

## Review Article

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# Factors affecting the expression of recombinant glycoproteins

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**N-glycosylation is both species and tissue specific with a series of membrane bound glycosidases and glycosyltransferases modifying the oligosaccharide as it moves through the endoplasmic reticulum (ER) and Golgi. Each of these individual enzymatic reactions may not go to completion; therefore giving rise to glycoforms of the polypeptide. Glycosylation patterns of recombinant proteins are relevant for the immunogenicity, the pharmacological activity, pharmacokinetic profile, solubility and stability of the protein.**

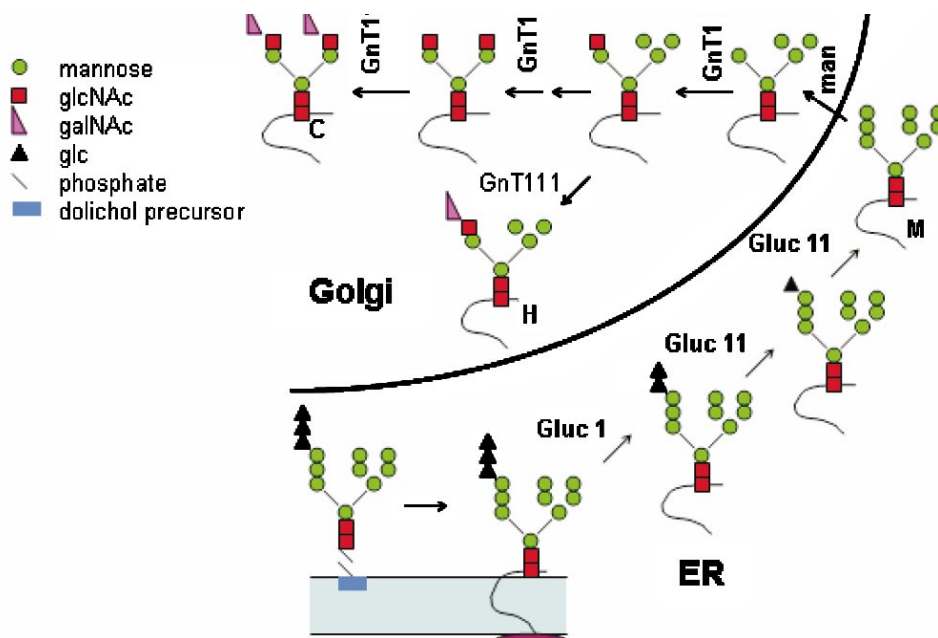
**This review describes the effect of primary and the 3-dimensional structure of the protein on sequon occupancy. Heterogeneity due to cell specific glycosylation and tissue culture conditions are discussed with main emphasis on N-glycosylation sequon occupancy. The review also discusses how fully glycosylated with total sequon occupancy glycoproteins which are of prime relevance in the expression of pharmaceutically relevant glycoproteins can be obtained.**

**Key words** Dithiothritol - glycoforms - glycoprotein - oligosaccharide - Thy-1

In eukaryotic cells the presence of carbohydrates on majority of cell surfaces and secreted proteins is important for a very wide range of functions. Carbohydrate recognition events serve a general function in the folding, modification and transport of secretory proteins, preventing aggregation of folded intermediates by increasing their solubility and enabling interaction with chaperones like calnexin<sup>1,2</sup>. The role of glycosylation as being mandatory for secretion is ambiguous as many “non-glycoprotein” proteins are also secreted<sup>3</sup>. Generally the requirements for glycosylation are more stringent for membrane proteins than for secretory proteins. The modification of the attached oligosaccharide is important in development, where alterations in cell surface oligosaccharides are associated

with various pathological conditions such as rheumatoid arthritis and cancer.

Glycosylation is of two types: N- and O-linked, which is both species and tissue specific. N-linked glycans are attached to the Asn of the consensus Asn-X-Ser/Thr sequon<sup>4</sup>. A series of membrane bound glycosidases and glycosyltransferases process the oligosaccharide as it moves through the endoplasmic reticulum (ER) and Golgi modifying it (Fig.). Each of these individual enzymatic reactions may not go to completion, giving rise to glycoforms of the polypeptide<sup>4</sup>. The enzymatic activity of modifying enzymes, reflects the physiological environment of the cell in which the glycoprotein is modified. Glycosylation patterns of recombinant proteins are relevant for the immunogenicity, the pharmacological



**Fig.** Attachment and processing of N-linked oligosaccharides in the ER and Golgi<sup>41</sup>. The abbreviations used are as follow: Gluc I,  $\alpha$ -glucosidase I; gluc II,  $\alpha$ -glucosidase II; Man II,  $\alpha$ -1,2 mannosidase; GnT I, N- acetylglucosaminyltransferase I; GnTII, N-acetylglucosaminyltransferaseII, GnT III; N-acetylglucosaminyltransferase III.

activity, pharmacokinetic profile, solubility and stability of the protein<sup>5</sup>.

