A Tight-Knit Group: Protein Glycosylation, Endoplasmic Reticulum Stress and the Unfolded Protein Response

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Abstract

Although the dependence upon glycosylation for protein folding and function is understood to be a key part of protein maturation within the endoplasmic reticulum (ER), details concerning the interconnected nature of pathways associated with protein glycosylation, ER stress and the unfolded protein response are only now beginning to come to light. Changes in glycosylation may induce ER stress or may be induced by ER stress. It has been established that glycosylation and ER stress are essential in a variety of cellular processes and diseases. *N*-linked glycosylation within the ER is necessary for monitoring the state of protein folding and the state of glycosylation in the ER is a determinant for further processing of proteins in Golgi or destruction of improperly folded proteins in the ER associated degradation (ERAD) process. This chapter explores the interdepen-

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dence of ER stress and glycosylation during protein quality control and cellular response to physiological stress.

Keywords

Golgi · Endoplasmic reticulum · *N*-glycan · *O*-glycan · Biopharmaceutical · Oligosaccharide · Lectin · Glycosidase · Chaperone · Glycosyltransferase · Calnexin · Calreticulin

Abbreviations

2dGlc	2-deoxy-Dglucose
Asn	Asparagine
BiP	immunoglobulin heavy chain-Binding Protein
CMPST	CMP-sialic acid transporter
DTT	Dithiothreitol
eIF2α	eukaryotic Initiation Factor-2α
ER	Endoplasmic Reticulum
ERAD	ER Associated Degradation
ERManI	ER α1–2 mannosidase I
EMC	ER membrane protein complex
GalNAc	N-acetyl-galactosamine
GDPFT	GDP-fucose transporter
Glc	Glucose
GlcNAc	N-actylglucosamine
GRP78	Glucose Regulated protein 78
HA	influenza hemagglutinin A
Man	Mannose
Man2C1	cytoplasmic α-mannosidase
MANEA	Golgi resident endomannosidase
PTMs	Post Translational Modifications
TM	Tunicamycin
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UPR	Unfolded Protein Response
XBP1	XBP1

1 Introduction

In eukaryotic cells, the majority of proteins are modified during or soon after translation. These covalent modifications are collectively called post-translational modifications (PTMs) and serve to provide an additional level of regulation for proteins as well as to allow selective participation in multiple processes [1–3]. One of the

most prevalent PTMs is glycosylation which is the attachment of oligosaccharide structures (glycans).

With few exceptions, glycosylation of proteins occurs in the eukaryotic secretory pathway and is carried out in discrete biosynthetic steps divided between the endoplasmic reticulum (ER) and Golgi apparatus [4–9]. Unlike nucleic acids and proteins, glycan structures are not directly determined by genes, are not synthesized in a template-based manner, and may be linear or branched in structure. In some instances, glycans may be further modified by acetylation, sulfation or phosphorylation.

Glycosylation is also a fundamental part of the ER's protein quality control system, which sorts improperly folded proteins for systematic recycling. The secretory pathway, beginning with the ER and ending at the *trans*-Golgi, is charged with the delivery of properly folded and glycosylated proteins to the cell surface and this activity is vital in the development and homeostasis of all eukaryotes as well as cell-to-cell communication within multi-cellular organisms [3, 5]. Glycan moieties on glycoproteins are also involved in a wide array of functions ranging from increasing the solubility and stability of proteins to extending their circulatory half-life in the serum and also have roles in most chronic and infectious diseases [10]. It is therefore unsurprising that glycosylation pathways are profoundly influenced by ER stress [11]. Situations, which induce ER stress contribute to the altered regulation of pathways associated with the unfolded protein response (UPR), endoplasmic reticulum associated protein degradation (ERAD) and protein secretion as well as underlying levels of transcription and translation [12, 13].

The purpose of this chapter is to introduce the process of glycosylation and explore some of the recent studies and findings instrumental in connecting observed changes in glycosylation with respect to ER stress, ERAD and the UPR.

