

Glycosylation and Bioinformatics: current status for glycosylation prediction tools

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REVIEW

ABSTRACT

Glycosylation is an important co- and post-translational modification involved in a variety of critical biological processes. The development of computational algorithms for protein glycosylation prediction has been propelled in the latest years. The localization of potential glycosylated sites facilitates the rational alteration of glycosylation-related functions in cells. This manuscript gives an overview of current available bioinformatics resources and databases for glycobiology, focusing on glycosylation predictors. As a complement, general features about the different glycosylation types are also exposed.

Keywords: glycosylation, post-translational modification, bioinformatics, prediction, glycobiology, carbohydrate, databases

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RESUMEN

Glicosilación y Bioinformática: estado actual de las herramientas para predecir glicosilación. El desarrollo de algoritmos computacionales para la predicción de sitios potenciales de glicosilación en las proteínas ha sido impulsado en los últimos años. La glicosilación constituye una modificación co- y post-traducciona involucrada en una gran variedad de procesos biológicos críticos. La localización de los sitios potenciales de glicosilación facilita la modificación racional de las funciones relacionadas con la glicosilación en las células. Este manuscrito resume el estado actual de las herramientas bioinformáticas y las bases de datos disponibles para la glicobiología, haciendo énfasis en los predictores de glicosilación. Además, como complemento se incluyen las principales características de los diferentes tipos de glicosilación.

Palabras clave: glicosilación, modificación post-traducciona, bioinformática, predicción, glicobiología, carbohidrato, bases de datos

Introduction

Glycosylation is an essential co- and post-translational modification occurring in cells. It involves the selective attachment of carbohydrate molecules (also referred as glycans, sugars or saccharides) to proteins and lipids. Glycans are composed of monosaccharides covalently linked by glycosidic bonds, adopting linear and branched structures. There are two major types of glycosylation: N-glycosylation and O-glycosylation. Besides, another three unusual glycosylation forms have been described, known as C-glycosylation (or C-mannosylation), Glypiation (or glycosylphosphatidylinositol (GPI) anchoring) and Glycation (or non-enzymatic glycosylation). All glycosylation types are enzyme-directed site-specific processes, with the exception of glycation. Glycation is a non-enzymatic reaction of glucose and other saccharide derivatives with proteins, nucleotides and basic phospholipids [1]. Since glycation is not an enzymatic post-translational modification process it will not be covered in this review.

The biological roles of glycosylation are diverse and influence both at cellular and protein levels, for example, protein folding and oligomerization [2], protein degradation [3], protein solubility and stability [4], epitope recognition [5], cell-cell interactions [6] and protein transport [7]. Hence, as may be expected, the glycoproteins are involved in the development and progression of several diseases, such as cancer [8], autoimmune diseases [9] and congenital disorders [10].

Among post-translational modifications, glycosylation may be considered the most complex of all [11]. First, carbohydrate structures are indirectly encoded in the genome. Both sequence and structure of glycan highly depends on the action of enzymes (*e.g.*, glycosyltransferases, carbohydrate-modifying enzymes and glycosidases) that create, modify or degrade glycosidic bonds [11]. Then, carbohydrate structure is well determined by the enzymes expressed in a particular cell or tissue. Second, glycoproteins can be modified with different carbohydrates in the same glycosylated site (leading to several glycoforms). Besides, not all potential glycosylation sites are simultaneously occupied [11].

Glycans bind to specific motifs within protein sequence depending on the glycosylation type, as will be described below. The recognition of glycan-occupied sites can be experimentally determined but it is an expensive and laborious process [12]. Thus, the number of verified glycosylated residues is still limited in relation with the growing number of known protein sequences [13]. The merely knowledge of glycosylated site locations may be a valuable tool. For example, to improve the 3D protein structure prediction, ensuring the appearance of glycosylated residues surface-exposed, as well as to modify the protein pharmacokinetic properties by changing protein-associated carbohydrate (glycoengineering). In this scenario, the development of bioinformatics tools to predict glyco-

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sylation sites is playing an increasing important role. Recently, bioinformatics resources for glycomics and glycobiology-related databases have been nicely reviewed elsewhere [14-16]. The application of bioinformatics tools in any biology field certainly demands a general understanding of the biological processes involved. Hence, this manuscript gives an overview of available glycosylation prediction methods, supported by a description of essential features for the different known glycosylation types.

