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Stimulation of N-Linked Glycosylation and Lipid-Linked Oligosaccharide Synthesis by Stress Responses in Metazoan Cells

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ABSTRACT Endoplasmic reticulum (ER) stress responses comprising the unfolded protein response (UPR) are activated by conditions that disrupt folding and assembly of proteins inside the ER lumenal compartment. Conditions known to be proximal triggers of the UPR include saturation of chaperones with misfolded protein, redox imbalance, disruption of Ca^{2+} levels, interference with N-linked glycosylation, and failure to dispose of terminally misfolded proteins. Potentially, ER stress responses can reprogram cells to correct all of these problems and thereby restore ER function to normal. This article will review literature on stimulation of N-linked glycosylation by ER stress responses, focusing on metazoan systems. The mechanisms involved will be contrasted with those mediating stimulation of N-linked glycosylation by cytoplasmic stress responses. This information will interest readers who study the biological roles of stress responses, the functions of N-linked glycans, and potential strategies for treatment of genetic disorders of N-linked glycosylation.

KEYWORDS endoplasmic reticulum stress, unfolded protein response, cytoplasmic stress, congenital disorder of glycosylation

INTRODUCTION

Cellular homeostasis requires mechanisms that monitor essential processes, and then regulate reactions and pathways involved in those processes. This concept is reflected in cellular responses to stress, defined here as a rapid molecular change of physiological or pathological nature, in either the lumenal compartment of the endoplasmic reticulum (ER) or the cytoplasm. Perturbations of reducing potential, protein folding, or metabolic pathways in either of these two compartments can trigger stress-sensitive signals that result in compensatory reactions, such as chaperone synthesis due to protein folding abnormalities, or ATP production in response to energy depletion. This review will explore the influences of cellular stress responses upon protein N-linked glycosylation (a process intimately associated with the ER) in metazoans, but some examples from non-metazoans will be cited to emphasize important ideas and novel concepts. In earlier reviews, effects of psychological/physiological stress (Lauc *et al.*, 1998) and heat shock (Henle *et al.*, 1995) on glycosylation were examined. This review

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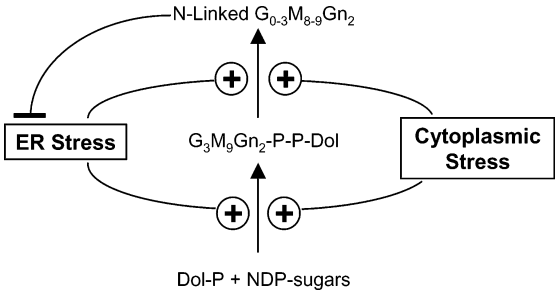

FIGURE 1 **General relationships of N-linked glycosylation to stress responses. ER stress occurs if nascent proteins are insufficiently N-glycosylated with $G_3M_9Gn_2$, or if processing of $G_3M_9Gn_2$ is impaired. Hence, N-linked $G_3M_9Gn_2$ processing intermediates can suppress ER stress. This article discusses evidence for stimulation of synthesis of $G_3M_9Gn_2$-P-P-Dol, transfer, and processing of N-linked $G_3M_9Gn_2$ by stress responses of the ER lumen and cytoplasm. Since deficiencies of N-linked glycosylation are not known to cause cytoplasmic stress, no feedback relationship is indicated.**

will be limited to stress responses for which the required components can be contained within a single cell. Work on other forms of stress (such as inflammation, acute phase, and psychological stress) will not be covered.

It is clear that N-linked glycosylation is important for ER function and maintenance of cellular homeostasis. Its interruption can result in potent ER stress and activate the unfolded protein response (UPR, discussed below). Abrogation of other ER-associated processes, especially those involved in protein folding, also trigger the UPR. Much is already known about the mechanisms used by the UPR to deal with protein misfolding. This article will focus on mechanisms by which the UPR can compensate for interference with protein N-linked glycosylation, a subject that has yet to be reviewed in depth. Evidence for modulation of N-linked glycosylation by cytoplasmic stress responses will also be addressed, even though interference with N-linked glycosylation does not cause robust stress in the cytoplasmic compartment. These paradigms are illustrated schematically in Figure 1. The subject matter requires understanding of the process of protein N-linked glycosylation, of the roles of N-glycans for protein folding in the ER, and the mechanisms of ER stress responses. This introductory chapter will cover essential aspects of these subjects.

Synthesis and Processing Asparagine-Linked (N-Linked) Glycans in the Endoplasmic Reticulum

N-linked glycosylation begins at the ER membrane with synthesis of precursor glycans attached to the carrier polyisoprenoid lipid dolichol phosphate (Dol-P), typically 95 carbons (19 isoprenyl units) in mammals. Starting with the terminal phosphate of Dol-P, a 14-sugar oligosaccharide unit is assembled in pyrophosphate linkage, composed of three residues of glucose, nine of mannose, and two of N-acetylglucosamine ($Glc_3Man_9GlcNAc_2$; $G_3M_9Gn_2$), forming a lipid-linked oligosaccharide (LLO). The linkages and anomeric configurations in $G_3M_9Gn_2$ are highly conserved (Figure 2), and with only rare exceptions (Samuelson *et al.*, 2005) $G_3M_9Gn_2$-P-P-Dol is found in all eukaryotes. Initially, at the cytoplasmic face of the ER membrane, a residue of GlcNAc-1-P is transferred by GlcNAc-1-P-transferase (GPT) from UDP-GlcNAc to Dol-P with release of UMP. GPT is selectively inhibited by tunicamycin (TN). The product GlcNAc-P-P-Dol is then modified with an additional residue of GlcNAc from UDP-GlcNAc and five residues of mannose from GDP-mannose, all of which are added by cytoplasmically-oriented enzymes. The product (M_5Gn_2-P-P-Dol) then "flips" to the lumenal leaflet of the ER membrane, and is extended to $G_3M_9Gn_2$-P-P-Dol in reactions requiring the sugar donors mannose-P-Dol (MPD) and glucose-P-Dol (GPD). These sugar-P-dolichols are themselves synthesized from Dol-P with GDP-mannose and UDP-glucose, respectively, by cytoplasmically-oriented enzymes, and then flip to the lumenal leaflet to act as sugar donors. Genetic defects in the synthesis of LLOs are responsible for the Type I congenital disorders of glycosylation (CDG) (Marquardt & Freeze, 2001; Jaeken & Matthijs, 2001). Twelve distinct genetic disorders (CDG Types Ia-Il) have been reported to date, affecting specific LLO glycosyltransferases as well as enzymes and factors involved in the synthesis and use of donor substrates. With the diversity of functions of N-linked glycans, it is not surprising that Type I CDG patients suffer from abnormalities of many organ systems.

In a reaction catalyzed by the multi-subunit enzyme oligosaccharyltransferase (OT), lumenally oriented glycans from $G_3M_9Gn_2$-P-P-Dol are transferred to nascent lumenal ER polypeptides, with concomitant release of Dol-P-P. OT transfers glycans to sterically accessible asparaginyl residues in the tripeptide context Asn-X-Ser/Thr, with X being any amino acid residue except proline, usually co-translationally. Dol-P-P is recycled to replenish the pool of cytoplasmically oriented Dol-P for additional rounds of synthesis of

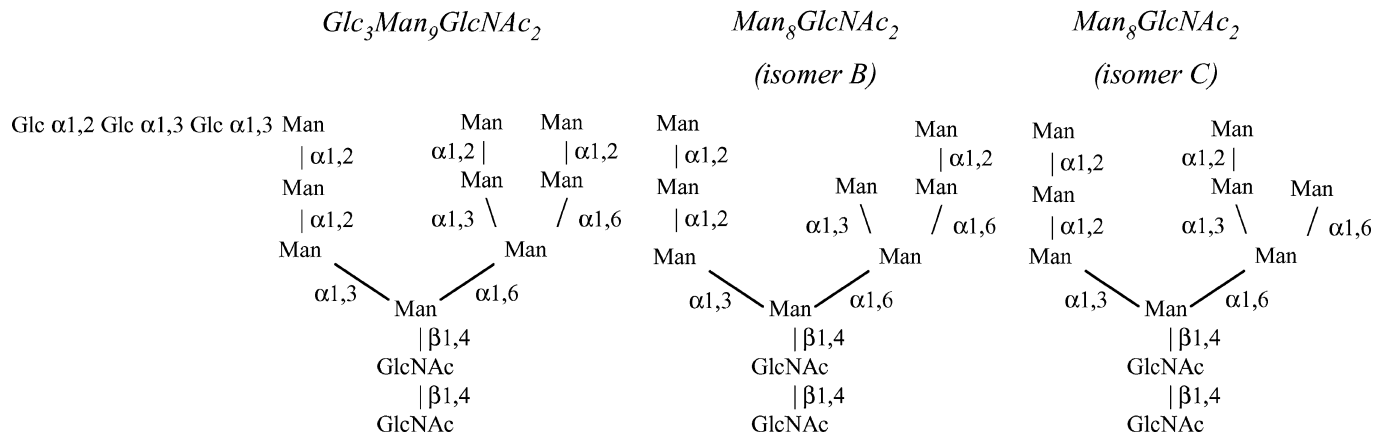

FIGURE 2 **Structures of $G_3M_9Gn_2$ and M_8Gn_2 (B and C isomers).**

$G_3M_9Gn_2$-P-P-Dol, MPD, and GPD (Schenk *et al.*, 2001; Gao & Lehrman, 2002b).