2 The *N*-linked Glycosylation Process

Oligosaccharides attached through the amine of asparagine (Asn) within a polypeptide chain are termed *N*-glycans. Because *N*-linked glycosylation begins while the polypeptide chain is still being folded in the ER and continues in the Golgi after the protein is folded, it is a co-translation as well as post-translational modification. The initial steps of *N*-linked oligosaccharide biosynthesis in the ER are similar in all eukaryotic cells. However, there are distinct differences in the processing and elongation of *N*-glycans in the Golgi complex which lead to organism-specific oligosaccharide chains (glycoforms).

N-linked glycosylation requires the consensus amino acid sequence Asn-X-Ser/Thr within nascent polypeptide chains for attachment where X can be any amino acid except proline. Transfer of the fourteen-sugar structure consisting of glucose (Glc), mannose (Man) and *N*-acetylglucosamine (GlcNAc) as a complete, preformed oligosaccharide unit ($Glc_3Man_9GlcNAc_2$) from an ER-membrane lipid do-

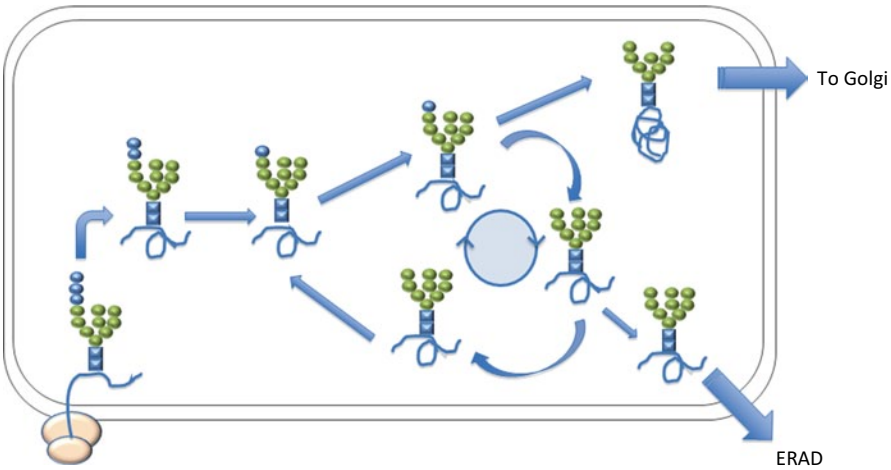

Fig. 1 N-Linked glycosylation process in the ER. GlcNAc; Man; Glc; calnexin and calreticulin mediated protein folding and reglucosylation by UGGT

nor to the sequon marks the initiation of *N*-linked glycosylation [9, 14]. Further steps of *N*-glycan processing in the ER (Fig. 1) are catalyzed by specific glycosylhydrolases (glycosidases) and glycosyltransferases. Glucosidase I removes the first Glc residue (furthest from the reducing end of the oligosaccharide) and glucosidase II removes the second Glc residues from the oligosaccharide chain. During this process two chaperon proteins, the soluble calreticulin and the membrane-bound calnexin, play a critical role in assessing the state of protein folding prior to allowing further glycosylation modification. Both calreticulin and calnexin are carbohydrate-binding proteins (lectins) and their chaperoning mechanism relies on their ability to specifically recognize the monoglucosylated oligosaccharide on the nascent protein while it remains within the ER. Normally, removal of the third and last Glc by glucosidase II is a signal of proper protein folding and exit to Golgi, however, further mechanisms ensure that misfolded proteins are not exported. For example, UDP-glucose:glycoprotein glucosyltransferase (UGGT) acts as a folding sensor by interacting with both the Glc-free *N*-glycan and the backbone of the protein being folded. If the protein is still unfolded after the removal of the last Glc, UGGT re-glucosylates the oligosaccharide chain and allows the protein another attempt at folding by associating with calreticulin and calnexin. In most cases, this cycle of deglycosylation by glucosidase II and reglycosylation by UGGT continues until the target protein is correctly folded. If it fails to fold, the target protein is sent to ERAD after association with additional chaperones such as EDEM and OS9 [15].

