells that are the most commonly used cell lines for the production of recombinant proteins. However, both CHO and BHK show differences in their potential for glycosylation compared to human cells. The absence of a functional α1,3 fucosyltransferase in CHO cells prevents the addition of peripheral fucose residues and the absence of N-acetylglucosaminyltransferase III (Gn TIII) prevents the addition of bisecting GlcNAc to glycan structures (Jenkins and Curling 1994).

The sialic acid variant – N-glycolylneuraminic acid – is prevalent in mouse-derived cells (Raju et al. 2000) and is potentially immunogenic in humans (Noguchi et al. 1995). For NS0-derived glycoproteins, the NGNA can be $>50\%$ of the total sialylation, whereas in CHO cells the proportion is lower but not insignificant; 1–5% for one study (Hokke et al. 1995) and 15% in another (Baker et al. 2001).

Functional glycomics

The production of specific protein glycoforms may allow the possibility of even more efficacious drugs

(Shriver et al. 2004). Functional glycomics is an expanding area of science that attempts to understand the physiological function of specific carbohydrate groups. This approach established the importance of the sialylation of EPO with the discovery that the removal of sialic acid groups from the glycans resulted in a significantly reduced half-life in the blood stream (Erbayraktar et al. 2003). Therapeutic recombinant EPO is normally enriched with highly sialylated glycoforms by selection using ion exchange chromatography of the product secreted from CHO cells. Protein engineering has allowed the creation of a modified EPO with two extra glycan attachment sites and with the potential to incorporate 8 extra sialic acid groups per molecule. This has led to a new generation EPO called *darbepoetin*, which has a three times higher drug half-life (Egrie et al. 2003). This strategy of enhancing the half-life of a biotherapeutic has also been successful for other recombinant proteins such as follicle stimulating hormone (Perlman et al. 2003) and thyroid stimulating hormone (Thotakura et al. 1991)

Structural changes of glycans can also be brought about by metabolic engineering of the host cell line. This includes gene-knockout of already expressed glycosyltransferases or the insertion of novel activities (Weikert et al. 1999). The presence of a bisecting N-acetylglucosamine (Umana et al. 1999; Davies et al. 2001) or the absence of fucose (Shields et al. 2002; Shinkawa et al. 2003; Okazaki et al. 2004) in the conserved glycan of an IgG antibody has been shown to enhance attachment to Fc receptors and result in an increase in antibody-dependent-cell-mediated cytotoxicity (ADCC). This has been of value in the design of antibody therapeutics. For example, recent work with Herceptin®, which is a novel humanized antibody approved for the treatment of breast cancer, has shown that a glycoform with no fucose has a 53× higher binding capacity to an Fc receptor that triggers its therapeutic activity (Shinkawa et al. 2003). This enhancement of ADCC allows the antibody to be effective at lower doses. Afucosylated antibodies can be produced from cells in which the gene for fucosyl transferase has been removed by gene knock-out technology.

Complete glycosylation of recombinant proteins is usually associated with maximisation of galatosylation and sialylation. Often these two processes are incomplete and this gives rise to considerable glycan structural variation. CHO cells can be engineered with a combination of human β1,4-galactosyltransferase and α2,3-sialyltransferase to ensure high activities of these enzymes. The recombinant proteins produced by these cells exhibited greater homogeneity compared to controls and increased terminal sialic acid residues (Weikert et al. 1999). An alternative approach involves glycoengineering of the proteins *in vitro* (Raju et al. 2001). Preparations of these terminal transferase enzymes can be immobilized so that glycoproteins can be galactosylated and sialylated in the presence of appropriate galactose and sialic acid donors.

Conclusion

The importance of the glycosylation process to the production of biopharmaceuticals has increased with the realisation that the glycan structures can have a major effect on the biological activities of glycoproteins. The strategic control of a bioprocess is certainly important to ensure high yields of product but also to ensure consistency of quality manifested by a consistent glycosylation profile. The study of functional glycomics is rapidly revealing examples of desirable biological activities that are expressed only by specific glycoforms. This is likely to lead to the future design of bioprocesses that allow synthesis of glycoprotein with only pre-selected glycans. However, before this becomes a reality there is a need for further fundamental understanding of the parameters that control protein glycosylation in host cells as well as in bioreactors.