Within minutes after transfer to protein, a family of ER-resident glycosidases begins to digest the N-linked $G_3M_9Gn_2$ unit. Sequentially, the terminal glucose residue is removed by ER glucosidase I, and the remaining two glucose residues are removed by ER glucosidase II. ER mannosidase I then typically removes a single mannosyl residue (under certain conditions this mannosidase can remove multiple residues [Herscovics *et al.*, 2002]), with the resulting Man_8Gn_2 oligosaccharide, known as isomer B, depicted in Figure 2. The processing intermediates $G_1M_9Gn_2$, M_9Gn_2, and Man_8Gn_2 are particularly important for folding and processing of glycoproteins (Table 1), as will be discussed later. Cell-permeant inhibitors can block both of the ER glucosidases (castanospermine (CSN) and deoxynojirimycin (DN)) and ER mannosidase I (kifunensine (KF) and deoxymannojirimycin (DMN)). N-linked oligosaccharides normally found within the ER are classified as *high mannose-type*, given their high proportion of mannosyl residues. Glycans on proteins exported from the ER to the Golgi apparatus typically have eight mannosyl residues with no glucosyl residues. However,

TABLE 1 **Direct interactions between N-linked glycans and ER quality control components**

N-linked glycan	Role and binding partner
$G_3M_9Gn_2$ $G_2M_9Gn_2$	Binding to glucosidase II to promote its action *in trans* on proteins with multiple glycans (Deprez *et al.*, 2005)
$G_1M_9Gn_2$	Interaction with lectin-chaperones calnexin (Ou *et al.*, 1993; Hammond *et al.*, 1994; Ware *et al.*, 1995), calreticulin (Spiro *et al.*, 1996), and [a]calmegin (Ikawa *et al.*, 1997)
M_9Gn_2	Substrate on misfolded glycoproteins for UDP-Glc:unfolded glycoprotein glucosyltransferase (UGGT) (Parodi, 2000) Substrate for ER mannosidase I to initiate proteasomal ERAD of misfolded glycoproteins (Hosokawa *et al.*, 2003) Binding to ERGIC-53 for export of glycoproteins from ER (Nichols *et al.*, 1998; Moussalli *et al.*, 1999; Appenzeller *et al.*, 1999)
M_8Gn_2 (isomer B)	Substrate for ER mannosidase II to initiate non-proteasomal ERAD of misfolded glycoproteins (Cabral *et al.*, 2000; Mancini *et al.*, 2003) Binding to EDEM1 for proteasomal ERAD (Hosokawa *et al.*, 2001) [a]binding to EDEM2 for proteasomal ERAD (Olivari *et al.*, 2005; Mast *et al.*, 2005)
$M_{7-9}Gn_2$	Binding to VIP36 for polarized glycoprotein secretion (Fiedler *et al.*, 1994; Hara-Kuge *et al.*, 1999)
[b]$M_{3-9}Gn_2$	Binding to Fbs1/Fbx2 during ERAD (Yoshida *et al.*, 2002; Yoshida *et al.*, 2005) Binding to Fbs2/Fbx6b during ERAD (Yoshida *et al.*, 2003b; Yoshida *et al.*, 2005)

[a]Binding activities inferred from properties of highly related proteins.
[b]$M_{5-7}Gn_2$ glycans have been reported on ERAD substrates (Hosokawa *et al.*, 2003; Frenkel *et al.*, 2003).

in the event of export before glucosidase digestion is completed, a Golgi endomannosidase can remove any attached glucosyl residues plus the underlying mannosyl residue as a single oligosaccharide (Lubas & Spiro, 1988). These processes are similar for all eukaryotes. In contrast, remodeling of glycans within the Golgi apparatus results in extensive variations in the ultimate structures of N-linked oligosaccharides, depending upon the species, organ system, and cell type. Single polypeptides can give rise to multiple different glycoproteins (glycoforms) as a result of variations in the processing of glycans.

Historical Evidence for a Link Between N-Linked Glycosylation, Protein Folding, and ER Stress Responses

The first indication for an ER stress response, which also demonstrated the importance of protein glycosylation, appeared in the mid-1970s. Searching for novel cancer-associated factors, investigators noticed that samples of total cellular protein from virally transformed cells sometimes exhibited enhanced expression of proteins of approximately 78 kDa and 94 kDa. However, this was shown convincingly to be unrelated to the process of transformation *per se*. Rather, the appearance of these proteins was caused by depletion of glucose from the culture medium, which was accelerated by viral transformation (Pouyssegur *et al.*, 1977; Shiu *et al.*, 1977). Proteins induced by glucose deprivation were operationally designated as glucose-regulated proteins, or "GRPs," with indications that the induction was at the level of increased translatable mRNA (Shiu *et al.*, 1977). Similar results were obtained by adding glucosamine or 2-deoxyglucose to interfere with N-linked glycosylation, as well as with a mutant line defective for synthesis of GlcNAc (Pouyssegur *et al.*, 1977). Although these proteins were also induced by infection of cells with paramyxovirus (Peluso *et al.*, 1978), a class of nontransforming virus, in this particular case the induction was unrelated to depletion of glucose and instead appeared to be a response to synthesis of viral proteins.

Early ideas for GRP function included roles in metabolism or transport of glucose. However, the most abundant of these (GRP78) was shown to be identical to the immunoglobulin heavy-chain binding protein BiP (Munro & Pelham, 1986; Hendershot *et al.*, 1988). Many subsequent studies have demonstrated the role of GRP78/BiP (as well as other GRPs) as a chaperone for newly synthesized proteins in the ER, and its induction as a result of ER stress. It is thus interesting to reconsider the results of paramyxovirus infection (Peluso *et al.*, 1978), which involved a burst of viral glycoprotein synthesis expected to exceed the folding capacity of the ER. In contrast, in addition to affecting energy metabolism, one of the consequences of glucose depletion is likely to be interference with the supply of hexose precursors for synthesis of LLO (discussed later). If so, this would probably cause accumulation of truncated LLO intermediates. These are poor substrates for glycosylation of nascent ER proteins by OT, resulting in either unglycosylated proteins or glycoproteins with atypical N-glycans, which do not effectively mediate glycan-dependent steps in protein folding and quality control. This is consistent with the induction of unglycosylated GRP94 (reported as migrating as a 97 kDa polypeptide when glycosylated and a 95 kDa polypeptide when unglycosylated) preceding induction of GRP78/BiP during glucose deprivation (Shiu *et al.*, 1977). It is therefore straightforward to see how, in these seminal studies, glucose deprivation could be expected to lead to ER stress, trigger the UPR, and cause the enhanced expression of GRPs that was observed in virally transformed cultures (Pouyssegur *et al.*, 1977; Shiu *et al.*, 1977).

The general importance of N-linked glycosylation in protein folding is also apparent from studies with TN, which blocks the first step of LLO synthesis (Lehrman, 1991) to prevent protein N-linked glycosylation, as well as with CSN, which prevents processing of newly transferred $G_3M_9Gn_2$ chains by glucosidases. Although both agents interfere with protein folding, the UPR obtained with TN is considerably stronger than that seen with CSN (Doerrler & Lehrman, 1999; Shang *et al.*, 2002). This finding suggests that unprocessed N-linked $G_3M_9Gn_2$ may facilitate protein folding by physical means, such as by increasing water solubility of the nascent polypeptide. However (as discussed below and indicated by Table 1), processed forms of N-linked $G_3M_9Gn_2$ probably play the major roles in ER protein folding.