As properly folded proteins are prepared for export to Golgi, ER α1–2 mannosidase I (ERManI) removes one Man to generate the $Man_8GlcNAc_2$ structure, thus ensuring that the oligosaccharide can no longer be glucosylated [16]. Upon arrival in the Golgi, glycoproteins bearing the $Man_8GlcNAc_2$ structure are then further trimmed by glycosidases prior to the addition of monosaccharides which extend

the oligosaccharide chains. These sequential monosaccharide additions are carried out by sugar- and linkage-specific glycosyltransferases. In mammalian cells, *N*-linked oligosaccharide chains often possess penultimate galactose and terminal sialic acid residues at the non-reducing end (distal to the polypeptide to which they are attached). These reactions are carried out by galactosyltransferases and sialyltranferases, respectively.

3 The *O*-linked Glycosylation Process

Addition of glycans to the hydroxyl group of serine or threonine amino acid residues is known as *O*-linked glycosylation [8, 17, 18] and is thought to be an exclusively post-translational modification. Most *O*-glycosylation (initiation and maturation) of fully-folded proteins occurs entirely within the Golgi complex. Each monosaccharide is added in a sequential manner to generate linear or branched oligosaccharide structures. There are different types of *O*-linked glycosylation in eukaryotic cells but among them, addition of *N*-acetyl-galactosamine (GalNAc) to serine/threonine is the most common type in mammals which is commonly known as mucin type glycosylation [17].

4 Protein Glycosylation in Relation to ER Stress and the UPR

Together, the *N*- and *O*-linked glycosylation processes generate a very large repertoire of physiologically responsive glyco-epitopes. Even though protein folding and the influence of glycosylation during ER quality control is considered to be one of the better-understood cellular processes [11, 19, 20], elongation of *N*- and assembly of *O*-glycans in the Golgi is less completely understood and continues to complicate efforts to fully understand the complex ER/UPR/Glycosylation relationship. Only recently have the first findings concerning the effects of Golgi glycosylation on ER stress and the UPR been reported [21]. ER stress inducers that are routinely used to directly alter protein folding, such as the reducing agent dithiothreitol (DTT) or genetic mutation and resulting amino acid substitution, will ultimately alter glycosylation within the ER or suppress export to Golgi causing prolonged ER stress and activation of the UPR [22, 23]. Alternatively, agents that either block transfer of *N*-glycans to nascent proteins or inhibit completion of glycosylation, such as tunicamycin ™ derived from fungus or plant alkaloids which block glycosyltransferases, will lead to a surplus of unfolded proteins, thus creating prolonged ER stress, activation of the UPR and ultimately cell death [24, 25]. From these examples, it is clear that glycosylation will be affected in either situation and it is this duality that fogs the understanding of the specific mechanisms of glycosylation in relation to the UPR and how the occurrences of individual glycoforms are influenced by the onset and persistence of ER stress and *vice versa*.

The current state of understanding with respect to ER stress and UPR cell biology has recently been reviewed in detail (see Chap. 3). ER stress and initiation of the UPR are defined not only by the accumulation of unfolded proteins within the ER itself, but also by a surplus of incompletely glycosylated secreted proteins which are exported to expected and unexpected targets, a change in Ca^{2+}levels, occupation of resident chaperone proteins, and phosphorylation state of UPR signaling proteins [26, 27]. As these changes may ultimately manifest themselves as differential expressions of surface glycosylation, they may therefore be central to a variety of physiological phenomena [5, 22].

ER stress is associated with a wide variety of conditions and pathologies [28]. The list includes dystroglycanopathies [29], protein mis-localization and mis-trafficking [30, 31], microvascularization and tumorigenesis [32], Epstein-Barr virus replication [33], cardiovascular disease [34], diabetes [35], kidney disease [36, 37] and neuro-degenerative disorders [38], among others. It is the evidence supporting the association of ER stress with several human diseases, especially diabetes [35, 39] and cancer [40], that is largely driving interest in understanding ER stress and related mechanisms.

5 Glycosylation, ER Stress and the Biopharmaceutical Industry

The complex relationship between glycosylation, ER stress and the UPR is also an important focus of the biopharmaceutical products industry [41–45]. Many commercial therapeutic products are glycoproteins. Excessive levels of ectopic protein expression, like those encountered in large-scale biopharmaceutical production, can lead to ER stress and undesirable consequences for host cells, ultimately affecting yield and quality of recombinant products [43, 45]. Central to revealing more about ER stress, glycosylation and biopharmaceutical quality control, new analytical technologies and instrumentation have begun to enhance the ability of scientists and engineers to probe deeper into the cell's biochemistry. The enhanced measurement of genetic expression, transcription and translational controls, protein covalent modifications and secretion, could allow fine-tuning of metabolic pathways and bioenergetics to limit stress and help ensure complete glycosylation.