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References

Allen S., Naim H.Y. and Bulleid N.J. 1995. Intracellular folding of tissue-type plasminogen activator. Effects of disulfide bond formation on N-linked glycosylation and secretion. J. Biol. Chem. 270: 4797–4804.

Andersen D.C. 2004. Cell Culture Effects on the Glycosylation of Therapeutic Proteins. Bioprocess International. IBC Life Sciences, Boston.

Andersen D.C., Bridges T., Gawlitzek M. and Hoy C. 2000. Multiple cell culture factors can affect the glycosylation of Asn-184 in CHO-produced tissue-type plasminogen activator. Biotechnol. Bioeng. 70: 25–31.

Andersen D.C. and Goochee C.F. 1994. The effect of cell-culture conditions on the oligosaccharide structures of secreted glycoproteins. Curr. Opin. Biotechnol. 5: 546–549.

Andersen D.C. and Goochee C.F. 1995. The effect of ammonia on the O-linked glycosylation of granulocyte colony-stimulating factor produced by Chinese hamster ovary cells. Biotechnol. Bioeng. 47: 96–105.

Andersen D.C. and Krummen L. 2002. Recombinant protein expression for therapeutic applications. Curr. Opin. Biotechnol. 13: 117–123.

Angata T. and Varki A. 2002. Chemical diversity in the sialic acids and related α-keto acids: an evolutionary perspective. Chem. Rev. 102: 439–469.

Apweiler R., Hermjakob H. and Sharon N. 1999. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim. Biophys. Acta. 1473: 4–8.

Backstrom M., Link T., Olson F.J., Karlsson H., Graham R., Picco G., Burchell J., Taylor-Papadimitriou J., Noll T. and Hansson G.C. 2003. Recombinant MUC1 mucin with a breast cancer-like O-glycosylation produced in large amounts in Chinese-hamster ovary cells. Biochem. J. 376: 677–686.

Baker K.N., Rendall M.H., Hills A.E., Hoare M., Freedman R.B. and James D.C. 2001. Metabolic control of recombinant protein N-glycan processing in NS0 and CHO cells. Biotechnol. Bioeng. 73: 188–202.

Barasch J., Kiss B., Prince A., Saiman L., Gruenert D. and al-Awqati Q. 1991. Defective acidification of intracellular organelles in cystic fibrosis. Nature 352(6330): 70–73.

Borys M.C., Linzer D.I. and Papoutsakis E.T. 1993. Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells. Biotechnology (NY) 11: 720–724.

Bragonzi A., Distefano G., Buckberry L.D., Acerbis G., Foglieni C., Lamotte D., Campi G., Marc A., Soria M.R., Jenkins N. and Monaco L. 2000. A new Chinese hamster ovary cell line expressing alpha2,6-sialyltransferase used as universal host for the production of human-like sialylated recombinant glycoproteins. Biochim. Biophys. Acta. 1474(3): 273–282.

Breton C., Oriol R. and Imberty A. 1998. Conserved structural features in eukaryotic and prokaryotic fucosyltransferases. Glycobiology 8: 87–94.

Butler M. 2004. Animal Cell Culture and Technology, 2nd ed. Bios Scientific, Oxford.

Butler M., Quelhas D., Critchley A.J., Carchon H., Hebestreit H.F., Hibbert R.G., Vilarinho L., Teles E., Matthijs G., Schollen Argibay, P., Harvey D.J., Dwek R.A., Jaeken J. and Rudd P.M. 2003. Detailed glycan analysis of serum glycoproteins of patients with congenital disorders of glycosylation indicates the specific enzyme defect and, coupled with proteomics, provides an insight into pathogenesis. Glycobiology 13: 601–622.

