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Glycoprotein reglucosylation

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Abstract

Proteins following the secretory pathway acquire their proper tertiary and in certain cases also quaternary structures in the endoplasmic reticulum (ER). Incompletely folded species are retained in the ER and eventually degraded. One of the molecular mechanisms by which cells achieve this conformational sorting is based on monoglucosylated *N*-glycans ($Glc_1Man_{5\text{-}9}GlcNAc_2$) present on nascent glycoproteins in the ER. This chapter discusses two of the steps that regulate the abundance of such *N*-glycan structures, including glycoprotein deglucosylation (by glucosidase II) and reglucosylation (by the UDP-Glc:glycoprotein glucosyltransferase), as well as an overview of methods to evaluate the *N*-glycans prevalent during glycoprotein biogenesis in the ER.

1. Protein folding in the endoplasmic reticulum

Protein folding in living cells is a complex, error-prone process. Numerous mechanisms, referred to as "quality control," are in place to ensure that newly synthesized proteins reach their properly folded functional form. The concept of quality control in the secretory pathway emerged after a number of reports in the late 1970s and early 1980s showed that for some proteins, insertion in the endoplasmic reticulum (ER)[1] did not necessarily result in their appearance at the expected final destination, intra- or extracellular [1,2]. It became clear then that the secretory pathway released proteins in their native conformations, and those that failed to fold properly were degraded [1,2] (see chapter by Brodsky, this volume).

The ER lumen has certain features that differentiate this sub-cellular compartment from others that also support protein folding, such as the cytosol or the mitochondria [3]. The ER lumen is particularly rich in Ca^{2+}, which is required by several chaperones and folding facilitating enzymes for activity. The ER also provides an oxidizing environment, where proteins rich in disulfide bonds are assisted by several enzymes belonging to the protein disulfide isomerase family that facilitate proper formation of disulfide bridges. Enzymes that catalyze *cis–trans* proline isomerization and several classical chaperones (Grp78/BiP, Grp94, and Grp170) are also present in the ER lumen.

1.1. The contribution of N-glycans to protein folding in the ER

The quality control functions in the ER also rely on the addition of *N*-glycans to nascent glycoproteins, one of the most prevalent post-translational modifications [4–7]. The beneficial effect of covalently linked *N*-glycans on protein folding can be observed in cell-free assays, where they seem to provide bulky hydrophilic domains that maintain molecules in solution while protein moieties successively adopt a variety of different conforma-

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[1] *Abbreviations used:* ConA, concanavalin A; CNX, calnexin; CRT, calreticulin; Dol, dolichol; DMJ, deoxymannojirimycin; Endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum; GI, glucosidase I; GII, glucosidase II; Grp78/BiP, an endoplasmic reticulum chaperone of the Hsp70 family; GT, UDP-Glc:glycoprotein glucosyltransferase.

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tions before reaching their final structures. In addition, the hydrophilic nature of *N*-glycans forces the asparagine units to which they are linked and neighboring amino acids to be at or near the protein surface.

While these effects most likely occur in vivo, *N*-glycans also contribute to folding efficiency in the ER by a series of oligosaccharide processing and lectin-binding reactions, that contribute to the folding, ER retention, or eventual targeting for degradation of the glycoprotein to which they are attached. These interactions are mediated by specific *N*-glycan structures, which are generated by the concerted action of processing enzymes.

2. Deglucosylation of *N*-glycans

The removal of glucose residues from newly synthesized glycoproteins occurs predominantly in the ER, immediately after *N*-glycans have been transferred to protein (Fig. 2). The terminal glucose residue *n* is removed cotranslationally by glucosidase I, a membrane bound enzyme in the ER [8–10]. Although glucosidases are confined to the ER, some tissues in higher eukaryotes absent in lower eukaryotes [11] express an endomannosidase that allows Glc removal in the *cis*-Golgi [12,13]. The endomannosidase cleaves after one of the terminal mannose residue that is not hydrolyzed by α-mannosidases in the ER (Fig. 1, residue *g*).

Another deglucosylating enzyme, glucosidase II (GII), then removes the two remaining Glc residues (*m* and *l*, Fig. 1). Unlike typical glycosidases in the secretory pathway, which are type II membrane proteins [14], glucosidase II is a soluble enzyme resident in the ER. It is composed of one catalytic subunit (α) and one accessory subunit (β) assembled into a highly asymmetric heterodimer [15,16]. The catalytic subunit is fully active in vitro in the absence of the β subunit [16], but both subunits are required for glucosidase II activity in vivo [17–19]. Alternatively spliced forms of both chains have been described [19–21] but the catalytic core appears conserved in all of them. Mutations in the β-subunit are implicated in polycystic liver disease [22,23]. The methodology to assay GII activity is described below, to allow the evaluation of structure and activity properties of the enzyme. The isolation of GII form rat liver is also described (see part 4).