Regulatory agencies have increased pressures requiring companies to better understand and more tightly control bioprocesses that produce commercial biotherapeutics. As a result, the biopharmaceutical industry has increased its efforts to explore pathways which impact glycoprotein quality using systems biology approach combining transcriptomic, proteomic, glycomic and metabolomic measurements. A more thorough understanding of how inputs affect output parameters in both upstream and downstream process steps is necessary. Conventional bioreactor experiments exploring the impacts of soluble gas ratios and changing nutrients have already lead to clues about the cellular biochemical pathways affected and how these in turn influence the quality and quantity of the biotherapeutic product.

6 Recent Examinations of Glycosylation with Respect to ER Stress and the UPR

At the genetic level, there is significant overlap of key players in ER stress/UPR and glycosylation machinery [46–48]. RNA-based expression studies have been aimed at establishing links to individual secretory pathway genes for specific characteristics induced by ER stress. Glycosylation pathway genes ('glycogenes') account for approximately 5 % of the total expressed genome and ER-stress related expression changes of these are generally moderate across studies. Although the percentage and fold changes may appear small, their impact is widespread because the majority of the eukaryotic proteome is glycosylated. Altered expression of glycogenes has structural and functional implications on a large number of carrier proteins as well as lipids. Several expression studies [49–54] conducted over the past few years have aimed at establishing links to individual secretory pathway genes for specific characteristics induced by ER stress. Some more recent examples of these transcriptomic studies have examined gene expression changes associated with ER stress in fungi [55, 56], plants [57–61] and animals or cultured animal cells [45, 62]. Variations in the chemical treatments used to induce ER stress, such as thapsigargin, tunicamycin [50, 54], salubrinal [33], antimycin, buformin, metformin, phenformin, rotenone, versipelostatin [63] have produced different responses reflecting the slightly different mechanism(s) of inducing ER stress. Finally, individual cell lines may also demonstrate different responses to a single UPR modulator. For example, similar thapsigargin treatment of human medullablastoma cell lines [54] and lymphoblastoid cell lines [33] produced differing glycogene regulation (Table 1). All of these factors have contributed to a poor consensus about particular responses among existing data.

Within the network of genes related to ER stress and the UPR, there is still a number of uncertainties with respect to the necessity of individual components. A recent study of yeast by Bircham *et al.* [64] has essentially suggested that the UPR is non-essential within a range of non-optimal conditions because a wide margin of compensation mechanisms can still function to maintain homeostasis in the absence of a functioning UPR network. The ER membrane protein complex genes (*EMC*) associated with the UPR are believed to be part of the early chaperone-driven protein folding functions of the ER and four of these have been previously shown to produce UPR activation when deleted. To evaluate the necessity of *EMC* genes 1 through 6 and additional genes in the UPR, an automated confocal microscopy protocol was developed to monitor changes in localization of GFP-labeled plasma membrane proteins as a result of UPR gene deletion and these results were compared to microarray analyses. Not surprisingly, the authors discovered that yeast cells were hypersensitive to TM and DTT treatment as a consequence of removing the additional homeostatic regulation afforded by the UPR, but the mutant cells still had the ability to recover homeostasis at low doses of these ER stress agents. Specifically pertaining to glycosylation, one of the most interesting outcomes of the study was the observation that GFP-modified carboxypeptidase Y produced in *EMC1–6* double mutant cells experiencing significant ER stress had no detectable changes in glycosylation.