Butler M. and Spier R.E. 1984. The effects of glutamine utilisation and ammonia production on the growth of BHK cells in microcarrier cultures. J. Biotechnol. 1: 187–196.

Chee Furng Wong D., Tin Kam Wong K., Tang Goh L., Kiat Heng C. and Gek Sim Yap M. 2005. Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. Biotechnol. Bioeng. 89: 164–177.

Chotigeat W., Watanapokasin Y., Mahler S. and Gray P.P. 1994. Role of environmental conditions on the expression levels, glycoform pattern and levels of sialyltransferase for hFSH produced by recombinant CHO cells. Cytotechnology 15: 217–221.

Clark K.J., Griffiths J., Bailey K.M. and Harcum S.W. 2005. Gene-expression profiles for five key glycosylation genes for galactose-fed CHO cells expressing recombinant IL-4/13 cytokine trap. Biotechnol. Bioeng. 90: 568–577.

Curling E.M., Hayter P.M., Baines A.J., Bull A.T., Gull K., Strange P.G. and Jenkins N. 1990. Recombinant human interferon-gamma. Differences in glycosylation and proteolytic processing lead to heterogeneity in batch culture. Biochem. J. 272: 333–337.

Davidson S.K. and Hunt L.A. 1985. Sindbis virus glycoproteins are abnormally glycosylated in Chinese hamster ovary cells deprived of glucose. J. Gen. Virol. 66: 1457–1468.

Davies J., Jiang L., Pan L.Z., LaBarre M.J., Anderson D. and Reff M. 2001. Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. Biotechnol. Bioeng. 74: 288–294.

Dawson G., Moskal J.R. and Dawson S.A. 2004. Transfection of 2,6 and 2,3-sialyltransferase genes and GlcNAc-transferase genes into human glioma cell line U-373 MG affects glycoconjugate expression and enhances cell death. J. Neurochem. 89(6): 1436–1444.

Doyle C. and Butler M. 1990. The effect of pH on the toxicity of ammonia to a murine hybridoma. J. Biotechnol. 15: 91–100.

Egrie J.C., Dwyer E., Browne J.K., Hitz A. and Lykos M.A. 2003. Darbepoetin alfa has a longer circulating half-life and greater *in vivo* potency than recombinant human erythropoietin. Exp. Hematol. 31: 290–299.

Ellgaard L. and Helenius A. 2003. Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. 4(3): 181–191.Review.

Erbayraktar S., Grasso G., Sfacteria A., Xie Q.W., Coleman T., Kreilgaard M., Torup L., Sager T., Erbayraktar Z., Gokmen N., Yilmaz O., Ghezzi P., Villa P., Fratelli M., Casagrande S., Leist M., Helboe L., Gerwein J., Christensen S., Geist M.A., Pedersen L.O., Cerami-Hand C., Wuerth J.P., Cerami A. and Brines M. 2003. Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. Proc. Natl. Acad. Sci. U S A 100: 6741–6746.

Follstad B.D. 2004. A Method for Increasing Glycoprotein Sialylation in Mammalian Cells. Cell Culture Engineering IX Poster.

Freeze H.H.J. 2002. Sweet solution: sugars to the rescue. Cell Biol. 158: 615–616.

Gavel Y. and von Heijne G. 1990. Sequence differences between glycosylated and non-glycosylated Asn-*X*-Thr/Ser acceptor sites: implications for protein engineering. Protein Eng. 3(5): 433–442.

Gawlitzek M., Valley U., Nimtz M., Wagner R. and Conradt H.S. 1995. Characterization of changes in the glycosylation pattern of recombinant proteins from BHK-21 cells due to different culture conditions. J. Biotech. 42: 117–131.

Gawlitzek M., Valley U. and Wagner R. 1998. Ammonium ion and glucosamine dependent increases of oligosaccharide complexity in recombinant glycoproteins secreted from cultivated BHK-21 cells. Biotechnol. Bioeng. 57: 518–528.

Goochee C.F. 1992. Bioprocess factors affecting glycoprotein oligosaccharide structure. Dev. Biol. Stand. 76: 95–104.Review.