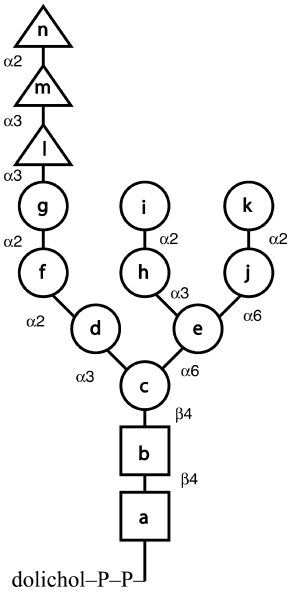

Fig. 1. Structure of the oligosaccharide that initiates N-glycosylation. The cartoon depicts the oligosaccharide assembled on the ER membrane, bound to dolichol-P-P and transferred en bloc to Asn residues in glycosylation sites on nascent polypeptides. The letters (*a*–*n*) that identify each residue correspond to the order in which they are incorporated into the lipid-bound precursor. Squares, circles, and triangles represent GlcNAc, Man, and Glc, respectively.

2.1. Assay for GII

GII activity can be measured using two types of substrates, radioactive glycans or artificial substrate analogs. Radioactive [^{14}C]Glc- or [^{3}H]Glc-labeled $Glc_1Man_9GlcNAc$ can be prepared in vitro by incubating rat liver microsomes (20 mg/ml) with 10–40 μM UDP-Glc (0.8–3.2 μCi of UDP-linked [^{14}C]Glc or [^{3}H]Glc should be added), 1 mM castanospermine or 1-deoxynojirimycin (Sigma, Roche, Calbiochem), 10 mg/ml denatured thyroglobulin (Sigma) (see assay for GT below), 10 mM $CaCl_2$, 1% Triton X-100, 5 mM of 2-mercaptoethanol, 20 mM Hepes buffer, pH 7.4, in a final volume of 200 μl for 60 min at 37 °C. Under these conditions the radiolabeled Glc is incorporated into *N*-glycans on glycoproteins directly via reglucosylation (mostly on the denatured thyroglobulin added as exogenous acceptor) or, additionally, via the dolichol pathway. At the end of the incubation samples are extracted with chloroform/methanol/water as described above for isolation of radio labeled *N*-glycans from cells (see Section 5.2.2. Extraction of protein-bound *N*-glycans). $Glc_1Man_9GlcNAc$ has to be chromatographically purified from $Glc_3Man_9GlcNAc$ and $Glc_2Man_9GlcNAc$ that might have been formed via the Dol-P pathway.

To measure GII activity, radiolabeled glycans are incubated for 5–60 min at 37 °C with test samples in a final volume of 50–100 μl containing 10 mM Hepes buffer, pH 7.4 (1% Triton X-100 has to be added when microsomal vesicles are used as enzyme source). At the end of the incubation the released radioactive Glc ([^{14}C]Glc or [^{3}H]Glc) can be detected in a number of ways. One possibility is to separate the released Glc from the intact oligosaccharide by paper chromatography [24,25]. Another approach to separate the released Glc is to add 100 μl of concanavalin A (ConA) (1 mg/ml, Sigma) in 200 mM Tris–HCl buffer, pH 8.0, 1 mM $CaCl_2$ to bind all the undegraded glycan, followed by polyethylene glycol addition to precipitate the glycan–lectin

complex [26]. The Glc liberated by GII in the assay is not bound by ConA and therefore remains in the supernatant after centrifugation, and is then quantified by liquid scintillation counting.

GII activity can also be measured with the artificial substrate *p*-NO_2-phenyl-α-D-glucopiranoside (Sigma). GI does not cleave this substrate, and therefore GII is the main enzyme capable of cleaving this substrate at pH 8.0, since most lysosomal glycosidases are unstable and/or inactive at such pH. In this case, Glc removal is followed by Abs405 nm to detect the free *p*NO_2-phenol released. Assays can be conducted in a final volume of 100 μl containing 1 mM *p*-NO_2-phenyl-α-D-glucopiranoside in 20 mM Tris–HCl buffer, pH 8.0, 1 mM EDTA in a thermostatized cuvette, monitored continuously at 405 nm.

3. Glycoprotein reglucosylation

Deglucosylated glycoproteins can be transiently reglucosylated on mannose residue *g* by a soluble glucosyltransferase in the ER (GT, Fig. 2) [5,6,27–29]. Although the membrane bound glucosyltransferases utilize Dol-P-Glc to add Glc residues to the Dol-P-P oligosaccharide precursor, GT specifically utilizes UDP-Glc as sugar donor. A transport system allows entrance of UDP-Glc into the mammalian cell ER lumen coupled to exit of UMP [30]. Moreover, two UDPase/GDPase activities have been described in the same sub-cellular location, believed to convert the product of UDP-Glc consumption (UDP) into UMP to promote further UDP-Glc transport [31,32].

The reaction products generated by GT are the respective monoglucosylated derivatives that were previously deglucosylated by GII, as the single Glc unit is added to Man *g* (Fig. 1) in an α(1,3) linkage [25]. The Glc added by GT are also removed by glucosidase II, and this seems to be the only function of this enzyme in trypanosomatids, since they do not synthesize Glc containing Dol-P-P-oligosaccharide precursors [25,33]. Interestingly, mannosidases in the ER do not remove the mannose to which Glc units are added by reglucosylation (Fig. 1, residue *g*), maximizing the chances for reglucosylation before exiting the ER. However, this Man residue can be removed in some cell types by the Golgi endomannosidase, terminating reglucosylation cycles.