### Variable sequon occupancy - glycoforms

The enzymes involved in modification of N-linked glycosylation of glycoproteins are found in the ER and the Golgi (Fig.)<sup>6</sup>. Processing of oligosaccharides begins in the ER by  $\alpha$ -glycosidases I and II and a collection of processing mannosidases both in the ER and Golgi complex<sup>6</sup>. The endoplasmic reticulum contains a unique pathway for the folding and retention of glycoproteins, central to which is calnexin<sup>7</sup> which assists in the initial folding of glycoproteins, retains transport-incompetent misfolded glycoproteins, and may function in assembling of oligomeric proteins in the ER<sup>8</sup>.

Each glycosylation site may further exhibit variable occupancy, in that a site may be fully, partially or totally unoccupied. Each occupied site in turn exhibits heterogeneity in the attached glycan structures<sup>9</sup>. Site heterogeneity is reproducible although the glycans at each site may or may not belong to the same structural class<sup>10</sup>. The composition and structure of the carbohydrate attached to a glycoprotein are determined by the glycosylation machinery available in a specific cell type, changes in which can significantly alter the

structure of the oligosaccharide. The term glycoform essentially describes this structural diversity which is a result of cell specific biosynthesis<sup>11</sup>, and leads to functional diversity as seen in the case of ribonuclease<sup>12</sup>.

### Effect of primary and 3-D structure of the protein on sequon occupancy

The presence of Pro on either side of the sequon inhibits glycosylation as does Pro/Asp in the middle of the sequon<sup>13</sup>. The amino acid in the X position of the Asn-X-Ser/Thr sequon is an important determinant of core glycosylation efficiency as seen in Rabies virus glycoprotein variants<sup>14</sup>. Amino acids with hydroxy and sulphhydryl groups (Ser and Thr and Cys) are associated with high glycosylation efficiency in contrast to those with amide groups (Asn and Gln) which show suboptimal glycosylation. Large hydrophobic amino acids (Trp, Leu, Phe and Tyr) inhibit core glycosylation in contrast to Gly. The negatively charged amino acids (Asp and Glu) inhibit while the positively charged (Lys, Arg and His) favour glycosylation<sup>14</sup>. The charge of the X amino acid may influence the ability of oligosaccharyltransferase to bind simultaneously to the sequon and the negatively charged dolichol-PP-oligosaccharide precursor<sup>15</sup>.

Amino acids in the close vicinity of the sequon have an effect on glycosylation. In particular, folding of the protein that involves disulphide bridges hinders glycosylation, as seen in interleukin 6, where the elimination of the disulphide bridge between Cys 45 and Cys 51 increases the efficiency of glycosylation at Asn 46<sup>16</sup>.

The position of the glycosylation site in the protein is important. The sites on the exposed turns of the  $\beta$  sheets, though close to Pro residues are usually occupied while in general those near the amino or carboxy terminal are less efficiently glycosylated. For example, IL-1 $\beta$ , which has a glycosylation site at Asn 7 is 50 per cent glycosylated when expressed in *Saccharomyces cerevisiae*. Moving the Asn site 9 amino acids away from the signal processing site results in almost 100 per cent glycosylation of the sequon<sup>17</sup>.

### Cell type specific glycosylation

Variation in topology (localization within the ER and Golgi) and levels of the glycosyltransferases provides an explanation for differing glycosylation of the same glycoprotein expressed in different cells. This however, cannot account for the difference among different glycoproteins synthesized within the same cell.

For cells in stasis, a steady state exists and therefore the final population of glycoforms is reproducible and not random. Under cell culture conditions both enzyme and substrate levels vary, with proteins being glycosylated differently when compared to conditions *in vivo*. Glycoproteins secreted eutopically and ectopically by tumour cells *in vivo* and by cell lines *in vitro* show glycosylation characteristic of cell type which may reflect either a mutational event or genetic polymorphism. Therefore different cell lines containing different repertoires of enzymes will glycosylate the same protein in a cell specific manner<sup>18</sup>. For instance, the oligosaccharides at Asn-289 of recombinant human plasminogen expressed in both Chinese hamster ovary (CHO) and *Mamestra brassicae* cell lines have been compared with human serum plasminogen. While plasminogen derived from human tissue has sialylated complex biantennary oligosaccharides, both cell lines express oligomannose structures with the CHO cell line in addition demonstrating the presence of triantennary complex oligosaccharides<sup>19,20</sup>.