Goto M., Akai K., Murakami A., Hashimoto C., Tsuda E., Ueda M., Kawanishi G., Takahashi N., Ishimoto A., Chiba H. and Sasaki R. 1988. Production of recombinant human erythropoietin in mammalian cells: Host–cell dependency of the biological activity of the cloned glycoprotein. Bio/Technology 6: 67–71.

Grabenhorst E., Hoffmann A., Nimtz M., Zettlmeissl G. and Conradt H.S. 1995. Construction of stable BHK-21 cells coexpressing human secretory glycoprotein and human Gal (β1–4)GlcNAc-R α2,6-sialyltransferase. Eur. J. Biochem. 232: 718–725.

Grammatikos S.I., Valley U., Nimtz M., Conradt H.S. and Wagner R. 1998. Intracellular UDP-N-acetylhexosamine pool affects N-glycan complexity: a mechanism of ammonium action on protein glycosylation. Biotechnol. Prog. 14: 410–419.

Gu X. and Wang D.I. 1998. Improvement of interferon-gamma sialylation in Chinese hamster ovary cell culture by feeding of N-acetylmannosamine. Biotechnol. Bioeng. 58(6): 642–648.

Hayter P.M., Curling E.M.A., Baines A.J., Jenkins N., Salmon I., Strange P.G., Tong J.M. and Bull A.T. 1992. Glucose-limited chemostat culture of chinese hamster ovary cells producing recombinant human interferon-γ. Biotechnol. Bioeng. 39: 327–335.

Heidemann R., Lutkemeyer D., Buntemeyer H. and Lehmann J. 1998. Effects of dissolved oxygen levels and the role of extra- and intracellular amino acid concentration upon the metabolism of mammalian cell lines during batch and continuous cultures. Cytotechnology 26: 185–197.

Hirschberg C.B. 2001. Golgi nucleotide sugar transport and leukocyte adhesion deficiency II. J. Clin. Invest. 108: 3–6.

Hokke C.H., Bergwerff A.A., Dedem G.W.Van, Kamerling J.P. and Vliegenthart J.F. 1995. Structural analysis of the sialylated N- and O-linked carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. Sialylation patterns and branch location of dimeric N-acetyllactosamine units. Eur. J. Biochem. 228: 981–1008.

Jan D.C., Petch D.A., Huzel N. and Butler M. 1997. The effect of dissolved oxygen on the metabolic profile of a murine hybridoma grown in serum-free medium in continuous cultures. Biotech. Bioeng. 54: 153–164.

Jenkins N. and Curling E.M. 1994. Glycosylation of recombinant proteins: problems and prospects. Enzyme Microb. Technol. 16: 354–364.

Jenkins N., Parekh R.B. and James D.C. 1996. Getting the glycosylation right: implications for the biotechnology industry. Nat. Biotechnol. 14: 975–981.

Jones M.B., Teng H., Rhee J.K., Lahar N., Baskaran G. and Yarema K.J. 2004. Characterization of the cellular uptake and metabolic conversion of acetylated N-acetylmannosamine (ManNAc) analogues to sialic acids. Biotechnol. Bioeng. 85(4): 394–405.

Kagawa Y., Takasaki S., Utsumi J., Hosoi K., Shimizu H., Kochibe N. and Kobata A. 1988. Comparative study of the asparagine-linked sugar chains of natural human interferon-beta 1 and recombinant human interferon-beta 1 produced by three different mammalian cells. J. Biol. Chem. 263: 17508–17515.

Kornfeld R. and Kornfeld S. 1985. Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 54: 631–664.

Kunkel J.P., Jan D.C., Jamieson J.C. and Butler M. 1998. Dissolved oxygen concentration in serum-free continuous culture affects N-linked glycosylation of a monoclonal antibody. J. Biotechnol. 62: 55–71.

Kunkel J.P., Yan W.Y., Butler M. and Jamieson J.C. 2003. Decreased monoclonal IgG_1 galactosylation at reduced dissolved oxygen concentration is not a result of lowered galactosyltransferase activity *in vitro*. Society for Glycobiology Meeting, San Diego, CA, Glycobiology 13: 875.