N-glycosylation

N-glycosylation consists in the attachment of a sugar moiety to the amide side chain of an Asn residue within any of the following consensus sequences: Asn-X-Ser and Asn-X-Thr (and in some rare cases, Asn-X-Cys), where X could be any amino acid except Pro [17]. These tripeptide sequences are known as sequon. For many years, it was thought that N-glycosylation was present just in eukaryotes. But today, N-glycosylated proteins in prokaryotes is a fact [18]. Several differences have been observed in the biosynthesis of eukaryotic N-glycans compared with bacteria and archaea [19, 20]. In eukaryotes, N-linked protein glycosylation occurs in the endoplasmic reticulum (ER) [21]. It begins by transferring the oligosaccharide portion ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) from a lipid-linked precursor (dolichol phosphate) to the protein that is being translated in the ribosome (Figure 1) [22]. Next, the oligosaccharide immediately undergoes trimming and processing. First, two terminal glucose residues are removed leading to $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ -Asn linked protein. In this form, the newly synthesized glycoprotein enters to the calnexin/calreticulin cycle [23]. Calnexin (membrane-bound) and calreticulin (soluble) are lectin proteins residing in the ER. They specifically interact with the monoglucosylated glycoproteins to assist their folding and quality control. Once the glycoprotein has acquired its native conformation, it exits calnexin/calreticulin cycle and continues along the secretory pathway. Instead, the glycoprotein is re-glucosylated and re-sent to the calnexin/calreticulin cycle. Upon the deletion of the remaining glucose residue, one mannose is trimmed leading to $\text{Man}_9\text{GlcNAc}_2$ -Asn linked protein (Figure 1). This emerging N-glycosylated protein is transported to the Golgi apparatus for other mannose trimmings [22]. Finally, the $\text{Man}_5\text{GlcNAc}_2$ -Asn linked protein is the starting point to generate a huge repertory of N-glycan types in the Golgi apparatus [24].

In bacteria, the transference of the glycan portion to the nascent protein is a reaction similar to that occurring in eukaryotes [19]. However, both the enzyme catalyzing such reaction, named as oligosaccharyltransferase enzyme (OST) and the initial lipid-linked precursor differ in prokaryotes and eukaryotes [19]. The eukaryotic OST is a complex containing several membrane-associated protein subunits anchored in the lumen of the ER. The OST complex is involved in other functions besides oligosaccharide transfer reaction [23]. For example: (1) scanning of the polypeptide for possible N-linked glycosylation sites bearing the tripeptide sequon Asn-X-Ser/Thr, (2) directing the nascent polypeptide chain to the OST active site in the proper conformation, (3) positioning the acti-