### Effect of tissue culture conditions on glycosylation

Glycosylation of protein molecules is affected by the cell culture environment. The presence of

Man<sub>4</sub>GlcNAc<sub>2</sub> oligosaccharide in sThy-1 expressed from COS-7 cells indicated degradation of the oligomannose oligosaccharides, suggesting  $\alpha$ -mannosidases were secreted by these cells. There is no documented report of the accumulation of  $\alpha$ -mannosidases in CHO-K1 or COS-7 cell supernatants.  $\alpha$ -Fucosidase<sup>21</sup> and sialidase activity have been shown in CHO-K1 cells<sup>22</sup>. However,  $\alpha$ -mannosidases are present in serum and are used as a marker in the diagnosis of  $\alpha$ -mannosidosis<sup>22,23</sup>. Even if cells do secrete  $\alpha$ -mannosidases, their activity is not known which would depend on a number of factors such as tissue conditions, depletion of nutrients, amount of serum used and pH of the medium *etc.* The longer the cells are left under culture conditions resulting in over growth of the cells, the more acidic the medium and perhaps nearer the optimal pH activity of these lysosomal  $\alpha$ -mannosidases<sup>21,22</sup>.

### Expression of recombinant glycoproteins in mammalian cell lines

The most effective production of pharmaceutically active glycoproteins is using mammalian expression systems like Chinese hamster ovary cells (CHO), mouse fibroblast cells (C127-BPV) and transgenic animals expressing the cDNA or genomic DNA encoding the protein of interest<sup>5,24</sup>. Various recombinant molecules have been expressed in CHO cell lines. CHO-K1 cells in common with human cells contain a large repertoire of oligosaccharide processing enzymes and can therefore process a wide variety of glycans<sup>18</sup>. With the exception of minor variations in sialylation and fucosylation the carbohydrate profiles for a glycoprotein produced in CHO-K1 are constant and reproducible<sup>18</sup>. For example,  $\beta$ -interferon and erythropoietin, molecules have been reported to express  $\alpha$ 2, 3 but not  $\alpha$ 2, 6 linked sialic acids which is characteristic of proteins expressed in CHO cell lines<sup>25,26</sup>. On examining the total population of Asn-linked oligosaccharides synthesized by CHO cells, a spectrum of structures were found: di-, tri-, tetra-branched and poly-lactosamine complex type oligosaccharides with sialic acid in  $\alpha$ 2,3 and not  $\alpha$ 2,6 linkage<sup>25,26</sup>. Thus CHO cell lines appear to express the  $\alpha$ 2,3-sialyltransferase but not a functional  $\alpha$ 2,6 sialyltransferase<sup>27</sup>. As seen for erythropoietin, the oligosaccharide structures seen in the recombinant form are found that extracted from urine and therefore this recombinant molecule can be used clinically<sup>28</sup>. CHO-K1 cell line as yet has not been shown to produce deleterious carbohydrate linkages as seen in other cell lines. Glycans derived from other cell lines like mouse epithelial cells (C127) and human lung adenocarcinoma

cells (PC8) are dissimilar to the native and CHO derived glycans of IL- $\beta$ 1. Recombinant human interferon from C127 cells shows the presence of a Gal $\alpha$ 1-3Gal linkage which is present in glycoproteins from several mammalian species but not in humans<sup>25</sup>. Since human serum contains naturally occurring antibody which recognizes the Gal $\alpha$ 1-3Gal linkage, recombinant protein from these cell lines (C127 and PC8) cannot be used clinically<sup>29</sup>. Expression of CD4 is of interest as it is an important molecule in major histocompatibility complex (MHC) class II restricted T cell responses and also because it is the receptor for the human immunodeficiency virus. Oligosaccharides of CD4 expressed in CHO-K1 cells are of the biantennary complex, hybrid, or oligomannose type with low levels of tri-antennary or poly-N-acetylglucosamine structures found<sup>30</sup>. This is in contrast to the glycosylation of other recombinant proteins expressed in CHO cell lines, indicating the effect of the polypeptide chain on processing of oligosaccharide. In addition, CD48 the proposed ligand for CD2, shows at least 47 different glycan structures<sup>18</sup>. This further confirms the diversity seen in carbohydrates of glycoproteins expressed in CHO cell lines.