Specific Roles of N-Glycans in ER Protein Folding and Quality Control

Processes involving glycan recognition begin as glycosylated polypeptides emerge from the translocation pore. They continue until folding is achieved, and, if

necessary, the glycosylated polypeptide is assembled into multisubunit complexes. The first clear evidence for interaction between a specific N-linked processing intermediate and a component of the ER chaperone system came in the mid-1990's from a series of papers demonstrating a specific and relevant interaction between the monoglucosylated glycan $G_1M_9Gn_2$ and the chaperone calnexin (Ou *et al.*, 1993; Hammond *et al.*, 1994; Ware *et al.*, 1995). Successfully folded and assembled glycoproteins are packaged for export to the Golgi apparatus. However, terminally misfolded glycoproteins are degraded by ER-associated processes, some involving interactions of specific glycan processing intermediates on the misfolded proteins with ER glycosyltransferases and glycosidases, or with ER proteins structurally related to lectins and glycan-metabolizing enzymes. Since many excellent review articles on this subject are available, including those in this series (Anken & Braakman, 2005; Sayeed & Ng, 2005), this subject will not be reviewed here, although selected ER proteins will be discussed to illustrate specific concepts dealing with stress responses. To impress upon the reader the broad spectrum of roles of N-glycans in ER function, Table 1 lists resident proteins of the ER and ER-to-Golgi intermediate compartment (ERGIC) which have been implicated as having important interactions with specific N-linked processing intermediates.

Endoplasmic Reticulum Stress and the Unfolded Protein Response

"Unfolded protein response" (UPR) is an umbrella term that describes various ER-associated signaling mechanisms which are responsive to stress in the lumenal compartment, *i.e.*, "ER stress." Table 2 lists

TABLE 2 **Reagents used to study N-linked glycosylation and cellular stress responses**

Reagent	Relevant Target or Reaction	Effect on N-linked glycosylation pathway	Causes ER stress?[a]	Causes cytoplasmic stress?[a]
tunicamycin	inhibitor of UDP-GlcNAc:Dol-P GlcNAc-1-P transferase (ALG7)	Direct: blocks synthesis of lipid-linked oligosaccharide	Yes	No
glucosamine	multiple effects	Indirect: Inhibits multiple steps in LLO synthesis	Yes	(No)
castanospermine; deoxynorjirimycin	inhibitors of ER glucosidases I and II	Direct: prevents glucosidase processing of glycoproteins	Yes	No
deoxymannojirimycin	inhibitor of ER mannosidase I and Golgi mannosidase I	Direct: prevents mannosidase processing of glycoproteins	(Yes)	(No)
kifunensine	inhibitor of ER mannosidase I			
dithiothreitol	disulfide reductant	Indirect: via ER stress response pathways	Yes	No
thapsigargin	depletes ER Ca^{2+} by blocking the ER Ca^{2+}-ATPase			
azetidine-2-carboxylic acid	inhibits prolyl isomerization	Indirect: via ER stress response and cytoplasmic stress response pathways	Yes	Yes
geldanamycin	inhibits HSP-90 and GRP-94			
arsenite	inhibits enzymes containing vicinal thiols	Indirect: via cytoplasmic stress response pathways	No	Yes
diamide; disulfiram	sulfhydryl oxidant			
metformin	stimulation of mannose transport	Indirect: increased production of LLO biosynthesis substrates	No	No
5-aminoimidazole-4-carboxamide riboside (converted intracellulary to ribotide)	stimulates AMP-activated protein kinase	unknown		
	stimulates glycogen phosphorylase	Indirect: increased production of LLO biosynthesis substrates		

[a] responses in parentheses indicated anticipated result

many of the reagents used to induce the UPR. The discovery and characterization of the proteins that mediate the UPR have been recently reviewed (Rutkowski & Kaufman, 2004; Schroder & Kaufman, 2005). Briefly, in metazoans there are three types of ER stress sensors, IRE1α/β, PERK, and ATF6α/β. Each is an ER resident single-transmembrane span protein, and in their quiescent states their lumenal stress-sensing domains are bound to GRP78/BiP. Interactions of nascent polypeptides with ER chaperones are normally transient, but associations with malfolded proteins are long lasting. Thus, an excess of malfolded protein results in a decrease in free (unassociated) GRP78/BiP, creating a signal for ER stress. This promotes dissociation of GRP78/BiP (possibly by dynamic competition) from the lumenal stress-sensing domains of IRE1α/β and PERK, which then dimerize or oligomerize, respectively, through homotypic interactions (Bertolotti *et al.*, 2000). Consequently, their cytoplasmic kinase domains cross-phosphorylate and become activated. The cytoplasmic domain of IRE1 also has an RNAase function which initiates regulated splicing of pre-existing mRNA encoding the transcription factor XBP1 in metazoans, or in yeast, the analogous mRNA for HAC1. This favors synthesis of active XBP1 protein, which then enters the nucleus and induces transcription of UPR-responsive genes. In contrast, the activated PERK kinase domain attenuates translation by phosphorylating eIF2α, effectively reducing ER stress by lightening the load on the protein-folding and degradation machinery. This translation attenuation affects the synthesis of most proteins, but translation of mRNA encoding the transcription factor ATF4 is actually stimulated under such conditions. ATF4 then initiates a transcriptional program for genes involved in the integrated stress response (Harding *et al.*, 2003).

ATF6α/β appear to be activated by a different mechanism. The lumenal domain of ATF6 binds GRP78/BiP, but dissociation of GRP78/BiP subsequent to ER stress likely involves an active mechanism based upon control of ATP hydrolysis, rather than competitive binding of GRP78/BiP by misfolded protein (Shen *et al.*, 2005a). After dissociation of GRP78/BiP, ATF6$\alpha\beta$ do not dimerize, but instead exit the ER and are cleaved by the S1P/S2P protease system of the Golgi apparatus (Ye *et al.*, 2000; Shen *et al.*, 2002). This releases the ATF6 cytoplasmic domain, which enters the nucleus and stimulates transcription of UPR-dependent genes. Thus, all three types of ER stress sensors are able to initiate transcriptional programs. One layer of complexity derives from the ability of the ATF6 cytoplasmic domain to activate transcription of the XBP1 gene (Lee *et al.*, 2002).

Curiously, ATF6 itself senses conditions that limit N-linked glycosylation of proteins, causing it to activate UPR-responsive promoters more efficiently (Hong *et al.*, 2004). After thapsigargin (TG)-induced ER stress, an electrophoretically faster-migrating variant of the ER (uncleaved) form of ATF6 was identified that retained sensitivity to endoglycosidase H, consistent with an ER location. This form, designated ATF6(f), was missing an N-glycan at Asn^{643} of the lumenal domain, and was formed by hypoglycosylation of newly synthesized ATF6, rather than by deglycosylation of pre-existing ATF6. By mimicking ATF6(f) with a $Thr^{645}Ile$ mutation, $ATF6^{T645I}$ had greatly reduced association with calreticulin (although associations with calnexin and GRP78/BiP were not diminished). Compared with native ATF6, under unstressed (constitutive) conditions $ATF6^{T645I}$ had accelerated transit to the Golgi apparatus (possibly due to reduced ER retention by calreticulin), more S2P-dependent formation of cleaved/nuclear $ATF6^{T645I}$, and greater activity toward the GRP78/BiP promoter. With ER stress, the activities of native ATF6 and $ATF6^{T645I}$ were comparable (*i.e.*, ATF^{T645I} had greater basal activity but similar maximal activity). ATF6 has two other N-linked glycosylation sites, and hypoglycosylation caused by mutating these sites also increased GRP78/BiP promoter activity in unstressed cells. These results suggest that ATF6 and its role in the UPR may be particularly sensitive to conditions that interfere with N-linked glycosylation. By extension, this implies the existence of physiological or pathological conditions that limit protein glycosylation.

PERK-dependent translation attenuation is a post-translational event occurring within minutes of an appropriate ER stress signal, while transcriptional effects of the UPR require several hours to fully manifest. Robust ER stress can initiate apoptotic programs (Xu *et al.*, 2005), presumably in cases where activation of transcriptional programs and other facets of the UPR are not sufficient to restore ER function to normal. As will be discussed, stimulation of N-linked glycosylation requires mild ER stress, and involves transcriptional controls as well as apparently novel and relatively rapid forms of regulation.

N-LINKED GLYCOSYLATION STIMULATED BY ER STRESS: REGULATION?