Leonard C.K., Spellman M.W., Riddle L., Harris R.J., Thomas J.N. and Gregory T.J. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. 265: 10373–10382.

Longmore G.D. and Schachter H. 1982. Product-identification and substrate-specificity studies of the GDP-L-fucose:2-acetamido-2-deoxy-beta-D-glucoside (FUC goes to Asn-linked GlcNAc) 6-alpha-L-fucosyltransferase in a Golgi-rich fraction from porcine liver. Carbohydr. Res. 100: 365–392.

Meynial-Salles I. and Combes D. 1996. *In vitro* glycosylation of proteins: an enzymatic approach. J. Biotechnol. 46: 1–14.

Miyoshi E., Noda K., Ko J.H., Ekuni A., Kitada T., Uozumi N., Ikeda Y., Matsuura N., Sasaki Y., Hayashi N., Hori M. and Taniguchi N. 1999. Overexpression of alpha1–6 fucosyltransferase in hepatoma cells suppresses intrahepatic metastasis after splenic injection in athymic mice. Cancer Res. 59: 2237–2243.

Nabi I.R. and Dennis J.W. 1998. The extent of polylactosamine glycosylation of MDCK LAMP-2 is determined by its Golgi residence time. Glycobiology 8: 947–953.

Narhi L.O., Arakawa T., Aoki K.H., Elmore R., Rohde M.F., Boone T. and Strickland T.W. 1991. The effect of carbohydrate on the structure and stability of erythropoietin. J. Biol. Chem. 266: 23022–23026.

Noda K., Miyoshi E., Uozumi N., Gao C.X., Suzuki K., Hayashi N., Hori M. and Taniguchi N. 1998. High expression of alpha-1–6 fucosyltransferase during rat hepatocarcinogenesis. Int. J. Cancer. 75: 444–450.

Nyberg G.B., Balcarcel R.R., Follstad B.D., Stephanopoulos G. and Wang D.I. 1999. Metabolic effects on recombinant

interferon-gamma glycosylation in continuous culture of Chinese hamster ovary cells. Biotechnol. Bioeng. 62: 336–347.

Ohyama C., Smith P.L., Angata K., Fukuda M.N., Lowe J.B. and Fukuda M. 1998. Molecular cloning and expression of GDP-D-mannose-4,6-dehydratase, a key enzyme for fucose metabolism defective in Lec13 cells. J. Biol. Chem. 273: 14582–14587.

Okazaki A., Shoji-Hosaka E., Nakamura K., Wakitani M., Uchida K., Kakita S., Tsumoto K., Kumagai I. and Shitara K. 2004. Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcgammaRIIIa. J. Mol. Biol. 336: 1239–1249.

Pels Rijcken W.R., Overdijk B., Van den Eijnden D.H. and Ferwerda W. 1995. The effect of increasing nucleotide-sugar concentrations on the incorporation of sugars into glycoconjugates in rat hepatocytes. Biochem. J. 305: 865–870.

Perlman S., van den Hazel B., Christiansen J., Gram-Nielsen S., Jeppesen C.B., Andersen K.V., Halkier T., Okkels S. and Schambye H.T. 2003. Glycosylation of an N-terminal extension prolongs the half-life and increases the in vivo activity of follicle stimulating hormone. J. Clin. Endocrinol. Metab. 88: 3227–3235.

Petrescu A.J., Milac A.L., Petrescu S.M., Dwek R.A. and Wormald M.R. 2004. Statistical analysis of the protein environment of N-glycosylation sites: implications for occupancy, structure, and folding. Glycobiology 14: 103–114.

Prati E.G., Matasci M., Suter T.B., Dinter A., Sburlati A.R. and Bailey J.E. 2000. Engineering of coordinated up- and down-regulation of two glycosyltransferases of the O-glycosylation pathway in Chinese hamster ovary (CHO) cells. Biotechnol. Bioeng. 68(3): 239–244.