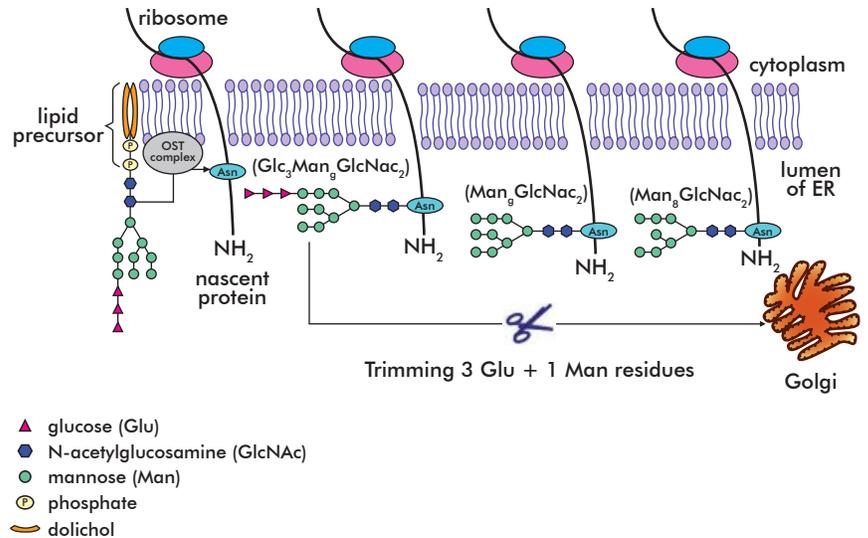


Figure 1. Schematic representation of the steps involved in the N-glycosylation process while protein resides in the endoplasmic reticulum (ER). OST- Oligosaccharyltransferase complex.

ve site of the OST complex near the retrotranslocon complex, and (4) recognizing and moving the lipid-linker precursor to the OST active site. Indeed, the availability and location of the OST complex relative to the nascent glycoprotein affects the N-glycan site occupancy [23]. However, in both bacteria and archaea, the OST enzyme is a single subunit homologous to the catalytic subunit of the multimeric OST eukaryotic complex [19]. There are other differences in the N-linked glycosylation process between eukaryotes and prokaryotes. For example, in bacteria, the N-glycosylation occurs in the periplasm and seems to be only a post-translational process, although it has not been confirmed yet [19]. Instead, N-glycosylation in eukaryotes is a co- and post-translational process [21].

N-glycosylation consensus sequence

The existence of the above described consensus sequon does not guarantee the occurrence of N-glycosylation [25-33]. For example, the N-glycan occupied sequences usually appear at points of change in secondary structure and on hydrophobic exposed patches in protein surface [30]. Instead, non-occupied asparagine residues are generally located on non-accessible surface areas and close to 60 residues from the C-terminal protein end [30, 34]. However, the influence of sequon distance to the protein C-terminal end in N-glycosylation site occupancy is still controversial. Since it was demonstrated that the same sequons in similar positions, located fewer than 60 residues of the C-terminal end, from two different proteins can be differentially utilized by OST complex in the same cell line [35]. Besides, the nature of amino acids both at position X and surrounding the sequon strongly modulate the occurrence of N-glycosylation. For example, occupied sequons from eukaryotes, bacteria or archaea never contain Pro residues at position X (also referred as position +1) [20, 30-33, 36]. In case of bacteria, the existence of Pro residues at position -1 also inhibits N-glycosylation [32]. Besides, the frequency of Pro residue is very low at position +3 in eukaryal