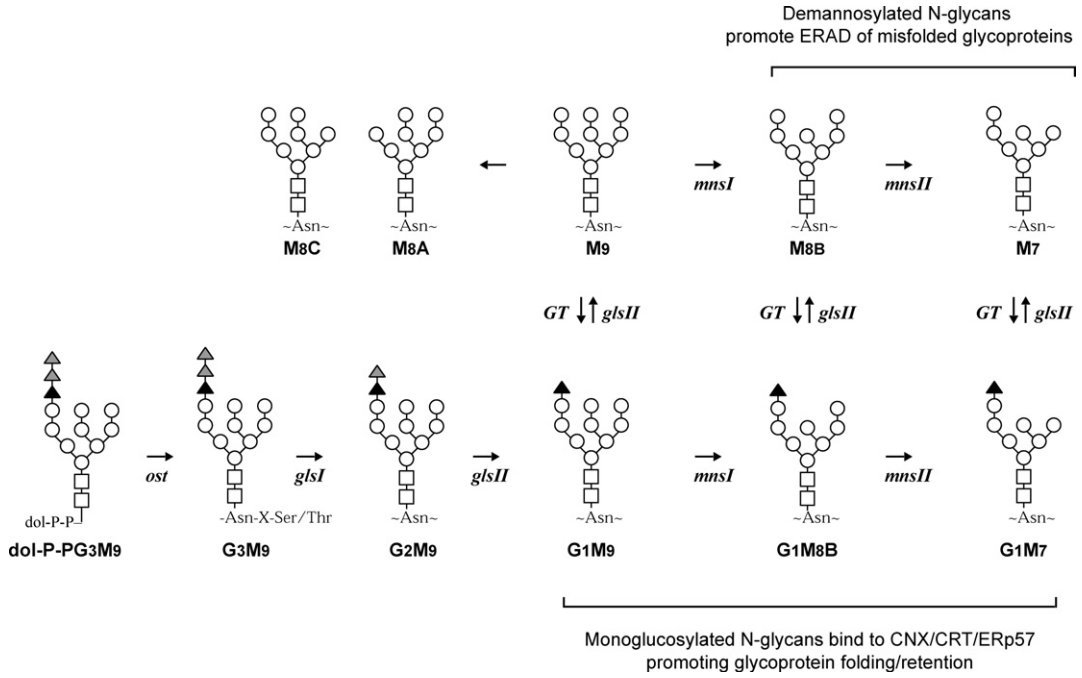

Fig. 2. Processing of *N*-glycans in the ER and their role in quality control. The first structure shown on the left (G3M9) is the dolichol-bound oligosaccharide precursor transferred to Asn residues on nascent polypeptides by the oligosaccharyltransferase (*ost*). Once this oligosaccharide is transferred to proteins, glucose residues are first trimmed by glucosidase I (*glsI*) to produce G2M9 and then by glucosidase II (*glsII*) generating G1M9 and M9. Protein bound G3M9 accumulates in the presence of glucosidase inhibitors, blocking binding to CNX/CRT. Fully deglucosylated *N*-glycans (M9, M8B, and M7) on glycoproteins that are not folded properly can be reglucosylated to regenerate the corresponding G1M9, G1M8B or G1M7. M9 glycans can be further trimmed by mannosidase I (*mnsI*) to generate the M8B structure. In some species such as yeasts and flies M8B is the main processing intermediate accumulating in the ER. In higher eukaryotes, M8C, as well as M7 or even M6 can arise by the action of mannosidase II (*mnsII*) or possibly mannosidase I. With the exception of the first two deglucosylation steps (glucosidase I followed by glucosidase II, generating G2M9 and G1M9), the subsequent processing reactions are not necessarily sequential. Demannosylated structures (M8A) that lack the terminal mannose residue utilized by GT can no longer be reglucosylated. In higher eukaryotes, these M8A, M8C, and smaller structures can be generated in the ER by mannosidase II. They can also be generated by α-mannosidases or the endomannosidase in the Golgi, on those glycoproteins that cycle between the ER and the Golgi during QC. The symbols represent: (△) glucose; (○) mannose; and (□) *N*-acetylglucosamine.

Such cycle of deglucosylation and reglucosylation can occur repeatedly on glycoproteins during their biosynthesis [34], and is now recognized as a central part of a quality control and chaperone pathway in the ER lumen (Fig. 2). The monoglucosylated *N*-glycans produced in this cycle are recognized by two lectins in the ER, calnexin (CNX), and calreticulin (CRT), as discussed by Williams in this volume.

3.1. Domain structure of GT

At least two domains can easily be identified in GT [29]. One of them comprises the N-terminal 80% of the molecule, has no homology to other known proteins and is probably involved in non-native conformer recognition. The other one is the C-terminal or catalytic domain binds [β-^{32}P]5N$_3$UDP-Glc and displays a similar size and significant similarity to glycosyltransferase family eight members [35]. The C-terminal domains of GT from different species share a significant similarity (65–70%), but no such similarity occurs between N-terminal domains [29]. For instance, the N-terminal domains of GT from rat and *Drosophila* share ≈33% similarity between them, but they only show ≈15% similarity with the same portion of *Schizosaccharomyces pombe* GT. Nevertheless, the N-terminal domains of the fly and yeast enzymes were found to be mutually interchangeable, showing that they probably share common structural and functional features [36].