Rademacher T.W., Jaques A. and Williams P.J. 1996. The defining characteristics of immunoglobulin glycosylation. In: Isenberg D.A. and Rademacher T.W. (eds), Abnormalities of IgG Glycosylation and Immunological Disorders. publ. Wiley, NY, pp. 1–44.

Raju T.S., Briggs J.B., Borge S.M. and Jones A.J. 2000. Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. Glycobiology 10: 477–486.

Raju T.S., Briggs J.B., Chamow S.M., Winkler M.E. and Jones A.J. 2001. Glycoengineering of therapeutic glycoproteins: in vitro galactosylation and sialylation of glycoproteins with terminal N-acetylglucosamine and galactose residues. Biochemistry 40: 8868–8876.

Rearick J.I., Chapman A. and Kornfeld S. 1981. Glucose starvation alters lipid-linked oligosaccharide biosynthesis in Chinese hamster ovary cells. J. Biol. Chem. 256: 6255–6261.

Restelli V. and Butler M. 2002. The effect of cell culture parameters on protein glycosylation. In: Al-Rubeai M (ed.), Glycosylation, vol 3. Kluwer, Dordrecht, pp. 61–92.

Reuter G. and Gabius H.J. 1999. Eukaryotic glycosylation: whim of nature or multipurpose tool? Cell Mol. Life Sci. 55: 368–422.Review.

Rijcken P.W.R., Overdijk B., Van den Eijnden D.H. and Ferwerda W. 1995. The effect of increasing nucleotide-sugar concentrations on the incorporation of sugars into glycoconjugates in rat hepatocytes. Biochem. J. 305: 865–870.

Rothman R.J., Warren L., Vliegenthart J.F. and Hard K.J. 1989. Clonal analysis of the glycosylation of immunoglobulin G secreted by murine hybridomas. Biochemistry 28: 1377–1384.

Rudd P.M. and Dwek R.A. 1997. Glycosylation: heterogeneity and the 3D structure of proteins. Critical Rev. Biochem. Mol. Biol. 32: 1–100.

Saitoh A., Aoyagi Y. and Asakura H. 1993. Structural analysis on the sugar chains of human alpha 1-antitrypsin: presence of fucosylated biantennary glycan in hepatocellular carcinoma. Arch. Biochem. Biophys. 303: 281–287.

Sburlati A.R., Umana P., Prati E.G. and Bailey J.E. 1998. Synthesis of bisected glycoforms of recombinant IFN-beta by overexpression of beta-1,4-N-acetylglucosaminyltransferase III in Chinese hamster ovary cells. Biotechnol. Prog. 14(2): 189–192.

Schweikart F., Jones R., Jaton J.C. and Hughes G.J. 1999. Rapid structural characterisation of a murine monoclonal IgA alpha chain: heterogeneity in the oligosaccharide structures at a specific site in samples produced in different bioreactor systems. J. Biotechnol. 69: 191–201.

Sears P. and Wong C.H.. 1998. Enzyme action in glycoprotein synthesis. Cell. Mol. Life Sci. 54: 223–252.

Seppala R., Lehto V.P. and Gahl W.A. 1999. Mutations in the human UDP-N-acetylglucosamine 2-epimerase gene define the disease sialuria and the allosteric site of the enzyme. Am. J. Hum. Genet. 64: 1563–1569.

Sheeley D.M., Merrill B.M. and Taylor L.C. 1997. Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal alpha-linked galactose. Anal. Biochem. 247: 102–110.

Shelikoff M., Sinskey A.J. and Stephanopoulos G. 1994. The effect of protein synthesis inhibitors on the glycosylation site occupancy of recombinant human prolactin. Cytotechnology 15: 195–208.

Shields R.L., Lai J., Keck R., O'Connell L.Y., Hong K., Meng Y.G., Weikert S.H. and Presta L.G. 2002. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J. Biol. Chem. 277: 26733–26740.