Table 1 Differentially regulated glycogenes involved in ER stress. Six different transcriptomic studies relevant to ER stress were retrieved from Gene Expression Omnimus (GEO, NCBI) and analyzed. Negative (−) sign in fold change columns indicates down regulated genes and blank columns are non differentially regulated glycogenes

Symbol	Description	GeneBank	Fold change							
			GSE8562 (Gomez *et al.* 2007)	GSE21979 (Pereira *et al.* 2010)	GSE2980 (Bridges *et al.* 2006)	GSE22097 (Kennedy *et al.* 2010)	GSE19519, (Dombroski *et al.* 2010)		GSE 31447, (Taylor *et al.* 2011)	
			XBP vs Control	Thapsigagrin vs Control	Exon4 mutant vs WT	hyperglycemic vs nondiabetic	Tunicamycin vs DMSO	Thapsigargin vs DMSO	Thapsigargin vs DMSO	Salubrinal vs DMSO
CALR	Calreticulin	NM_004343	1.26		−1.20	1.91	1.91	1.22		
CANX	Calnexin	NM_001746	–	1.57		−1.45	1.55	1.20	1.28	1.29
HPRT1	Hypoxanthine phosphoribosyltransferase 1	NM_000194			1.62		−1.26			
HSPA5	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	NM_005347		8.94		1.77	2.99	1.98		
OS9	Osteosarcoma amplified 9, endoplasmic reticulum lectin	NM_006812		1.82	−1.15	1.69	1.77	1.13		
RPN1	Ribophorin I	NM_002950	1.75	1.46	1.14		1.82	1.19		
USP14	Ubiquitin specific peptidase 14 (tRNA-guanine transglycosylase)	NM_005151				−1.55	1.11	1.20	1.27	

The carboxypeptidase Y expressed by the mutant yeast did, however, demonstrate a lower surface localization efficiency and a higher proportion of the tagged protein was improperly localized to intracellular membranes [64]. Because yeast naturally do not have the ability to assemble hybrid or complex *N*-glycan structures, it may be possible that similar UPR-gene deletions in higher eukaryotes would produce vastly different results with respect to glycosylation of proteins which normally display more advanced glycan structures.

In contrast, a complete and functional UPR network has been suggested to be essential for the proper induction of apoptosis in some studies, including in the case of breast cancer treatment in a mouse model, again with the inhibitor of glycosylation, TM [32]. As a potential cancer treatment mechanism, TM induction of the UPR via ER stress was shown to effectively cause tumor cell death [32]. Findings like these have turned attention toward anti-tumor strategies incorporating chemical modulation of ER stress and continued activation of the UPR in order to induce apoptosis [25, 32, 48, 65, 66, 67]. It is important to note that chemical agents which may alter glycosylation and induce ER stress, such as TM, have a more global influence on the cellular physiology and that the observations made by researchers may or may not be directly due to ER stress events.

Global feedback mechanisms controlling pathways directly associated with the building and transfer of the oligosaccharide structures as well as those governing translation of proteins which are being glycosylated work in concert. The already complex relationship between glycosylation and protein expression levels is further complicated during ER stress. When populations of oligosaccharide structures demonstrate changes in the presence of ER stress inducing chemicals, including DTT, deoxynorjirimycin, Brefeldin A or TM, defining whether the origin of change is a product of translational feedback or posttranslational feedback requires that both systems and even those which feed into them (*e.g.* glycolysis and the pentose-phosphate pathway) be monitored for their own respective changes [62, 68]. Glycoform inventory is affected not only by the control of glycosyltransporters, glycosyltransferases and glycosylhydrolases, it is also affected by the availability of substrate nucleotide sugars, and nascent polypeptides [5, 7, 69, 70]. For example, total oligosaccharide populations during ER stress may show that particular oligosaccharide structures across the cell population decrease or increase, but determining the specific origin of these changes often is not as straightforward as just being the result of up or down regulation of glycosyltransferase or glycosidase expression. The application of a systems biology approach to studies involving glycosylation, ER stress and the UPR will be necessary to fully address these issues.

Influences external to the secretory pathway are crucial to the ER, UPR, and glycosylation networks. Glycolysis, the breakdown of glucose for use as an energy source or material for anabolic processes, occurs in the cytoplasm of eukaryotic cells. Yet, treatment of immortalized cells with the glycolysis inhibitor 2-deoxy-D-glucose (2dGlc) directly impacts glycosylation and ER stress [25]. Autophagy is a cellular survival mechanism which is induced along with cell death (apoptosis) during extreme stress. In their work on autophagy and ER stress induced by 2dGlc, Xi and colleagues demonstrated that an increase in exogenous mannose had the ability to abolish autophagy and also reduce cell stress [25].