The notion that recognition of non-native conformers is mediated by the N-terminal domain is supported by the enzymatic activities of chimeras constructed with N- and C-terminal domains of human GT homologues [37]. Two GT homologues were identified in human cells, but only one them is able to glucosylate misfolded glycoproteins [38]. A chimera containing the catalytic domain of the inactive enzyme plus the N-terminal portion of the active one was found to glucosylate misfolded conformers [37]. The junction between both N- and C-terminal domains was found to be very sensitive to proteolysis, but both domains in the cleaved molecules could not be separated by a number of analytical procedures without losing enzymatic activity and the presence of the N-terminal domain appeared to be required for proper folding of the C-terminal part [36].

3.2. Specificity of GT

A key property of GT is that it does not glucosylate properly folded glycoproteins [25,28,39]. Such ability of GT to distinguish between native and non-native conformations was discovered and characterized in vitro, but a similar preference has been observed in living cells (see for example [34,40–42]). The early studies that identified the ability of GT to distinguish between native and non-native glycoproteins utilized chemically denatured substrates, that contained an ill defined mixture of non-native structures. Such heterogeneity made it difficult to study the elements recognized by GT. The analysis of GT specificity is better pursued using more defined substrates, whose structures are still not native but allow a better control of their properties [43,44], such as small monomeric proteins that remain soluble under experimental conditions, without aggregating. The use of such substrates allowed to establish that GT can sense very minor conformational differences, even when the substrate glycoprotein is very close to a native conformation [43]. Moreover, it was noticed that GT is able to not only distinguish between native and non-native glycoproteins, but it can further distinguish between different non-native structures of the same glycoprotein [44]. By direct comparison of GT recognition of different conformations of ribonuclease B, it was found that a fully unfolded form was much less reglucosylated that a largely structured form (although still non-native), even when the fully unfolded form was efficiently bound by Hsc70 [44]. A similar result was obtained with a fragment of chymotrypsin inhibitor-2, in which of two non-native forms, one was recognized by BiP but not by GT, and vice versa [45].

A preferential recognition by GT of compact, native-like structures, over fully unfolded forms was also observed in vivo. Addition of dithiothreitol to live *S. pombe* cells did not enhance GT-mediated protein glucosylation [46]. This result probably reflects the inability of most glycoproteins, which normally have disulfide bonds, to reach a compact, molten globule-like conformation when formation of those bonds is prevented. Furthermore, a lysosomal protease from *Trypanosoma cruzi* was not reglucosylated shortly after synthesis in vivo, but was selectively reglucosylated at more advanced stages of folding after most of its disulfide bonds were formed [41].

Although these studies showed that GT does not recognize the same determinants as chaperones of the Hsp70 family, GT seemed to detect exposure of hydrophobic amino acid patches in collapsed, molten globule-like conformers [45]. A preference for certain hydrophobic elements in the vicinity of the N-glycosylation site was also found in the glucosylation of glycopeptides [47].

The preference for structured conformations over more extensive unfolded ones was mapped to very advanced stages of folding, when substrates reach a highly compact structure close to the native state [48]. GT was proposed to glucosylate *N*-glycans present in the vicinity of structural perturbations of the protein backbone in ribonuclease B chimeras [49], but in the reglucosylation of exo(1,3)β-glucanase GT appeared to recognize more distal conformational changes [50].

The studies described above show how the analysis of different substrates with an increasing variety of conformational properties is beginning to illuminate the mech-

anism of recognition of glycoprotein substrates by GT. The methodology necessary to assay GT activity in vitro is described below. The purification of GT from rat liver is also described (see part 4). The enzyme can also be obtained recombinantly [35,37,38,45].

3.3. Assay for GT

The assay for GT is based on the incorporation of radioactive Glc from the sugar donor (UDP-[^{14}C]Glc or UDP-[^{3}H]Glc) into polymannose glycans on unlabeled denatured acceptor glycoproteins. The radiolabeled reaction product (glucosylated glycoproteins) is separated from the radiolabeled substrate (UDP-Glc) by trichloroacetic acid (TCA) precipitation and quantified by liquid scintillation counting. The acceptor glycoproteins (bovine thyroglobulin; soybean agglutinin, SBA; bovine pancreatic ribonuclease B (RNaseB; available from Sigma, Roche, Worthington) can be prepared by chemical denaturation [25,28]. Glycoproteins are dissolved at high concentrations (20–50 mg/ml) in 10 mM Hepes buffer, pH 7.4. One gram of solid urea is added per milliliter of protein solution, and the mixture is incubated at 60 °C for 4 h. The samples are then exhaustively dialyzed against 10 mM Hepes buffer, pH 7.4. The assay for GT is very specific, especially when solubilized extracts and subsequently purified fractions are used as source of enzyme. When microsomes are used, incorporation of radioactive Glc into proteins may potentially arise from the Dol-P pathway [51]. This involves the formation of Dol-P-Glc from UDP-Glc and endogenous Dol-P, leading to the formation of $Glc_3Man_9GlcNAc_2$-P-P-Dol followed by transfer of the entire glycans to Asn residues on vacant glycosylation sites on the denatured glycoproteins used as substrates [52]. The enzymes involved in this cascade of reactions are integral membrane proteins, and are poorly extracted in the conditions utilized to solubilize GT [25]. As a consequence, radiolabeling of the acceptor glycoproteins via the Dol-P pathway only occurs when crude microsomal membranes are used, and does not occur once GT is solubilized from microsomes. In some systems (such as those derived from yeasts or plants) incorporation of radioactive Glc into trichloroacetic acid (TCA) insoluble polysaccharides may also be observed when crude extracts are used. Reactions are conducted in 50–100 μl final volume, containing 10 mM Hepes buffer, pH 7.4, 10 mM $CaCl_2$, 5 mM of 2-mercaptoethanol, and 2.5 μM UDP-Glc (about 0.01–0.05 μCi of UDP-linked [^{14}C]Glc or [^{3}H]Glc should be added). Reactions are initiated by addition of the test sample and incubated for 5–30 min at 37 °C and stopped by addition of 1 ml of 10% TCA. After boiling the stopped reactions for 5 min to allow for complete protein insolubilization, the precipitated proteins are recovered by low speed centrifugation (5 min at 2000*g*) and washed three times with 1 ml of 10% TCA. The washed pellets are resuspended in 100 μl of 1 N KOH in methanol or other commercial solubilizers, diluted with 3 ml of scintillation cocktail and quantified by liquid scintillation counting. The incorporation of radioactive Glc into acceptor glycoproteins can also be detected by autoradiography [25,48–50].