Rationale for Regulation of the LLO Pathway

Sections below will discuss evidence that ER stress results both in increased synthesis of glycosylation factors and in metabolic conditions expected to favor LLO synthesis and N-linked glycosylation. However, as a prerequisite it is pertinent to consider whether any such enhancements would represent true regulation or would simply be manifestations of artificial experimental systems. In other words, is anything known about the LLO pathway or N-linked glycosylation to suggest that regulation is actually needed? Classical studies of the LLO pathway with many different normal metazoan cell lines, using defined culture media and metabolic labeling techniques, have consistently given a picture of very robust synthesis of the pathway's final product, $G_3M_9Gn_2$-P-P-Dol, with only small quantities of LLO intermediates being present. Similar results were obtained for LLOs in CHO-K1 cells and dermal fibroblasts using fluorophore-assisted carbohydrate electrophoresis (FACE), a non-radioactive and quantifiable technique for steady-state LLO analysis (Gao & Lehrman, 2002a). FACE showed that $G_3M_9Gn_2$-P-P-Dol represented about 70% of the LLO pool in these cells. Together, these results suggested that the LLO pathway is highly robust and operates at maximum efficiency at all times, and therefore is unlikely to be under careful regulatory control. In contrast, analyses of animal tissues–canine pancreas by HPLC (Badet & Jeanloz, 1988; Gibbs & Coward, 1999), and multiple mouse tissues by FACE (Gao & Lehrman, 2002a; Cho *et al.*, 2005)–show that the LLO pathway in a physiological setting does not operate as efficiently. Depending upon the tissue, $G_3M_9Gn_2$-P-P-Dol can be less than 50% of the LLO pool, with LLO intermediates being abundant. This finding suggests that LLO synthesis may be carefully regulated in tissues, balancing the steady-state accumulation of intermediates. That is, the LLO pathway does not seem to operate at maximum efficiency, but still generates amounts of $G_3M_9Gn_2$-P-P-Dol that are adequate for N-linked glycosylation.

This point is reinforced by analyses of LLO synthesis in CDG-I fibroblasts. Regardless of the genetic defect, CDG-I patients are never homozygous nulls, and therefore have at least one partially active allele. Thus, all CDG-I cells all express some residual activity from the defective locus, and would be expected to produce some $G_3M_9Gn_2$-P-P-Dol. For example, LLO analyses done with physiological glucose by FACE (Gao *et al.*, 2005) show that appreciable amounts of $G_3M_9Gn_2$-P-P-Dol are still made by CDG-Ib and CDG-Ic fibroblasts. This suggests that there are two thresholds for $G_3M_9Gn_2$-P-P-Dol, a lower one necessary for fetal viability and infantile life, and an upper one needed to achieve full N-linked glycosylation and normal health. It is therefore reasonable to anticipate that regulatory processes exist to keep $G_3M_9Gn_2$-P-P-Dol from falling below the upper threshold. If so, what mechanism constitutes the "thermostat," and what condition determines the "set-point"? Are either altered in disease? As reviewed below, the thermostat is proposed to be the UPR, and the set-point is proposed to be the extent of $G_3M_9Gn_2$-P-P-Dol synthesis needed to achieve full protein glycosylation, in order to support glycan-dependent functions in ER protein folding, quality control, and ERAD (Figure 3).

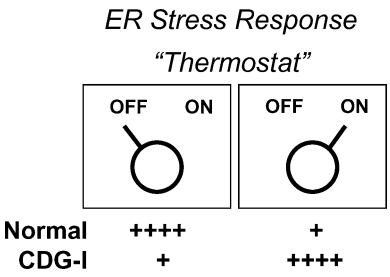

FIGURE 3 **Analogy of an ER stress "thermostat" in Congenital Disorder of Glycosylation Type I. The UPR is likened to a thermostat. Due to the LLO synthesis defect in CDG-I cells, the thermostat is "ON" a greater fraction of the time than for normal cells. This model is consistent with evidence of chronic ER stress in CDG-I cells.**

Evidence for Upregulation of the LLO Biosynthetic Pathway from Microarray Experiments

Several studies have identified UPR-dependent genes with microarray techniques, and a few cases reported increased expression of genes involved in LLO synthesis, glycan transfer, or glycan processing, listed in Table 3. This list is probably an underestimate: limitations of the microarray approach can result from inadequate representation of LLO synthesis genes on

TABLE 3 **Summary of microarray data indicating regulation of LLO synthesis or N-linked glycosylation by the Unfolded Protein Response**

		Cell type, stress inducer, and reference							
					Human dermal fibroblast[a]				
Reaction product	*Gene* or protein name	*C. elegans* (Shen *et al.*, 2005b) TN	Mouse embryonic fibroblast (Lee *et al.*, 2003) TN	Human dermal fibroblast (Lecca *et al.*, 2004) TN	TN	DTT	CS	TG	*S. cerevisiae* (Travers *et al.*, 2000) TN/DTT
M6P	*PMI140*								+
M1P	*PMM2*	+							
GlcN6P	Gln-Fru-6-phosphoamido transferase	+			+			+	
GDP-Man	GDP-man pyrophosphorylase A	+							
GDP-Man	GDP-man pyrophosphorylase B	+			+	+		+	
Dol-P	*SEC59*								+
GPD	*ALG5*			+					
MPD	*DPM1*								+
GlcNAc-P-P-Dol	*ALG7/GPT*		+						+
M_6Gn_2-P-P-Dol	*ALG3/RHK1*								+
M_8Gn_2-P-P-Dol	*ALG12*			+	+	+	+	+	
$G_1M_9Gn_2$-P-P-Dol	*ALG6*								+
N-linked $G_3M_9Gn_2$	*OST1*			+					
	STT3			+					
	OST2								+
	OST3								+
	SWP1								+
	WBP1/OST48	+							+
N-linked $G_2M_9Gn_2$	Glucosidase I		+						
N-linked $G_1M_9Gn_2$	UGGT1			+					
N-linked M_8Gn_2	*MNS1*								+
n/a	*RAMP4*		+						
n/a	*EDEM*	+	+	+					

[a]Data deposited by author with Consortium For Functional Glycomics. (http://glycomics.scripps.edu/CFGad.html).
CS = castanospermine; DTT = dithiothreitol; TG = thapsigargin; TN = tunicamycin.

microarray chips, and poor mRNA expression resulting in statistically insignificant ("absent") microarray signals. Such results should be viewed with caution unless obtained with multiple independent ER stress inducers, to rule out an adventitious effect of any single stress inducer. Preferably, results should be obtained in at least two independent studies. The only metazoan genes listed in Table 3 that meet this criterion encode glutamine:fructose-6-phosphoamidotransferase, on the pathway leading to UDP-GlcNAc; GDP-mannose pyrophosphorylase B, which synthesizes GDP-mannose; and MPD:Man_7Gn_2-P-P-Dol mannosyltransferase (ALG12), which adds the eighth mannosyl residue and forms an LLO intermediate bearing the "C" isomer of Man_8Gn_2 (Figure 2) (Helenius & Aebi, 2004). The product of ALG12 is efficiently converted to M_9Gn_2-P-P-Dol by ALG9 (Burda *et al.*, 1999; Frank & Aebi, 2005). ALG12 is interesting because glycosidic processing of N-linked $G_3M_9Gn_2$ to Man_8Gn_2 isomer "B" is thought to mediate interaction of misfolded

glycoproteins with the ERAD factor EDEM1 (itself a *bona fide* UPR-regulated gene [Yoshida *et al.*, 2003a]), and probably with EDEM2 (also UPR-regulated [Olivari *et al.*, 2005]). However, at least in *S. cerevisiae*, N-linked Man_7Gn_2 glycans derived from the acceptor substrate of ALG12 apparently cannot mediate ERAD (Jakob *et al.*, 1998) or interaction with Yos9p ERAD complexes (Szathmary *et al.*, 2005). Regulated co-expression of ALG12 and the EDEMs by the UPR would therefore help ensure the efficiency of this aspect of ER function.

Experiments Investigating Roles of Specific Factors in UPR Regulation of N-Linked Glycosylation

Although a positive result in a microarray study may implicate a role for a gene product in the UPR, measurements of the protein and its biochemical activity are necessary to show that the increase in mRNA does increase functional activity. Consequently, if an enzyme does not catalyze a rate-limiting step, or its synthesis is limited by translational controls, it is unlikely that transcriptional activation alone will result in enhanced function. Notable examples of genes involved in LLO synthesis or N-linked glycosylation, for which UPR-dependent transcription or biochemical activity has been reported, are discussed below.