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N-glycosylated sequons [30]. An acidic residue (Asp or Glu) at position -2 is required for N-glycosylation in bacteria [32]. Hence, bacterial N-linked sequon was extensive to Asp/Glu-Y-Asn-X-Ser/Thr, where X and Y are not Pro residues [32]. However, negatively charged amino acids are disfavored at position -2 in both eukaryal and archaeal N-glycosylated proteins. This position is preferred for non-polar residues, particularly for aromatic amino acids in the case of eukaryotes [30]. The position X contains a high incidence of small amino acids (Gly, Ala, Val) in eukaryal modified sequons [20]. By contrast, Ser or Thr residues are found at such position in archaeal N-linked sequences [20]. Large hydrophobic residues (Ile, Leu, Met, Phe, Trp or Tyr) are located at position +3 in eukaryal occupied sites [30]. However, such position is occupied by small amino acids (Ala and Gly) in archaeal modified sequons [20]. Moreover, in both archaeal and eukaryal N-glycoproteins, basic residues are poorly represented at position +3. The frequency of N-glycosylation also varies between sequons types; Asn-X-Thr sequon is the most glycosylated one [30]. The roles of Ser and Thr residues at position +2 in Asn-X-Ser/Thr sequons have been already discussed [36, 37]. The hydroxyl group of Ser and Thr residues interacts with the amide side chain of the Asn residue via hydrogen bond, accepting a hydrogen atom [17, 37]. This interaction is facilitated when sequons are placed on beta-turn or other loops because the hydroxyl group of Ser or Thr residues may be oriented close to the Asn amide group [38]. It was also noted that, the side chain of the amino acid at position X is opposite to the hydroxyl and amide groups from Ser/Thr and Asn residues, respectively. Thus, it was suggested that Pro residue is not favored at position X because its unusual rigid structure disrupts the turn structure [38]. Additional studies correlating the frequencies of N-glycosylation with other sequon characteristics have been done. For example, it was demonstrated that overlapping sequons in the yeast invertase (*e.g.*, Asn-Asn-Ser-Ser sequons) can be both clearly glycosylated [39]. Such evidence discharged the idea that steric hindrance might prevent the N-glycosylation of overlapping sequon, at least in yeast invertase [39].

N-glycosylation prediction tools

Currently, three softwares are capable of predicting N-glycosylation; they are known as NetNGlyc [40], EnsembleGly [41] and GPP (Glycosylation Prediction Program) [42]. EnsembleGly and GPP were recently developed and can be used not only for N-glycosylation prediction, but also for the prediction of other glycosylation types (Table 1). However, the web-online NetNGlyc server is still the most used predictor for N-glycosylation [43-45]. All prediction methods use machine learning techniques trained on amino acid sequences. These methods examine the sequon vicinity to discriminate between possible modified and non-modified asparagines, since the amino acid composition flanking potential N-glycosylation sequon is determinant [30].

O-glycosylation

O-linked glycosylation involves the binding of glycans to hydroxyl side chains of serine and threonine resi-

Table 1. Summary of available web-online glycosylation predictors

| Server | Glycosylation | URL |
|-----------------|---|---|
| NetNGlyc | N-glycosylation | http://www.cbs.dtu.dk/services/NetNGlyc/ |
| EnsembleGly | N-glycosylation O-glycosylation C-glycosylation | http://turing.cs.iastate.edu/EnsembleGly |
| GPP | N-glycosylation O-glycosylation | http://comp.chem.nottingham.ac.uk/glyco/ |
| NetOGlyc | O-glycosylation | http://www.cbs.dtu.dk/services/NetOGlyc/ |
| Oglyc | O-glycosylation | http://www.biosino.org/Oglyc |
| CKSAAP_OGlySite | O-glycosylation | http://bioinformatics.cau.edu.cn/zzd_lab/CKSAAP_OGlySite/ |
| YinOYang | O-glycosylation | http://www.cbs.dtu.dk/services/YinOYang/ |
| DictyOGlyc | O-glycosylation | http://www.cbs.dtu.dk/services/DictyOGlyc/ |
| Big-PI | GPI-anchor | http://mendel.imp.ac.at/gpi/gpi_server.html |
| DGPI* | GPI-anchor | http://129.194.185.165/dgpi/ |
| GPI-SOM | GPI-anchor | http://gpi.unibe.ch/ |
| FragAnchor | GPI-anchor | http://navet.ics.hawaii.edu/~fraganchor/NNHMM/NNHMM.html |
| MemType-2L | GPI-anchor | http://www.csbio.sjtu.edu.cn/bioinf/MemType/ |
| PredGPI | GPI-anchor | http://gpcr.biocomp.unibo.it/predgpi/ |
| NetCCGlyc | C-mannosylation | http://www.cbs.dtu.dk/services/NetCCGlyc/ |

* The url for this database was unavailable at the time of publishing.