Shinkawa T., Nakamura K., Yamane N., Shoji-Hosaka E., Kanda Y., Sakurada M., Uchida K., Anazawa H., Satoh M., Yamasaki M., Hanai N. and Shitara K. 2003. The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J. Biol. Chem. 278: 3466–3473.

Shriver Z., Raguram S. and Sasisekharan R. 2004. Glycomics: a pathway to a class of new and improved therapeutics. Nat. Rev. Drug Discov. 3: 863–873.

Spellman M.W. 1990. Carbohydrate characterization of recombinant glycoproteins of pharmaceutical interest. Anal. Chem. 62: 1714–1722.

Srikrishna G., Varki N.M., Newell P.C., Varki A. and Freeze H.H. 1997. An IgG monoclonal antibody against *Dictyostelium discoideum* glycoproteins specifically recognizes Fucalpha1,6GlcNAcbeta in the core of N-linked glycans. Localized expression of core-fucosylated glycoconjugates in human tissues. J. Biol. Chem. 272: 25743–25752.

Stark N.J. and Heath E.C. 1979. Glucose-dependent glycosylation of secretory glycoprotein in mouse myeloma cells. Arch. Biochem. Biophys. 192: 599–609.

Storring P.L. 1992. Assaying glycoprotein hormones–the influence of glycosylation on immunoreactivity. Trends Biotechnol. 10: 427–432.

Stubbs H.J., Lih J.J., Gustafson T.L. and Rice K.G. 1996. Influence of core fucosylation on the flexibility of a biantennary N-linked oligosaccharide. Biochemistry 35: 937–947.

Sturla L., Etzioni A., Bisso A., Zanardi D., De Flora G., Silengo L., De Flora A. and Tonetti M. 1998. Defective intracellular activity of GDP-D-mannose-4,6-dehydratase in leukocyte adhesion deficiency type II syndrome. FEBS Lett. 429: 274–278.

Tachibana H., Kim J. and Shivahata S. 1997. Building high affinity human antibodies by altering the glycosylation on the light chain variable region in N-acetyl glucosamine-supplemented hybridoms cultures. Cytotechnology 23: 151–159.

Takahashi T., Ikeda Y., Miyoshi E., Yaginuma Y., Ishikawa M. and Taniguchi N. 2000. Alpha1,6fucosyltransferase is highly and specifically expressed in human ovarian serous adenocarcinomas. Int. J. Cancer. 88: 914–919.

Takeuchi M., Takasaki S., Miyazaki H., Kato T., Hoshi S., Kochibe N. and Kobata A. 1988. Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. J. Biol. Chem. 263: 3657–3663.

Thotakura N.R., Desai R.K., Bates L.G., Cole E.S., Pratt B.M. and Weintraub B.D. 1991. Biological activity and metabolic clearance of a recombinant human thyrotropin produced in Chinese hamster ovary cells. Endocrinology 128: 341–348.

Tonetti M., Sturla L., Bisso A., Benatti U. and De Flora A. 1996. Synthesis of GDP-L-fucose by the human FX protein. J. Biol. Chem. 271: 27274–27279.

Umana P. and Bailey J.E. 1997. A mathematical model of N-linked glycoform biosynthesis. Biotechnol. Bioeng. 55: 890–908.

Umana P., Jean-Mairet J., Moudry R., Amstutz H. and Bailey J.E. 1999. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. Nat. Biotechnol. 17: 176–180.

Uozumi N., Yanagidani S., Miyoshi E., Ihara Y., Sakuma T., Gao C.X., Teshima T., Fujii S., Shiba T. and Taniguchi N. 1996. Purification and cDNA cloning of porcine brain GDP-L-Fuc:N-acetyl-beta-D-glucosaminide alpha1–6 fucosyltransferase. J. Biol. Chem. 271: 27810–27817.

Valley U., Nimtz M., Conradt H.S. and Wagner R. 1999. Incorporation of ammonium into intracellular UDP-activated N-acetylhexosamines and into carbohydrate structures in glycoproteins. Biotechnol. Bioeng. 64(4): 401–417.