PER5/RFT1

By a novel colony-sectoring approach, mutagenized *S. cerevisiae* were selected for cells which required a functional UPR to maintain viability (Ng *et al.*, 2000). Thus, the screen was designed to identify essential genes that were involved in ER function, with activities diminished by mutation and compensated by UPR activation. The role of the UPR in such mutants was verified by a requirement for IRE1 for viability. This approach yielded 16 *per* complementation groups, including 5 associated with N-linked glycosylation defects and 2 with GPI anchoring defects. This reaffirmed the requirement for these two forms of glycosylation for efficient ER quality control in *S. cerevisiae*, suggested by a prior microarray study (Travers *et al.*, 2000). One complementation group in particular, *per*5, was selected for more detailed analysis due to the severity of its N-linked glycosylation defect. The open reading frame of the *Per5* gene revealed identity with a previously identified gene of unknown function, *Rft1*. *Rft1* mRNA was induced 2.5-fold after TN-induced ER stress. Induction required IRE1, *i.e.*, a functional UPR signaling pathway, demonstrating that TN induced *Per5* because it was a true UPR-responsive gene (Ng *et al.*, 2000). This ruled out the alternative possibility that, as a likely N-linked glycosylation factor, *Rft1* was induced because it responded directly to diminished LLO or N-glycans caused by TN treatment. M_5Gn_2-P-P-Dol was accumulated, and $G_3M_9Gn_2$-P-P-Dol was sharply diminished, in the LLO pool of the *per5* mutant (Ng *et al.*, 2000), but only mature carbohydrates were found on protein, consistent with only $G_3M_9Gn_2$-P-P-Dol being oriented in the ER lumen and available for protein glycosylation. This finding led to the intriguing hypothesis that RFT1 might be involved in cytoplasm-to-lumenal flipping of M_5Gn_2-P-P-Dol in the ER membrane, with direct support provided in a subsequent study (Helenius *et al.*, 2002). Since highly similar open-reading frames for *Per5/Rft1* exist in metazoan genomes, these are likely to encode homologues with analogous functions.

PER9/COD1

Per9 was another gene identified in the aforementioned *S. cerevisiae* screen (Ng *et al.*, 2000) was later found identical to the *Cod1/Spf1* gene (Vashist *et al.*, 2002). Although moderately similar open-reading frames (such as *C.elegans* NM_069764.3; *H. sapiens* AK056420.1) are found in metazoan genomes, it is not clear whether true metazoan homologs of COD1 exist. COD1 appears to encode an ER-resident P-type ATPase with a role in Ca^{2+} homeostasis. COD1 mRNA is increased after TN-induced stress. A functional UPR involving the IRE1 pathway was required because induction was abrogated in a *hac1* mutant. Incorporation of [^{35}S]-labeled amino acids into COD1 was also increased with TN treatment. In mutant cells lacking COD1, ER-associated degradation was hindered for a form of carboxypeptidase Y (CPY*) which has a $Gly^{255}Arg$ mutation that prevents folding. Further, extension of N-glycans on CPY and both N- and O-glycans on Gas1p was diminished. ERAD of CPY* is greatly enhanced with an active ER mannosidase (product of the *Mns1* gene), which catalyzes conversion of M_9Gn_2 N-glycans to Man_8Gn_2, and mannosidase activity in the ER is Ca^{2+}-dependent (Kuznetsov *et al.*, 1993). These authors therefore tested whether loss of COD1 might result in diminished ER

mannosidase function, by examining the N-glycans on total newly synthesized glycoproteins. Wild-type and COD-1 deficient *S. cerevisiae* were labeled briefly with [^{3}H]-mannose, the glycoprotein fractions were recovered, and N-glycans were fractionated by HPLC. While a glycan eluting as $Man_8Gn_2$was the predominant N-glycan in wild-type cells, an equal mixture of glycans eluting as $Man_8Gn_2$and M_9Gn_2 was observed in the *cod1* mutants. In a *cod1/pmr1* double mutant (see below), the M_9Gn_2-like glycan was more abundant than the Man_8Gn_2 glycan. These results suggested that *Cod1*, a UPR-responsive gene, promotes mannosidase processing of M_9Gn_2 in *S. cerevisiae* (consistent with its detection in a screen for UPR function [Ng *et al.*, 2000] and diminished ERAD of CPY* in *cod1* mutants). However, since the oligosaccharide structures were assigned solely upon chromatographic elution, there is a formal possibility that $G_1Man_8Gn_2$ rather than M_9Gn_2 accumulated in *cod1* mutants, as these two glycans would be expected to elute similarly. Since *S. cerevisiae* lacks UGGT (see below), accumulation of $G_1Man_8Gn_2$ could occur if glucosidase II (instead of ER mannosidase) was dependent upon COD1. This, in turn, might be consistent with the observation of diminished Golgi processing of N-glycans on CPY and Gas1p (although it would not explain the diminished processing of Gas1p O-glycans), and slower ERAD of CPY*. These possibilities could be resolved by assessing the products of digestion of the putative M_9Gn_2 glycan with jack bean α-mannosidase, or by distinguishing between accumulation of Man_9Gn_2 or $G_1M_9Gn_2$ in a *cod1/mns1* double mutant. In any case, it is clear that COD1 significantly influences N-glycan processing (Vashist *et al.*, 2002), and its action is enhanced by activation of the UPR (Ng *et al.*, 2000). The COD1-deficient phenotypes regarding N-glycan processing, CPY* degradation, and Golgi processing of CPY and Gas1p are all mimicked by disruption of PMR1, also a P-type ATPase involved in transport of divalent cations. These phenotypes are more severe in *cod1/pmr1* double mutants, suggesting additive functions of the two proteins. In contrast to *Cod1*, the *Pmr1* gene is not UPR-responsive (Vashist *et al.*, 2002).

UDP-Glucose: Glycoprotein Glucosyltransferase

UDP-glucose: Glycoprotein Glucosyltransferase (UGGT), which is found in most eukaryotes (*S. cerevisiae* is a notable exception), is a resident ER enzyme that attaches a single glucose residue to the high-mannose glycans (typically Man_9Gn_2) of glycoprotein folding intermediates (Parodi, 2000; Caramelo *et al.*, 2003; Caramelo *et al.*, 2004). In this reaction, the $G_1M_9Gn_2$ recognition marker for the ER lectin-chaperones calnexin (Ware *et al.*, 1995) and calreticulin (Spiro *et al.*, 1996) is generated to direct transiently misfolded (normal) and terminally malfolded (abnormal) glycoproteins to calnexin and/or calreticulin for additional folding opportunities. Consequently, interference with UGGT function by disruption of its gene, by interference with the synthesis of the high-mannose acceptor glycan, or by depletion of UDP-glucose would likely cause ER stress. Conversely, UGGT function in *S. pombe* is important for resistance to ER stress (Fanchiotti *et al.*, 1998).

ER stress stimulates synthesis of at least two mRNAs that might aid UGGT function. First, expression of UGGT mRNA itself is stimulated by ER stress. In *S. pombe* (Fernandez *et al.*, 1996), UGGT mRNA was increased three- to nine fold by treatments that affected Ca^{2+} homeostasis (Ca^{2+} ionophore A23187), redox potential (β-mercaptoethanol), and N-linked glycosylation (TN or 2-deoxyglucose) in the ER, and thus expected to activate the UPR. The *S. pombe* UGGT gene was also responsive to heat shock (Fernandez *et al.*, 1996). Human cells contain two UGGT-like open reading frames, designated HUGT1 and HUGT2 (Arnold *et al.*, 2000). Although mRNAs for both were broadly expressed among tissues, in transfection assays only HUGT1 was shown to encode an enzymatically active protein. Using human nucleic acid probes, the HUGT1 homologue in COS-1 (monkey) cells was induced 3- to 4-fold by ER stress caused by treatments with A23187 or TN, while the HUGT2 homologue in the same cells underwent little or no induction. In *C. elegans*, mRNA encoding UGGT was induced by the same ER stress conditions that increased expression of mRNA encoding UDA-1.

UDA-1 is lumenally oriented enzyme found in the ER and/or in vesicles of density intermediate between ER and Golgi, and is capable of hydrolysing UDP and GDP to corresponding nucleoside monophosphates. In the ER, UDP is generated as a byproduct of the reaction catalyzed by UGGT. Conversion of UDP to UMP is beneficial because UDP is a competitive inhibitor of UGGT, while export of lumenal UMP drives import of cytoplasmic UDP-glc. Expression of UDA-1 mRNA, as well as UGGT mRNA,

was enhanced three- to fourfold by 6-hour treatments (5 μg/mL TN; 7% ethanol; 16 to 25 deg. thermal shift) that triggered the UPR in *C. elegans*, but UDA-1 expression was blocked in *ire1* mutants indicating transcription was mediated by XBP1 (Uccelletti *et al.*, 2004). UDP-glc consumption and UDP production by UGGT would be expected to increase under conditions that cause protein misfolding. This suggests that UDA-1 induction may ensure efficient hydrolysis of the UDP to UMP, both to eliminate the potentially inhibitory nucleoside diphosphate and to replenish the ER pool of UDP-glc used by UGGT. In the cases of both UGGT and UDA-1 it remains to be determined how the amounts of the enyzmes themselves, and their activities, are increased by ER stress.