4. Purification of GII and GT from rat liver

Both GII and GT are soluble proteins of the lumen of the ER, and are therefore soluble in the absence of detergents. Low concentration of detergents may nevertheless be used to release the soluble content of the microsomes at the start of the purification, but detergents are not used in the subsequent purification steps. GT and GII are minor components of the ER, and typically less than 1 mg GT or GII is obtained from 100 to 200 g of liver, with yields below 10%. Both enzymes are highly susceptible to proteases, and therefore it is critical to include protease inhibitors in the homogenization buffers, to maintain the pH above 7.0 and to process rapidly the microsomal extracts. Microsomes can be kept frozen for a few weeks, but it is important to go from the microsomes to the final step with minimal delays. Both enzymes are relatively stable after purification. GT is composed of a single polypeptide that runs at approximately 160 kDa in SDS–PAGE [28]. GII is composed of two different subunits, one running at approximately 110 kDa, and a second subunit that runs as a slightly diffuse band at approximately 80 kDa [15].

4.1. Buffers and chromatography media

Buffer A: 0.25 M sucrose, 2 mM EDTA, 20 mM Tris–HCl, pH 8.0, and 5 mM of 2-mercaptoethanol. Buffer B: 150 mM NaCl, 20 mM Tris–HCl buffer, pH 8.0, and 5 mM of 2-mercaptoethanol. Buffer C: 20 mM Tris–HCl buffer, pH 8.0 and 5 mM of 2-mercaptoethanol. Buffer D: 1 M NaCl, 20 mM Tris–HCl buffer, pH 8.0, and 5 mM of 2-mercaptoethanol. Buffer E: 1 M ammonium sulfate and 5 mM of 2-mercaptoethanol. Buffer F: 0.5 M sucrose, 10 mM imidazol buffer, pH 7.0, and 5 mM of 2-mercaptoethanol. Chromatography media are from Pharmacia or Sigma.

4.2. Preparation of rat liver microsomes

Rats (male or female, 4–12-week-old) are starved overnight. They are euthanized and their livers removed and rinsed on ice cold buffer A. From this point, all procedures are carried on ice or in a cold room, except for the elution step from the ConA–Sepharose column. Livers are weighted and minced in a blender with two to four volumes of buffer A containing protease inhibitors (1 mM EDTA, 10 μM leupeptin, 10 μM pepstatin, 10 μM E-64, 10 μM TLCK, 10 μM TPCK, and 100 μM PMSF).

The homogenate thus obtained is centrifuged at 10,000*g* for 10 min. The supernatant is further centrifuged at 100,000*g* for 60 min. The pellet containing the microsomal fraction is resuspended in buffer A and stored at −80 °C.

4.3. Extraction of GT and GII from microsomes

Since both GT and GII are soluble proteins, they can be extracted in the absence of detergents by mechanical disruption of microsomes (10 mg/ml) in buffer C using sonication, French press or equivalent. After mechanical disruption, the soluble fraction is recovered from the supernatant after high speed centrifugation (60 min at 100,000*g*) and precipitated with ammonium sulfate at 50% saturation (by addition of one volume of saturated ammonium sulfate solution). The insoluble pellet is resuspended and dialyzed against buffer C. Alternatively, the microsomal fraction can also be solubilized with detergents at low concentration. For this, microsomes are resuspended at 10 mg/ml in buffer C and extracted with 0.1% Triton X-100 for 30 min on ice. The homogenate is then centrifuged at 100,000*g* for 60 min and the supernatant of the microsomal extraction containing most of GT and GII activity is saved. Detergent extracts are not fractionated with ammonium sulfate.

4.4. Chromatographic steps

4.4.1. DEAE–cellulose

The solubilized fractions obtained either by detergent extraction of microsomes or the ammonium sulfate cut are loaded onto a DEAE–cellulose column equilibrated in buffer C, and washed in the same buffer until the Abs_{280} reaches background. Notice that no detergents are needed from this point on, since both GT and GII are soluble proteins. The column is then eluted with a gradient from 100% buffer C to 50% buffer D over 20 column volumes. The enzymatic activities for GT and GII are measured in the eluate of the DEAE–cellulose column (GT typically elutes between 300 and 400 mM NaCl, before GII which elutes between 400 and 500 mM NaCl). From this point, the fractions containing GT activity are separated from GII activity and are pursued separately.