Van den Steen P., Rudd P.M., Dwek R.A. and Opdenakker G. 1998. Concepts and principles of O-linked glycosylation. Crit. Rev. Biochem. Mol. Biol. 33(3): 151–208.

Voynow J.A., Kaiser R.S., Scanlin T.F. and Glick M.C. 1991. Purification and characterization of GDP-L-fucose-N-acetyl beta-D-glucosaminide alpha 1–6 fucosyltransferase from cultured human skin fibroblasts. Requirement of a specific biantennary oligosaccharide as substrate. J. Biol. Chem. 266: 21572–21577.

Walmsley A.R. and Hooper N.M. 2003. Glycosylation efficiency of Asn-*X*aa-Thr sequons is independent of distance from the C-terminus in membrane dipeptidase. Glycobiology 13(9): 641–646.

Wang W.C., Lee N., Aoki D., Fukuda M.N. and Fukuda M. 1991. The poly-N-acetyllactosamines attached to lysosomal membrane glycoproteins are increased by the prolonged association with the Golgi complex. J. Biol. Chem. 266: 23185–23190.

Wang W., Li W., Ikeda Y., Miyagawa J.I., Taniguchi M., Miyoshi E., Sheng Y., Ekuni A., Ko J.H., Yamamoto Y., Sugimoto T., Yamashita S., Matsuzawa Y., Grabowski G.A., Honke K. and Taniguchi N. 2001. Ectopic expression of alpha1,6 fucosyltransferase in mice causes steatosis in the liver and kidney accompanied by a modification of lysosomal acid lipase. Glycobiology 11: 165–174.

Wasley L.C., Timony G., Murtha P., Stoudemire J., Dorner A.J., Caro J., Krieger M. and Kaufman R.J. 1991. The importance of N- and O-linked oligosaccharides for the biosynthesis and *in vitro* and *in vivo* biologic activities of erythropoietin. Blood 77: 2624–2632.

Weikert S., Papac D., Briggs J., Cowfer D., Tom S., Gawlitzek M., Lofgren J., Mehta S., Chisholm V., Modi N., Eppler S., Carroll K., Chamow S., Peers D., Berman P. and Krummen L. 1999. Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. Nat. Biotechnol. 17: 1116–1121.

Weiss P. and Ashwell G. 1989. The asialoglycoprotein receptor: properties and modulation by ligand. Prog. Clin. Biol. Res. 300: 169–184.

Wormald M.R., Rudd P.M., Harvey D.J., Chang S.C., Scragg I.G. and Dwek R.A. 1997. Variations in oligosaccharide-protein interactions in immunoglobulin G determine the site-specific glycosylation profiles and modulate the dynamic motion of the Fc oligosaccharides. Biochemistry 36: 1370–1380.

Xie L. and Wang D.I. 1997. Integrated approaches to the design of media and feeding strategies for fed-batch cultures of animal cells. Trends Biotechnol. 15: 109–113.

Yamashita K., Koide N., Endo T., Iwaki Y. and Kobata A. 1989. Altered glycosylation of serum transferring of patients with hepatocellular carcinoma. J. Biol. Chem. 264: 2415–2423.

Yan A. and Lennarz W.J. 2005. Unraveling the mechanism of protein N-glycosylation. J. Biol. Chem. 280: 3121–3124.

Yanagidani S., Uozumi N., Ihara Y., Miyoshi E., Yamaguchi N. and Taniguchi N. 1997. Purification and cDNA cloning of GDP-L-Fuc:N-acetyl-beta-D-glucosaminide:alpha1–6 fucosyltransferase (alpha1–6 FucT) from human gastric cancer MKN45 cells. J. Biochem. 121: 626–632.

Yang M. and Butler M. 2000. Effects of ammonia on CHO cell growth, erythropoietin production, and glycosylation. Biotechnol. Bioeng. 68: 370–380.

Zanghi J.A., Mendoza T.P., Knop R.H. and Miller W.M. 1998. Ammonia inhibits neural cell adhesion molecule polysialylation in Chinese hamster ovary and small cell lung cancer cells. J. Cell Physiol. 177: 248–263.