In general, ER stress results in hypoglycosylation of which observed effects include a decrease in glyco-diversity and an increase in the relative number of high-mannose structures on secreted proteins. As a result of interest in ER stress-related hypoglycosylation, ER-resident glycosidases and lectins are a popular subject of study [23, 71, 72]. There has been particular interest in identifying further details associated with the final sorting steps which ultimately decide the fate of a newly-translated and folded proteins and whether they will be exported or degraded.

The relationship between ERAD and the UPR as a result of oligosaccharide trimming by ERManI has recently been examined [23]. ERManI, as introduced at the beginning of this chapter, is responsible for trimming high mannose core *N*-linked oligosaccharide structures on newly-synthesized proteins which reside in the ER for extended lengths of time, as in situations of stalled protein export to the Golgi. HEK293 cells were transfected with a gene for α1-antitrypsin containing a mutation which causes the protein to misfold. Through pulse-chase experiments, the authors discovered that ERManI expression was stabilized during ER stress and the resulting UPR, allowing a greater percentage of misfolded α1-antitrypsin protein in the ER to enter the ERAD system for disposal and in turn reducing ER stress [64].

During ERAD, misfolded proteins are transported to the cytoplasm and tagged with ubiquitin. Proteins are then systematically deglycosylated by oligosaccharide-specific cytoplasmic amidases and glycosidases [73]. One consequence of excessive ERAD activity in response to ER stress is a cytoplasmic accumulation of neutral oligosaccharides containing glucose (these are collectively termed free oligosaccharides, or FOS) [74, 75]. By the action of select glycosidases, constituent monosaccharides (Glc, Man, GlcNAc) accumulate as well, and these directly affect both processing of precursor sugars and assembly of oligosaccharides destined for ER-localized attachment to proteins [68]. The cytoplasmic α-mannosidase, Man2C1, was shown to be a key component of feedback mechanisms related to the breakdown or accumulation of free oligosaccharide chains resulting from ERAD activity in response to ER stress [73]. Through the use of HeLa cells overexpressing Man2C1, the authors demonstrated that the regulation of such glycosidase activity is tightly connected to the *N*-linked glycosylation process. Underglycosylation of GFP-tagged Null Hong Kong α-antitrypsin protein and β1-integrin was observed in conjunction with a 2.5-fold increase in transformed HeLa cells overexpressing Man 2C1 [73].

Malectin is an ER-resident lectin discovered in an African frog and later found to be highly conserved across metazoans [15, 76]. Because of its co-localization with calnexin, malectin has been hypothesized as a part of a ‘backup’ quality control mechanism which recognizes carbohydrates structures and operates during ER stress. *In vitro*, malectin has been shown to bind to di-glucosyl oligossacharides with high affinity; however the exact role of the lectin is not yet understood. HEK293 cells did not show differential expression of calnexin as a result of increased malectin expression and there was no change in the processing of influenza hemagglutinin A (HA) in cells expressing high levels of the lectin [76]. Interestingly, the amount of HA/malectin association in the ER was affected by folding efficiency of the viral protein as fully oxidized, rapidly folding molecules showed a delayed association with malectin in comparison to partially-oxidized counterparts. Malectin was also

shown to preferentially associate with HA molecules sensitive to deglycosylation with *endo*-glycosidase H, thus further reinforcing that it is involved in recognition of specific carbohydrate structures within the ER [76].

Other mechanisms may lead to an accumulation of underglycosylated or un-glycosylated proteins in the cell. The proteasome is a protein complex dedicated to the degradation of ubiquinated proteins during ERAD which operates in the cytoplasm. Prolonged ER stress chemically induced by the application of the proteasome inhibitor MG132 to cultured cells resulted in mis-localization of immunoglobulins and type I transmembrane protein, US2, and the observation of a high percentage of these proteins lacking *N*-glycans [30].