Thy-1 a membrane glycoprotein belonging to the immunoglobulin superfamily having three N-glycosylation sites, is linked to the cell surface through a glycosyl phosphatidylinositol (GPI) anchor<sup>31</sup>. Soluble recombinant Thy-1 expressed in the African green monkey kidney fibroblast cell line (COS-7) gave almost all the oligosaccharides seen in sThy-1 expressed from CHO-K1 cells<sup>32,33</sup>. The presence of the Man<sub>6</sub>GlcNAc<sub>2</sub> oligomannose type oligosaccharides present in sThy-1 from COS-7 cells was consistent with that seen in rat brain Thy-1 but not in sThy-1 derived from CHO-K1 cells. This indicates that the protein primary structure does have an effect on the glycosylation of the protein in addition to the cell in which synthesized. As glycoproteins from COS-7 cells have not been characterized before, nothing is known about the spectrum of oligosaccharides expected<sup>32</sup>. Processing of oligosaccharides is specified by the processing enzymes present in the cell. Hence the glycoform populations for a glycoprotein are both species and cell specific<sup>18</sup>. Thus for cells in stasis a characteristic oligosaccharide profile of the glycoprotein is obtained.

### **Expression of functional recombinant glycoprotein in yeast**

Since most therapeutically relevant glycoproteins require sialylation, mammalian cell lines are preferred.

However, yeast demonstrates better protein titres, shorter fermentation times and can grow in chemically defined media. However, recombinant glycoproteins expressed from *Pichia Pastoris* have high mannose N-oligosaccharides which have reduced half life<sup>34</sup>. Recently it has been reported that human glycosylation pathways have been engineered into *Pichia Pastoris*<sup>35</sup>, the incorporation of 14 heterologous genes. By taking recombinant erythropoietin as an example, the presence of terminally sialylated, complex bi-antennary glycoproteins were observed<sup>35</sup>.

### **Expression of single domain glycoproteins**

The expression and stability of single recombinant domains ending in a Cys residue involved in disulphide bridge formation can be obtained by the addition of residues at either end of the domain<sup>31,36</sup>. Secretion of recombinant soluble Thy-1 was achieved by stabilizing the terminal Cys 111 residue which takes part in intramolecular disulphide bond formation, by the addition of the tripeptide GGS at the C-terminus<sup>32</sup>. This effect has also been seen in the expression of T cell receptor  $\alpha\beta$  domain where the addition of 10-15 amino acid residues at the C-terminus is necessary for secretion of this protein<sup>37</sup>.

### **Expression under reducing condition - 100 per cent sequon occupancy**

Tissue plasminogen activator (t-PA) was used as a model protein to study the effect of intracellular folding on sequon occupancy. Human t-PA is a 70kd serine protease that contains 17 disulphide bonds which when secreted by both human and as a recombinant glycoprotein show variable sequon occupancy<sup>38</sup>. Intracellular folding of the glycoprotein affects sequon occupancy as shown when t-PA is expressed under mild conditions of reducing agent dithiothreitol to prevent disulphide bond formation in the ER. Conditions that prevent disulphide bond formation lead to complete glycosylation of the sequon which showed variable glycosylation in untreated cells<sup>38</sup>.

### **Membrane bound vs secreted glycoprotein - homogenous band**

Secretion in terms of expression levels and variable site occupancy was protein dependent as observed in the expression of recombinant soluble Thy-1 in COS-7 cells and CHO-K1 cells<sup>32,33</sup> and soluble recombinant CD2<sup>39</sup>. However, recombinant GPI anchored Thy-1 in CHO-K1 cells does not show variable site occupancy in that the fully glycosylated form alone was expressed

on the cell surface<sup>32</sup>. The GPI anchored Thy-1 expressed on CHO-K1 cells when purified appeared as a single protein band by SDS-PAGE and was not expressed as glycoforms<sup>32</sup>. This indicates that (i) the attachment of the protein to the cell membrane increases its stability by presenting the protein to the glycosylation processing enzymes in a protein specific manner resulting in a homogenous glycoprotein population, (ii) glycoforms or unglycosylated protein are efficiently degraded by the cells quality control mechanism leading to the exclusive expression of the fully glycosylated glycoprotein.

### Replenishing spent nutrients

Recombinant human interferon- $\gamma$  expressed in CHO-K1 cells showed a decrease in fully glycosylated glycoprotein which could be prevented by batch feeding of the culture with glucose and glutamine<sup>40</sup>. Culture conditions of the recombinant glycoprotein have to be maintained during process optimization.

### Conclusion

The expression of recombinant glycoproteins as cell surface expressed molecules would result in the expression a homogeneously glycosylated population of molecules. Though of a lower yield than bulk secreted protein from a mammalian expression system/transgenics, the final yield of functionally relevant glycoproteins would be higher and the GPI anchor can be removed using phospholipase C resulting in the desired soluble recombinant protein<sup>31</sup>.

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