SERP1/RAMP4

Stress-associated endoplasmic reticulum protein (SERP1) is predicted to contain 66 amino acid residues, with a single obvious membrane-spanning region near the C-terminus. It is identical to ribosome-associated membrane protein 4 (RAMP4), a Sec61 complex interacting protein, with 29% identity to *S. cerevisiae* SecY6p. Although its distribution is consistent with ER location and it has a consensus N-linked glycosylation site, no N-linked glycosylation was detected. SERP1/RAMP4 expression is induced in mammalian cell cultures by hypoxia (alone or with reoxygenation) and other ER stresses (achieved with the calcium ionophore A23187 or TN), and in rats by cerebral artery occlusion with expression in neurons and astrocytes (Yamaguchi *et al.*, 1999). XBP1 appears to be both sufficient and necessary for ER stress induced expression of SERP1/RAMP4 (Lee *et al.*, 2003).

In cells expressing the receptor for advanced glycation end products (RAGE) or CD8, ER stresses caused by addition of TN or A23187 diminished the quantities of these membrane proteins (normally monomers), presumably due to ERAD because aggregated and ubiquitinated RAGE was also detected. These decreases in quantity were largely prevented by co-expression of SERP1/RAMP4, although expression of SERP1/RAMP4 in the absence of ER stress had no discernable effect on RAGE or CD8. This suggested that SERP1/RAMP4 is able to stabilize nascent ER membrane proteins that would otherwise misfold due to ER stress. Interestingly, in the absence of exogenous SERP1/RAMP4, if TN was removed and RAGE synthesis was followed for 3 hours by labeling with [^{35}S]-methionine, only about 5% of the immunoprecipitable RAGE appeared N-glycosylated. This atypical underglycosylation of RAGE likely indicated that residual TN remained in the cells for most of the 3-hour period, limiting LLO synthesis. Yet if cells also expressed exogenous SERP1/RAMP4, not only was more RAGE detected, but 20% to 30% of the RAGE was N-glycosylated. Since OT was reportedly not induced under these conditions, these results suggest that SERP1/RAMP4 helped to maintain nascent RAGE in a state that was amenable to N-linked glycosylation. Hence, with the LLO pool limited due to residual TN, the ability of SERP1/RAMP4 to enhance glycosylation could be detected. SERP1/RAMP4 interacted with Sec61α and Sec61β, and calnexin, perhaps to form a "rescue unit." The likely positioning of SERP1/RAMP4 at the Sec61 translocation complex, which is also tightly associated with OT (Yan & Lennarz, 2005), would be consistent with this idea.

Interaction of SERP1/RAMP4 with MHC II invariant chain also correlates with N-linked glycosylation of the latter, possibly by increasing the residence time of the nascent polypeptide at the Sec61/OT complex. Presumably, efficient co-translational glycosylation requires adequate expression of SERP1/RAMP4. The influence of ER stress was not investigated (Schröder *et al.*, 1999).

Although there is no evidence of UPR involvement, a seemingly analogous result was reported for osmotic stress of duckling salt glands (Ernst *et al.*, 1994). By allowing ducklings to drink salt water instead of pure water, salt glands differentiate, and CFTR (a transmembrane N-glycoprotein synthesized by the ER) expression increases. Curiously, CFTR from salt glands of ducklings given pure water is approximately 170 kDa, while CFTR from ducklings given 1% NaCl was approximately 180 kDa. After N-glycanase treatment, each CFTR polypeptide was reduced to 140 kDa, indicating that the size difference was attributable to salt-induced differences in N-linked glycosylation. The studies did not determine whether this was due to a greater number of glycan chains, or a change in glycan structure.

Systemic Acquired Resistance

In plants, exposure to pathogens activates a *systemic acquired resistance* (SAR) pathway which results in transcription of a group of genes under the control of the *nonexpressor of pathogenesis-related* (NPR1) transcription factor. One outcome of SAR pathway induction is

production of a set of secreted and vacuolar pathogenesis-related (PR) proteins, which mediate pathogen resistance. Curiously, gene expression profiling in *Arabidopsis* revealed that many secretory pathway genes were also activated by SAR in a specific NPR1-dependent manner (Wang *et al.*, 2005). Induction of this set of genes occurred by a UPR-like mechanism involving a common *cis*-acting promoter element designated *TL1*, and a *TL1*-binding protein that did not appear necessary for expression of PR proteins. Enhanced expression of GRP78/BiP appeared to be a direct result of SAR rather than a secondary consequence, because its mRNA increased prior to accumulation of PR proteins. This finding implies that expansion of the secretory pathway may be necessary to handle increased production of pathogenesis resistance proteins, a hypothesis supported by experiments employing disruption of particular secretory pathway genes. Notably, SAR caused NPR1-dependent increased expression, by two- to threefold, of mRNAs encoding phosphomannomutase, and the OT subunits ribophorin I (Kelleher *et al.*, 1992) and DAD1 (Kelleher & Gilmore, 1997). OT is a key enzyme for protein N-linked glycosylation in eukaryotes, and phosphomannomutase catalyzes conversion of M6P to M1P (on the pathway synthesizing GDP-mannose). Direct enzyme activity measurements remain to be performed, and since SAR can be triggered in *Arabidopsis* by exogenous application of salicylic acid, such experiments should be feasible. For technical reasons, mRNA profiling of the SAR pathway required the use of cycloheximide (Wang *et al.*, 2005). Since translation attenuation suppresses ER stress (Harding *et al.*, 1999; Harding *et al.*, 2000), it is plausible that these results underestimated the number of NPR1-responsive secretory-pathway genes. Nonetheless, it appears that plants have developed programs for enhanced expression of N-linked glycosylation enzymes that are UPR-like, yet are distinct from those in metazoans.

Experiments Evaluating Activity of the LLO Pathway in Response to ER Stress

Under normal conditions, LLO synthesis and N-linked glycosylation occur efficiently. To test the effects of the UPR, it is therefore necessary to impair the glycosylation process, and then ask whether ER stress responses can restore glycosylation efficiency.

Expression of Carboxypeptidase Y in S. Cerevisiae

CPY* was overexpressed in normal or IRE1-deficient *S. cerevisiae* to introduce ER stress, and then examined to discern whether it had acquired all four of its expected N-glycans (Spear & Ng, 2003). CPY* overexpressed in normal cells was fully glycosylated, but in IRE1-deficient cells it was extensively underglycosylated. CPY* glycoforms lacking 1 to 4 glycans exceeding the amount of fully glycosylated CPY*, in a manner highly reminiscent of CPY expressed in cells defective in LLO synthesis (Burda *et al.*, 1999). Similar results were obtained with endogenous Gas1p; underglycosylated Gas1p glycoforms accumulated in IRE1-deficient cells but not wild-type cells. This implied that overexpressed CPY* competed for the limited pool of LLO, and that the UPR (active in normal cells but disabled in IRE1-deficient cells) compensated by stimulating either LLO synthesis or OT activity. Although neither mechanism was examined directly, as listed in Table 3, UPR-responsive/IRE1-dependent *S. cerevisiae* genes have been identified for both. Since a short metabolic pulse was used to identify glycoforms, it is unlikely that they were actually de-N-glycosylated proteins that had escaped proteasomal degradation due to the absence of IRE1-dependent functions. Analogous studies with metazoans have yet to be reported, and would require consideration of the contributions of ATF6 and PERK in addition to IRE1. In particular, translation attenuation by PERK in metazoan cells would complicate comparisons of glycoforms observed in the absence or presence of ER stress.

Transient Low-Glucose Incubations of Dermal Fibroblasts

A rather different approach (used extensively in the author's laboratory) with primary cultures of human dermal fibroblasts provided the first experimental evidence for regulation of the LLO biosynthetic pathway by ER stress responses (Doerrler & Lehrman, 1999). The LLO pool in these cells is highly sensitive to the extracellular concentration of glucose. In conventional culture medium containing 5 to 10 mM glucose, the predominant LLO in dermal fibroblasts is $G_3M_9Gn_2$-P-P-Dol, as shown by analysis with FACE (Gao & Lehrman, 2002a; Gao *et al.*, 2005). However, for the widely used technique of analyzing LLOs by metabolic labeling with [2-^{3}H]mannose, it is usually necessary to drop the

glucose concentration about tenfold during the labeling step, typically to 0.5 mM. This minimizes both competition for [2-^{3}H]mannose uptake by glucose, and intracellular isotopic dilution of [2-^{3}H]mannose metabolites with glucose metabolites. Notably, as shown by both FACE and [2-^{3}H]mannose labeling, within 20 to 30 minutes in 0.5 mM glucose the LLO compositions of dermal fibroblasts can change dramatically: LLO intermediates ($Man_{2-5}Gn_2$-P-P-Dol) can predominate, with $G_3M_9Gn_2$-P-P-Dol becoming a minor species (Doerrler & Lehrman, 1999; Shang *et al.*, 2002; Gao *et al.*, 2005). As discussed previously (Doerrler & Lehrman, 1999), the magnitude of this effect varies from laboratory to laboratory and is probably dependent upon minor technical variations. Regardless, this allows LLO synthesis to be studied in a single cell-type, under conditions that are either favorable or unfavorable for LLO synthesis. In contrast to primary cultures of dermal fibroblasts, the permanent cell lines examined so far do not have this property, and produce mainly $G_3M_9Gn_2$-P-P-Dol when labeled with 0.5 mM glucose media (Doerrler & Lehrman, 1999).