dues. There is no well defined motif for the O-glycan acceptor site. The glycans bind to serine and threonine residues which are usually found in a beta conformation and in close vicinity to proline residues [46]. The O-linked glycosylation occurs in bacteria, archaea and eukaryotes [47, 48]. O-glycosylation is a stepwise process where one monosaccharide is added at a time, rather than N-glycosylation where the high-mannose oligosaccharide is transfer en bloc to the target protein. Examples of O-glycans include: O-N-acetyl-galactosamine (O-GalNAc), O-N-acetylglucosamine (O-GlcNAc), O-Fucose, O-Glucose, O-Mannose, O-Hexose, O-Xylose. The most abundant and better characterized O-glycosylation type is mucin-type glycosylation [49]. This reaction is catalyzed by the enzymes UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase which adds an N-acetyl-galactosamine molecule to serine and threonine residues. Mucin-type glycans are found on many secreted and membrane-bound mucin proteins, which are the mucus main components. The function of such proteins is to protect epithelial surfaces [50, 51]. Mucin-type O-glycosylation occurs in the ER and the Golgi apparatus after N-glycosylation, folding, and oligomerization [52].

O-glycosylation target sequence

There is no clear consensus sequence for O-glycosylation. Some studies have confirmed a higher frequency of residues like Pro, Ser, Thr and Ala neighboring mucin-type glycosylated sites [53, 54]. Such sites are preferentially found in coil, turn or linker regions connecting domains. Besides, the experimentally verified O-glycosylated sites are more surface exposed than the non-glycosylated ones [55].

O-glycosylation prediction methods

Several methods for O-glycosylation prediction have been developed (Table 1). Among them, NetOGlyc [55] and Oglyc [56] predictors have been the most

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Both are able to recognize a higher number of GPI-anchored proteins with a lower rate of false positive errors, in comparison with other earlier described algorithms. But, PredGPI outperforms all available prediction methods [75]. MemType-2L is another resource capable to predict not only GPI-anchored protein, but also other seven types of membrane proteins [76]. However, both FragAnchor and MemType-2L are not able to detect the ω -site [74, 76].

C-mannosylation

C-mannosylation was originally found in human ribonuclease protein (RNase 2) from urine [77]. This post-translational modification involves the attachment of a mannopyranosyl residue to the C2 atom indole moiety of a tryptophan residue via a C-C bond [77, 78]. The transfer of the mannose residue to the target protein is catalyzed by the enzyme C-mannosyltransferase [77]. It has been suggested that this reaction probably occurs in the ER since already folded proteins are poor substrates *in vitro* [79]. Although C-glycosylation appears to be common in mammalian proteins, it has not been observed in yeast and bacteria [80]. At present, the knowledge related with the C-mannosylation functions and disorders is still very limited. However, at least three functions have been recently described. For example, C-mannosylation is required for an adequate folding of Cys subdomains contained in two mucin proteins (MUC5AC and MUC5B)[79]. Also, C-glycosylation appears to control the secretion of the puntion-1 protein [81] and it may be involved in the development of diabetic complications under hyperglycemic conditions [82].

C-mannosylation consensus sequence

C-mannosylation generally occurs at the first tryptophan residue (position 0) contained in the Trp-X-X-Trp sequence motif, where X could be any amino acid [77]. However, other studies revealed that mannosylated tryptophan has also been detected in other sequences motifs, for example in Trp-X-X-Phe, Trp-X-X-Tyr and Trp-X-X-Cys [83-85]. Since some sequence motifs having another aromatic residue instead of Trp at the position +3 can also be mannosylated, the Trp-X-X-Trp pattern seems to be sufficient but not strictly required for C-mannosylation. Indeed, it was demonstrated that only two-thirds of known mannosylated sites are found in Trp-X-X-Trp motifs [83]. Besides, small and/or polar residues (Ala, Gly, Ser and Thr) are preferred for occupying X positions within the sequence motif while Phe and Leu residues are not well tolerated at the mentioned position [83]. Other general features related with the structure of C-mannosylation motif, have also been described. For example, modified tryptophan residues instead of non-modified ones, are partly solvent exposed [83]. Besides, a particular interaction takes place at the Trp-X-X-Trp motif, where both tryptophan residues interact via an aromatic stacking. It was suggested that this type of interaction may account for the recognition of the C-mannosyltransferase enzyme [83].