4.4.2. ConA–Sepharose

The fractions containing GT or GII activity eluted from the DEAE–cellulose are applied separately to ConA–Sepharose columns (5 ml) equilibrated in buffer B supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. After washing in buffer B until the Abs_{280} reaches background, the column is filled with one volume (approximately 5 ml) of buffer B supplemented with 0.5 M α-methyl-mannopyranoside (Sigma) pre-warmed at 37 °C. The column is then stopped, and kept at 37 °C for 15 min. The elution continues with more pre-warmed buffer B supplemented with 0.5 M α-methylmannopyranoside.

4.4.3. MonoQ

The fractions containing GT or GII activity eluted from their respective ConA–Sepharose columns are diluted 5-fold and loaded (separately) onto a MonoQ 5/5 columns equilibrated in buffer C. The column is then eluted with a gradient from 100% buffer C to 50% buffer D over 20 column volumes (20 ml) and 1 or 0.5 ml fractions are collected.

4.4.4. Gel filtration

The fractions eluted from the MonoQ step containing GT or GII activity are further purified by gel filtration chromatography on a Superdex S-200 column (or equivalent) equilibrated and run in buffer B. At this stage, GII is usually homogeneous. If necessary, the MonoQ or gel filtration steps can be repeated to achieve a homogeneous preparation. GII is stored in buffer B at −80 °C.

4.4.5. Phenyl-Superose

After the gel filtration step GT usually requires further purification using hydrophobic interaction chromatography. The fractions eluted form the gel filtration column containing GT activity are diluted 10-fold with buffer E, filtered, and loaded onto a phenyl-Superose column (1 ml) equilibrated in buffer E. The column is eluted at 0.5 ml/min with a gradient from 100% buffer E to 100% buffer F over 20 ml, and then further eluted with another 15 ml of buffer F. GT is strongly retained by the column and typically elutes during the beginning of the wash with buffer F. GT is usually homogeneous at this step and can be stored in buffer F at −80 °C. If necessary, the MonoQ or gel filtration steps can be repeated for further purification, but buffer exchange into buffer F is recommended for storage of GT.

5. Glycoprotein reglucosylation in vivo

A number of observations suggest that folding facilitation mediated by glycoprotein–CNX/CRT interactions is not required for the viability of single cells under normal growth conditions. Mammalian or *S. pombe* cells deficient in GI or GII activities, in which monoglucosylated glycans cannot be formed either by partial deglucosylation of $Glc_3Man_9GlcNAc_2$ or by GT-mediated glucosylation, do not present any discernable phenotype [17,53–55]. Cells appear to have alternative systems involving different chaperones for helping proteins to acquire their native structures. A deficient system may be replaced by an alternative one and consequently, a substantial proportion of many glycoproteins may fold correctly in the absence of interaction with CNX/CRT.

For instance, the amount of carboxypeptidase Y that reached the vacuole in GII-deficient *S. pombe* cells after a short pulse with [^{35}S]Met decreased by 50% with respect to wild type cells and about half of HA molecules folded properly when translated in a rabbit reticulocyte-dog pancreas microsome system in the presence of GI/GII inhibitors [17,56]. Furthermore, accumulation of misfolded glycoproteins in the ER caused by a total or reduced ability of glycoproteins to interact with CNX/CRT triggers an upregulation of chaperones and other folding-assisting proteins (unfolded protein response). This has been observed in mammalian and *S. pombe* cells lacking GI/GII activities as well as in *S. pombe* and *T. cruzi* mutants lacking GT [17,57,58]. Further, even though several glycoproteins in *T. cruzi* have been identified as essential components of differentiation and mammalian cell invasion processes, total hindering of monoglucosylated *N*-glycan formation caused by disruption of both GT-encoding alleles did not affect the rate of cell growth of epimastigote form parasites, and only partially affected differentiation and mammalian cell invasion [57].

The dispensable character of glycoprotein–CNX/CRT interaction for single cell viability may be highlighted by the fact that this folding facilitating mechanism is probably not operative in *S. cerevisiae*, as this yeast lacks most components of the CNX/CRT/GT pathway [59]. Also, contrary to what happens in *S. pombe*, *T. cruzi*, and mammalian cells, no induction of ER chaperones was observed under conditions that prevent formation of monoglucosylated *N*-glycans, thus indicating that the absence of glycoprotein–CNX interaction does not lead to an accumulation of misfolded species in the ER (both *S. cerevisiae* and *S. pombe* lack CRT).

Although glycoprotein reglucosylation is dispensable for cell growth in culture, most glycoproteins are reglucosylated in vivo [60–62]. Indeed, Glycoprotein–CNX/CRT interaction is essential for viability under conditions of excessive ER stress such as those caused by underglycosylation of glycoproteins and high temperature: *alg6*/*gpt1 S. pombe* double mutants in which $Man_9GlcNAc_2$ is transferred (inefficiently, see above) from lipid derivatives, and are devoid of GT activity grew at 28 °C but not at 37 °C. Growth at high temperature was rescued not only upon transfection with a GT-encoding expression vector but also by 1 M sorbitol addition, thus suggesting that the affected glycoprotein(s) might be involved in cell wall formation [63]. When the folding of certain glycoproteins was studied in detail, it was found to be quite dependent on reglucosylation [2,6,7]. For example, it was reported that GI/GII inhibitors prevented VSV maturation by interfering with G protein folding [64], as well as with formation of infectious human immunodeficiency virus (HIV) type I particles, probably due to misfolding of loop V1–V2 in gp120 [65]. In addition, the same inhibitors prevented folding of tyrosinase in melanoma cells and assembly of hepatitis B virus particles by blocking the correct folding of M glycoprotein [66,67].