Modulation of LLO Extension in Dermal Fibroblasts by ER Stress

Using conditions (glucose depletion) that caused $Man_{2\text{-}5}Gn_2$-P-P-Dol accumulation in dermal fibroblasts, application of the ER stress inducers CSN, azetidine-2-carboxylic acid, dithiothreitol (DTT), geldanamycin, or TG (Doerrler & Lehrman, 1999; Shang *et al.*, 2002) resulted in a robust shift in the LLO pool. In all cases, ER stress inducers caused $G_3M_9Gn_2$-P-P-Dol to become the most prevalent LLO. Similar results were obtained by thermal shock, typically thought to be an inducer of cytoplasmic stress, although it was shown to also induce synthesis of GRP78/BiP mRNA (Doerrler & Lehrman, 1999), a classic hallmark of ER stress (Table 2). These experiments included controls arguing against enhanced LLO extension due to stress-enhanced hexose uptake, or accumulation of $G_3M_9Gn_2$-P-P-Dol due to either an absence of functional polypeptide acceptor for OT or to OT inactivity. Glycoprotein synthesis continued during these ER stress treatments. Subsequently it was shown that these stress conditions were relatively mild because stress-induced translation arrest was minimal or undetectable (Shang *et al.*, 2002), explaining the continuation of glycoprotein synthesis. With DTT, the effects on LLO synthesis and GRP78/BiP mRNA were both mitigated by prior adaptation to DTT stress (Shang *et al.*, 2002). Prolonged (12-hour) incubation of fibroblasts in low glucose triggered the UPR (judged by GRP78/BiP mRNA levels), and was associated with increased hexose uptake. This resulted in both a return to normal rates of $G_3M_9Gn_2$-P-P-Dol synthesis, and restored transfer of $G_3M_9Gn_2$ to nascent proteins, and was attributed to the sum of the contributions of both the UPR and hexose uptake.

By applying various concentrations of the ER stress inducers DTT, TG, azetidine-2-carboxylic acid, and CSN, and carefully measuring $G_3M_9Gn_2$-P-P-Dol synthesis, GRP78/BiP mRNA synthesis, translation arrest, and apoptotic death that can result from ER stress, it was possible to prioritize each aspect of the ER stress response. Extension of LLO intermediates had the highest priority, followed by transcriptional activation, translation arrest, and apoptosis (Shang *et al.*, 2002). For example, low concentrations of DTT stimulated LLO extension without appreciable activation of the other aspects of the UPR. These results are consistent with the idea of LLO extension as part of a first-line of defense against ER dysfunction.

All of the effects of ER stress inducers on LLO extension could be observed within 1 hour of treatment. With DTT, full effects were observed after 20 minutes (Doerrler & Lehrman, 1999; Shang *et al.*, 2002). This was unexpected given the current understanding of ER stress signaling. Reactions controlled by transcriptional regulation (mediated by IRE1/XBP1, ATF6, or PERK/ATF4) should require hours to be fully manifested. This implied that the rapid enhancement of LLO synthesis could involve a novel signaling mechanism. The kinase activity of PERK is also activated on the order of minutes, but its only known substrates are eIF2α and Nrf2 (Cullinan *et al.*, 2003), regulation of which are not known to cause rapid changes in glycan synthesis. As mentioned above, ER stress conditions were identified that caused full stimulation of $G_3M_9Gn_2$-P-P-Dol synthesis, but had little or no effect on PERK-mediated translation arrest (Shang *et al.*, 2002). Unfortunately, it may not be feasible to dissect the pathways leading to enhanced LLO extension by testing inhibitors of transcription and translation. By eliminating nascent polypeptide acceptors for OT, these agents diminish the turnover of the LLO pool and, consequently, incorporation of [2-^{3}H]mannose (Gao & Lehrman, 2002b). Additionally, these compounds would be expected to lessen the load of transiently misfolded protein in the ER lumen and attenuate the effects of ER stress

inducers, similar to the role of translation arrest by PERK.

Evidence that ER Stress Modulates LLO Synthesis by Regulating Hexose Metabolism

Although ER stresses lasting no more than 1 hour could enhance LLO extension in dermal fibroblasts, they did not alter the activities of a number of microsomal LLO biosynthetic enzymes (Doerrler & Lehrman, 1999). Consequently, steps in hexose metabolism were considered as possible ER stress-responsive regulatory points (Gill *et al.*, 2002), especially steps known to be rapidly regulated by phosphorylation. This concept was supported by ER stress-induced increases of intracellular G1P and G6P (Gill *et al.*, 2002). Using DTT as the ER stress inducer, intracellular G6P increased with a time course and a DTT concentration dependence that were highly consistent with results for $G_3M_9Gn_2$-P-P-Dol synthesis (Shang *et al.*, 2002). Although TN is a widely used and specific ER stress inducer (Table 2), it could not be used in earlier studies of LLO regulation due to its direct inhibition of GlcNAc-P-P-Dol synthesis. However, TN treatment elevated intracellular G6P (Gill *et al.*, 2002), further supporting the idea that the UPR regulates hexose metabolism.

A number of enzymes involved in hexose metabolism were assayed with *in vitro* reactions using cytoplasmic extracts of dermal fibroblasts treated with DTT or TN (Gill *et al.*, 2002). For seven reactions, no effects were detected, indicating that ER stress did not cause widespread disruption of cytoplasmic metabolism. Only glycogen phosphorylase (GP), which catalyzes glycogenolysis (the conversion of glycogen to G1P in the presence of inorganic phosphate), responded to ER stress. The reaction was AMP-independent, a hallmark of the phosphorylated form of the enzyme (GPα). No activation was measured for GP in the presence of AMP (to detect both GPα and AMP-dependent GP*b*) suggesting that the total amount of GP enzyme did not increase. Direct addition of DTT to extracts did not activate GP (as expected given the reducing potential of the cytoplasmic compartment), indicating that GP activation required an ER stress effect on intact cells.

Activation of GP was consistent with the observed increases of G1P and G6P, which is generated from G1P by phosphoglucomutase. Curiously, the effects of ER stress on *in vitro* GP activity could be mimicked with unstressed cells by the addition of phosphatase inhibitors to the extraction buffer. The effects of stress inducers and phosphatase inhibitors on GP were equivalent, but not additive (Gill *et al.*, 2002). While the basis for this result remains unclear, it further implicates a role for GP phosphorylation regulated by ER stress. Direct measurements of fibroblast glycogen showed that it decreased upon treatment with either DTT or TN. The amount of hexose associated with the loss of glycogen was calculated to be in great excess over the amount of G6P detected, which in turn was in great excess over the units of hexose that would be needed to extend LLO intermediates to $G_3M_9Gn_2$-P-P-Dol (measured quantitatively by FACE). Taken together, elevation of G6P and loss of glycogen with DTT and TN treatments indicate that one or more steps in hexose metabolism are regulated by the UPR. Activation of GP is a plausible mechanism because the time course and concentration dependence for stimulation of LLO extension by DTT treatment were similar to those for GP activation. However, the effects of ER stress on LLO extension have yet to be mechanistically linked to increases in GP activity, G6P concentrations, or glycogenolysis. This might be accomplished, for example, with specific inhibitors of GP. The nature of the proximal ER stress signal affecting hexose metabolism is also unknown. For reasons discussed above, it is unlikely that the known activities of IRE1, ATF6, or PERK are involved, implicating a novel ER stress sensor.

Case Study: The ER Stress Response in CDG-I Fibroblasts

The Congenital Disorders of Glycosylation Type I (CDG-I) are inherited pediatric disorders characterized clinically by multi-system defects (notably with neurological and developmental abnormalities) and biochemically by diminished N-linked glycosylation of serum glycoproteins secreted from hepatocytes (Marquardt & Freeze, 2001; Jaeken & Matthijs, 2001). To date, 12 CDG-I subtypes, a-l, have been reported (Freeze & Aebi, 2005). Each is due to a mutation in a distinct gene responsible for either catalyzing LLO synthesis or providing donor substrates. Depending upon the specific defect, CDG-I serum proteins may be hypoglycosylated due to modification with fewer, yet structurally normal oligosaccharides, or with structurally truncated oligosaccharides. Most biochemical studies of CDG-I have used patients' dermal fibroblasts. Since many of the aforementioned studies on ER stress were

performed with normal dermal fibroblasts, it is possible to evaluate the concepts discussed above by direct comparison with CDG-I results. Specifically, aberrant LLO synthesis in CDG-I cells should result in chronic ER stress, and at the same time the ER stress response is expected to compensate for the primary LLO synthesis defects. By the analogy introduced earlier, there should be more frequent activation of the UPR "thermostat" as the CDG-I cells attempt to reach the desired LLO "set-point" needed for effective glycosylation and protein folding (Figure 3).