New evidence points to the existence of additional mechanisms of protein degradation related to quality control that operate outside of the ER. In metazoans, the Golgi resident endomannosidase (MANEA) acts to remove the terminal residues (Glc-α1–2−Man α1−) attached to incomplete *N*-glycans. This mechanism is separate from the ER-resident exo-mannosidase, ERMan1, that normally operates after the removal of the final Glc. The removal of the terminal Glc-α1–2−Man α1− allows glycoproteins to escape the calnexin/calreticulin cycle and can allow even glycoproteins which are improperly folded access to Golgi-resident glycosylation processing and elongation [77]. A MANEA knockout Chinese hamster ovary (CHO-K1) cell line was transfected with an HA-tagged version of the gene for the mannosidase (HA-MANEA) and the localization of the enzyme traced to the *medial*-Golgi. HA-MANEA expression restored Golgi processing in CHO-K1 cells and reduced the overall level of FOS. However, the authors were able to detect a relative increase in FOS without Glc upon the introduction of the plant-derived ER glucosidase inhibitor *N*-butyldeoxynorjirimycin, effectively showing that a mechanism for degradation of incompletely glycosylated proteins does exist outside of the ER. These observations and the implication that a second mechanism of quality control-related degradation is active in the Golgi also point to the existence of considerable antero/retrograde trafficking of glucosylated glycoproteins between the ER and Golgi during conventional calreticulin/calnexin cycling [77].

Until recently, research aimed at ER stress and the UPR had largely ignored the effects of glycosylation carried out exclusively in Golgi. Two transporters responsible for the import and localization of nucleotide sugar substrates critical for the assembly of elongated *N*- and *O*-glycans in the Golgi were silenced (87–95%) by short interfering RNA (siRNA) in HeLa cells to study the effects on protein accumulation and resulting stress responses [21]. The study targeted a GDP-fucose transporter (GDPFT) vital for the attachment of fucose (Fuc) and a CMP-sialic acid transporter (CMPST) necessary for transfer of acetylated sialic acid (Neu5Ac) to glycans inside the Golgi stacks. As a result of the reduced completion of glycans, proteins accumulated in the Golgi which ultimately led to activation of the ER stress response (introduced by a slowdown of traffic from ER to Golgi) and the induction of UPR as indicated by phosphorylation of the eukaryotic initiation factor 2 (eIF2α), splicing of X-box binding protein one (XBP1) mRNA and BiP/GRP78 upregulation. These effects were similar to the those seen with TM treatment of HeLa cells without siRNA silencing of CMPST and GDPFT [21]. These results further

call to attention the interconnected nature of all parts of the glycosylation and secretory process in relation to ER stress and the UPR.

The complete elimination of glycosylation by mutation of glycosylation attachment residues may also induce the UPR. E-cadherin is a cell-surface glycoprotein with four reported glycosylation sites. The elimination of one of these glycosylation sites through a site-directed mutation resulted in the protein being directed to ERAD [78] most likely due to the continued exposure of hydrophobic domains near the sequon normally controlled by the presence of the hydrophilic oligosaccharide at Asn 633. The necessity of *N*-glycosylation during the folding process has been demonstrated in a variety of additional studies [31, 79–82]. Not all glycoproteins require intact glycosylation for proper folding or to move through the secretory pathway, however. One recent study of the maturation of sodium/potassium ATPase subunits in canine kidney cells included the removal of all three glycosylation sites from the β1 subunit had no effect on the localization of the protein to the plasma membrane [83]. On the other hand, the authors demonstrated that its counterpart, the β2 subunit, required glycosylation and chaperone-mediated folding for proper maturation and localization. It is important to note that while proteins may be folded and directed to the proper location, they may have lost function simply because the glycosylation is a required part of the protein's functional apparatus such as in cell communication or adhesion [3].

7 Conclusions

Cellular functions which depend upon glycosylation (and their associated pathways) and their relationship to ER stress and protein folding are just beginning to be unraveled. Work to understand the intertwined nature of the ER/UPR/Glycosylation systems has been ongoing for some time, but individual studies still tend to focus on a single part of the network irrespective of the rest, often simply as a result of experimental necessity. It is now clear that glycosylation, which is among the most important PTMs, is closely associated with ER stress, ERAD, the UPR, autophagy and apoptosis. Considerable momentum for these areas of research has been generated by the realization that ER stress and the UPR are potential therapeutic targets to fight disease [40, 48, 67]. These promising areas of research will benefit from cross-validating several experimental approaches. By bringing systems biology approaches to these fields of research, integration of information about responses across all of the connected portions of the secretory pathway and the UPR will finally become a reality.

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