The results described above indicate that although not required for cell growth in culture, glycoprotein folding facilitation and irreparably misfolded glycoprotein ER retention mediated by glycoprotein–CNX/CRT interaction is indeed required under special conditions or for proper folding of particular proteins. The extent to which glycoprotein–CNX/CRT interaction is required for viability of multicellular organisms is presently unknown. Some methodologies to evaluate glucosylation status of nascent glycoproteins are described below.

5.1. Indirect analysis of *N*-glycans present in ^{35}S-labeled glycoproteins

The analysis of the *N*-glycans present on specific glycoproteins is a challenging task, particularly when one needs to focus on specific glycosylation sites. Such analysis requires a rather large amount of protein, that has to be fragmented to isolate individual peptides containing the desired *N*-glycosylation site, from which oligosaccharides are further released and characterized. It is even more challenging to perform this kind of analysis during the folding of a glycoprotein, since only minute amounts of sample can be obtained. A first simplification is to radiolabel the *N*-glycans and subsequently isolate the protein of interest, from which oligosaccharides can be released and analyzed, providing direct structural information of the average oligosaccharide structures present in the glycoprotein studied (see for example [68,69]).

An even simpler approach is to evaluate *N*-glycan structures on immunoprecipitated ^{35}S-labeled proteins using glycosidases and SDS–PAGE analysis. These methods do not provide detailed carbohydrate structures but have several advantages: labeling is quite efficient, multiple samples can be compared simultaneously, the detection limit is very low and relatively few cells are needed. When the protein under study is synthesized in large quantities, very short pulses are sufficient to produce an intense signal, and very early events (even co-translational) can be detected. The glycan structures are not analyzed directly, but can still be evaluated by their susceptibility to endo- and exoglycosidases, evidenced as small increases in mobility on SDS–PAGE. Each glycan removed by endo-β-*N*-acetylglucosaminidase H (Endo H) results in an increase in mobility of about 2 kDa, but exoglycosidases produce smaller shifts. For large proteins, the shifts are usually very small unless the glycosidases cleave *N*-glycans on several glycosylation sites.

The most common analysis is the evaluation of whether glycoproteins contain Endo H sensitive glycans. This enzyme does not remove complex-type glycans

generated in the Golgi but removes all *N*-glycoforms present in the ER. Therefore, when glycoproteins are sensitive to cleavage by Endo H they are considered to have *N*-glycan structures similar to those acquired in the ER. Some exceptions to this rule are glycoproteins that traverse the Golgi but carry *N*-glycans that are not modified and thus remain partially or completely sensitive to Endo H. Also, certain yeasts elongate *N*-glycans in the Golgi but generate structures that remain sensitive to Endo H. In these cases, Golgi modifications can be identified by mobility shifts (the Golgi modified forms run much slower and often diffuse) or with antibodies to *N*-glycans structures produced in the Golgi. To verify that the lack of Endo H cleavage is due to acquisition of complex-type *N*-glycans, parallel samples should be digested with Endo F or with peptide: *N*-glycosidase F. These enzymes cleave *N*-glycans irrespective of their structure, and are thus insensitive to *N*-glycan modifications acquired in the Golgi.

For those glycoproteins that remain Endo H sensitive due to their residence in the ER, the presence of glucosylated *N*-glycans can be evaluated by partial resistance to digestion with α-mannosidases that only display exoglycosidase activity. Eight of the nine Man residues in ER-glycoforms are linked in the α-configuration and can be removed by α-mannosidases. *N* -glycans that carry a terminal Glc residue contain three Man residues protected from α-mannosidases (see for example [70–72]). Therefore, glucosylated *N*-glycans are partially resistant to α-mannosidases, and a mobility difference between completely demannosylated and partially demannosylated proteins can often be detected by high resolution SDS–PAGE (the magnitude of the shift observed with α-mannosidase digestion is smaller than with Endo H). It is difficult to distinguish between mono-, di- or triglucosylated *N*-glycans by partial resistance to α-mannosidase. Pretreatment with GII renders di- and monoglucosylated *N*-glycans fully sensitive to α-mannosidases.

Except for GII, the other glycosidases are commercially available (Roche, Sigma, New England Biolabs, US biologicals, Calbiochem). When α-mannosidase is obtained as an ammonium sulfate precipitate, is should be dialyzed against 50 mM sodium acetate buffer, pH 5.0, 0.1 mM zinc acetate prior to use. The washed immunoprecipitates are resuspended in 10–50 μl of 10 mM Hepes buffer, pH 7.4, 0.5% SDS, 2 mM dithiothreitol and heated at 95 °C for 10 min. After a short centrifugation, 1–5 μl of 10% Nonidet-P40 is added to quench the SDS, and sufficient GII, Endo H, Endo F, *N*-glycanase or α-mannosidase is added (usually in 1 μl). For α-mannosidase digestion, about 2–10 μl of 500 mM sodium acetate buffer, pH 5.0, 0.1 mM zinc acetate should be added. When appropriate, GII digestion can be performed before α-mannosidase to evaluate further the presence of glucosylated *N*-glycans. The samples are resuspended and incubated for 60–120 min at 37 °C. Reactions are stopped by addition of SDS–PAGE sample buffer and analyzed by electrophoresis and autoradiography.