C-mannosylation prediction

There is only one computational method reserved for the solely prediction of C-mannosylation and it

is called NetCGlyc [83] (Table 1). It predicts not only the typical mannosylated site (Trp-X-X-Trp), but others like those previously mentioned (Trp-X-X-Phe, Trp-X-X-Tyr and Trp-X-X-Cys). However, better predictions are achieved for the first case [83]. Recently, EnsembleGly could be also used for C-mannosylation prediction [41] (Table 1).

Databases

Undoubtedly, the development of accuracy glycosylation prediction methods requires the existence of databases with experimentally verified glycosylated sites. Usually, the data is extracted from O-GlycBase database [86], but in some cases, the Swiss-Prot database has been also used [86, 87]. O-GlycBase contains proteins having at least one experimentally verified O- or C-glycosylation site. Glycosylation data may also be found in databases including different types of post-translational modifications, like dbPTM [88], SysPTM [89], RESID [90] and others (Table 2). Besides glycoprotein databases, carbohydrate molecules databases also exists. For example, the major available databases of complex carbohydrates are: Complex Carbohydrate Structure Database (CCSD or CarbBank) [91], Glycosciences.de [92], KEGG GLYCAN [93], GlycomeDB [94], Carbohydrate DB from the Consortium Functional Genomic [95] and others (Table 2). Other available databases include the carbohydrate tertiary structures (*e.g.*, 3D Disaccharides [96], GDB:Structures [97] and GlycoMapsDB [98]). Others like GlyTorsionDB, GlySeqDB and GlyVicinityDB are integrated in the Carbohydrate Structure Suite [99]. Each database comprises the carbohydrate torsion angles, the glycoprotein sequences and the carbohydrate-protein interactions, respectively [99].

Concluding remarks

Currently, about 15 web-online softwares for the prediction of all glycosylation types are available. The possibility to perform prediction analysis using at least two or three different methods in parallel now exists. However, in our opinion, with upcoming prediction methods, a critical comparative study would

Table 2. Summary of available web-online glycosylation predictors

| Databases | URL |
|--|--|
| <i>Carbohydrates</i> | |
| CarbBank | http://www.boc.chem.uu.nl/sugabase/carbbank.html |
| Glycosciences.de | http://www.glycosciences.de |
| KEGG GLYCAN | http://www.genome.jp/kegg/glycan/ |
| GlycomeDB | http://www.glycome-db.org/ |
| <i>Carbohydrate Binding Protein DB</i> | |
| SWEET-DB | http://web.mit.edu/glycomics/cbp/cbpdbs.shtml |
| EuroCarbDB | http://www.glycosciences.de/modeling/sweet2 http://www.ebi.ac.uk/eurocarb/ |
| <i>Glycoproteins</i> | |
| BCSD | http://www.glyco.ac.ru/bcsdb/start.shtml |
| dbPTM | http://dbPTM.mbc.nctu.edu.tw/ |
| GlycoProDB (GPDB) | http://riodb.ibase.aist.go.jp/rcmg/glycodb/ |
| GlycoSuiteDB | http://glycosuitedb.expasy.org/glycosuite/glycodb |
| O-GlycBase | http://www.cbs.dtu.dk/databases/OLYCBASE/ |
| RESID | http://www.ncicrf.gov/RESID/ |
| SysPTM | http://www.biosino.org.cn/SysPTM/ |

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be required to define the most accurate one. The combination of such prediction methods with other non-carbohydrate tools facilitates the characterization and rational modification of the native protein glycosylation pattern.

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