5.2. Direct analysis of N-glycans

5.2.1. Labeling of N-glycans

The most common way of radiolabeling *N*-glycans is by incubating cells in growth media containing radioactive Glc, Man or Gal [73]. To achieve the most efficient labeling, cells need to be depleted from unlabeled intermediates as nucleotide sugars and Dol-P derivatives. This can be done by incubating them in media devoid of those sugars for 15–30 min. If glucosidase and/or mannosidase activities need to be inhibited, suitable compounds (castanosperine or 1-deoxynojirimycin to inhibit glucosidases, or kifunensine and 1-deoxymannojirimycin to inhibit mannosidases) may be added at the onset of the starving period at ≈1 mM, as those inhibitors penetrate rather slowly into cells. Labeling is bet performed in media containing 2–5 mM Glc, as lower concentrations may result in synthesis of truncated Dol-P-P derivatives and the formation of non-physiological protein-linked glycans. When there are sufficient raiolabeled precursors, pulses can be kept short (5 min or less) and consequently *N*-glycan processing can be followed during early stages of glycoprotein biosynthesis.

When [U-^{14}C]Glc is used, radioactivity is not only incorporated onto Glc residues. [U-^{14}C]Glc can be converted in the cells into Man and GlcNAc (among other sugars), leading to Glc, Man, and GlcNAc labeled *N*-glycans. Cells also convert Glc rapidly into labeled lipids and amino acids. As a consequence, glycans have to be purified extensively to avoid detection of radioactivity in other molecules.

Glucose residues on *N*-glycans can also be labeled with radioactive galactose, since entrance of galactose to general metabolism requires the conversion to Gal 1-P and then to UDP-Gal and UDP-Glc, which results in the labeling of *N*-glycans with very efficiently. As a consequence, when cells are radiolabeled with [^{14}C]Gal or [^{3}H]Gal in the presence of 1–5 mM glucose the label goes almost exclusively to glucose and/or galactose residues [34,73–75].

When *N*-glycans are labeled with [2-^{3}H]Man, radioactivity is confined to Man and fucose residues only, since interconversion of Man into other sugars requires oxidation of the –OH group at position 2, and therefore radiolabeled glycans retain the label exclusively in the Man or fucose. Both Glc and Man internalized by cells can be incorporated into glycoproteins following incorporation into UDP-Glc or GDP-Man and further transfer to Dol-P-Glc or Dol-P-Man [76–78].

5.2.2. Extraction of protein-bound N-glycans

After the pulse/chase, cells are extracted by suspension in chloroform/methanol/water (3:2:1) (this is best

done in a glass tube, 12 × 75 mm). After vigorous mixing the sample is centrifuged at 3000*g* for 5 min. A proteinaceous interphase that forms between two liquid phases is carefully recovered discarding both the upper and lower liquid phases. The pellet is further washed twice with chloroform/methanol/water (3:2:1), followed by further washes with chloroform/methanol/water (10:10:3) to extract Dol-P-P-glycans (which can be saved for further analysis). The washed proteinaceous pellet is then digested exhaustively with Pronase (2 mg/ml, Sigma) for 24 h in 1 mM $CaCl_2$, 200 mM Tris–HCl buffer, pH 8.0. In some cases, it may be beneficial to predigest the pronase solution for 30 min at 37 °C before adding it to the test sample, thus allowing for the destruction of potential glycosidase activities in the pronase preparation. The digestion converts most of the insoluble pellets into amino acids, short peptides, and glycopeptides. The digest is cleared by centrifugation (10,000*g* for 5 min) and the supernatant is desalted on a Sephadex G-10 column (2 × 60 cm) equilibrated and run in 7% 2-propanol, in which the glycopeptides are excluded and separated from most amino acids and monosaccharide in the hydrolysate. The isolated glycopeptides are dried and resuspended in 50 mM sodium acetate buffer, pH 5.5, and digested with Endo H for 18 h. Tubes are boiled for 5 min and then passed over an Amberlite MB3-acetate column (0.5 × 5 cm) to retain amino acids and other charged contaminants. The neutral glycans (released by Endo H digestion) are recovered in the flow through of the ion exchange column and dried. They can be analyzed by a number of chromatographic techniques.

5.2.3. Chromatographic analysis of N-glycans

The most simple and arguably the one with highest resolving power is descending paper chromatography in 1-propanol–nitromethane–water (5:2:4) [79]. Another simple alternative is thin layer chromatography on silica plates [80]. *N*-glycans can also be analyzed by FACE (Gao, this volume) or by HPLC, which requires dedicated equipment but provides good resolving power for free glycans, either without modification [81] or after derivatization with 2-aminopyridine [82], 2-aminobenzamide [83,84] or perbenzoylation [85].

6. Concluding remarks

A role for carbohydrate processing in glycoprotein folding in vivo is now well established, and the precise mechanisms by which they operate are being elucidated. The way in which glycoprotein conformation is evaluated by GT is now being dissected in vitro using a broader range of substrates with increasing level of refinement. The contribution of reglucosylation to the biogenesis of specific glycoproteins in vivo continues to be a challenging task, but the integration of genetic, cell biological, and biochemical approaches will bring a better understanding, and potential avenues of manipulation of glycoprotein biogenesis.

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