The first study of ER stress in CDG-I cells was performed by the author's laboratory (Shang *et al.*, 2002) with dermal fibroblasts from patients with CDG-Ia (phosphomannomutase deficient), CDG-Ib (phosphomannose isomerase deficient), or CDG-Ic (GPD:M_9Gn_2-P-P-Dol glucosyltransferase deficient). The metabolic basis for hypoglycosylation in Types Ib and Ic appears to be reasonably well understood, based upon [2-^{3}H]mannose labeling studies (Niehues *et al.*, 1998; Körner *et al.*, 1998) and by FACE (Gao *et al.*, 2005). Because the Type Ib phosphomannose isomerase defect results in less M6P formed from F6P, synthesis of GDP-mannose is decreased, and lower quantities of otherwise structurally normal $G_3M_9Gn_2$-P-P-Dol are available for N-linked glycosylation. In Type Ic, the total amount of LLO is normal, but most of it is M_9Gn_2-P-P-Dol and is therefore not a good donor substrate for OT. Hence, both cell types are useful models to evaluate the interplay between ER stress responses and LLO synthesis.

The metabolic defect in CDG-Ia appeared to be well understood based upon a series of studies with [2-^{3}H]mannose labeling of LLOs and glycoproteins, which reported accumulation of M_5Gn_2-P-P-Dol and diminished protein glycosylation. Yet, recent studies from the author's laboratory using FACE analysis have cast uncertainty upon previous models for CDG-Ia (Gao *et al.*, 2005). *A priori*, the phosphomannomutase defect in CDG-Ia would be expected to lower the amount of GDP-mannose available for LLO extension due to weaker conversion of M6P to M1P. Nonetheless CDG-Ia cells maintained in physiological glucose do not exhibit LLO synthesis (Gao *et al.*, 2005) or glycoprotein (Marquardt *et al.*, 1995; Marquardt *et al.*, 1996; Dupre *et al.*, 2000; Gao *et al.*, 2005) abnormalities. An abnormal CDG-Ia LLO phenotype is detected, however, if cells are cultured in subphysiological glucose concentrations (2.5 mM or less), as was typically done in previous [2-^{3}H]mannose labeling experiments (Gao *et al.*, 2005). Thus, in physiological glucose the phosphomannomutase deficiency is not sufficient to cause obvious LLO synthesis or glycoprotein defects in cultured CDG-Ia dermal fibroblasts. It remains an open question whether the decrease in GDP-mannose synthesis is drastic enough to interfere with LLO synthesis in CDG-Ia hepatic tissue. In light of the results with dermal fibroblasts, an effort was made to determine consequences of phosphomannomutase deficiency, other than diminished GDP-mannose, that might also contribute to hypoglycosylation in hepatocytes. Since M6P should accumulate in parallel to decreases for M1P, it was proposed that M6P might have antimetabolic properties in hepatic tissue that interfere with glycosylation. This proposal was supported by *in vitro* experiments using multiple cell types, which demonstrated that M6P (but no other hexose phosphate) is able to induce OT to "misfire," causing selective loss of $G_3M_9Gn_2$-P-P-Dol from the LLO pool and appearance of free $G_3M_9Gn_2$ (Gao *et al.*, 2005).

Since LLO synthesis and protein glycosylation are not deficient in CDG-Ia fibroblasts cultured with physiological glucose, two competing scenarios are suggested: (a) The residual phosphomannomutase activity in CDG-Ia fibroblasts is fully adequate for LLO synthesis and protein glycosylation; or (b) the residual phosphomannomutase activity in CDG-Ia fibroblasts is not adequate for LLO synthesis and protein glycosylation, but the resulting hypoglycosylation chronically activates the UPR to compensate for the phosphomannomutase deficiency. To distinguish between these two scenarios, fibroblasts from patients with CDG-Ia, Ib, and Ic were evaluated for evidence of chronically activated ER stress responses.

Initially, ER stress in CDG-Ia and Ib cells was evaluated by measurement of GRP78/BiP mRNA (Shang *et al.*, 2002). In the absence of stress treatments, these cells had normal amount of GRP78 mRNA. However, both types of cells had attenuated GRP78/BiP mRNA responses to stress induced by DTT or TN. Since these results were highly reminiscent of those obtained with normal cells that had been stress-adapted with DTT (Shang *et al.*, 2002), the CDG-Ia and Ib cells appeared to have been stress-adapted due to long term effects of their respective metabolic defects.

Further evidence of low-level, chronic ER stress was observed by direct analysis LLOs from cells

incubated 20 minutes in 0.5 mM glucose medium with [2-^{3}H]mannose, with or without prior ER stress. As discussed above, normal fibroblasts under these conditions have mostly $Man_{2\text{-}5}Gn_2$-P-P-Dol, and these are extended to $G_3M_9Gn_2$-P-P-Dol with ER stress. Interestingly, for both CDG-Ib and Ic cells under low glucose conditions, most of the LLO were fully mannosylated without prior ER stress. The small degrees of undermannosylation observed were corrected with ER stress. These results mirrored the results of GRP78/BiP analysis, suggesting that CDG-Ib and Ic fibroblasts were adapted to chronic stress.

Results with CDG-Ia cells were less straightforward, but perhaps more revealing (Shang *et al.*, 2002). Without stress, the LLOs labeled with [2-^{3}H]mannose in low glucose medium were even more severely undermannosylated than LLOs of normal cells. There was little or no improvement after addition of ER stress inducers to CDG-Ia cells. Yet, CDG-Ia cells in physiological glucose had no apparent LLO or glycosylation defects (Gao *et al.*, 2005). CDG-Ia cells maintained in medium with at least 5 mM glucose have morphologically dilated ER and delayed glycoprotein transport (Marquardt *et al.*, 1995) and, as discussed above, stress induction of GRP78/BiP mRNA in such CDG-Ia cells is attenuated, all consistent with chronic ER stress. This suggests that the CDG-Ia phosphomannomutase deficiency caused chronic ER stress, but that the stress response was sufficient to compensate for the consequences of this deficiency on LLO synthesis as long as the cells were supplied with at least 5 mM glucose. In other words, the LLO pool in CDG-Ia fibroblasts may represent a balance between inhibitory effects of the enzyme defect and stimulatory effects of the resulting stress response (consistent with scenario "b" above). Hence, the "thermostat" is "on" more frequently than in normal cells (Figure 3) and is able to achieve the desired set point if the extracellular glucose concentration is adequate. Conversely, the compensatory mechanisms did not appear sufficient to correct for phosphomannomutase deficiency in combination with the detrimental effects of glucose deprivation.

To determine what genes, especially among those related to ER stress, were activated in CDG-I, a microarray study was undertaken with fibroblasts from patients with CDG types Ic (see above), Ie (MPD synthase deficiency), or Ig (MPD:Man_7Gn_2-P-P-Dol mannosyltransferase deficiency) (Lecca *et al.*, 2004). Results were compared to those from normal cells subjected to a robust (5 μg/mL, 24 hours) treatment with TN, or to glucose starvation (24 hours). Compared with TN-treated normal cells, CDG-I cells appeared to have a qualitatively similar, though quantitatively weaker, chronic stress response. However, GRP78/BiP mRNA was not induced, consistent with the analyses of CDG-Ia and Ib cells discussed above (Shang *et al.*, 2002), and suggesting that GRP78/BiP is not a good marker for chronic ER stress, although it is an excellent marker for acute ER stress. Interestingly, the most highly induced gene in CDG-I cells was DNAJC3/P58^{IPK}, shown previously to be an inhibitor of PERK signaling (van Huizen *et al.*, 2003). Measurement of incorporation of [^{35}S]methionine into CDG-I total protein indicated no inhibition compared with normal cells. This finding suggested that, if PERK was more highly stimulated (inhibitory for protein synthesis) in CDG-I cells due to chronic ER stress, this may have been compensated by corresponding increase in the PERK-inhibiting activity of DNAJC3/P58^{IPK}. It is unlikely that PERK was completely inactive in CDG-I cells because elevated expression was also found for genes involved in amino acid synthesis and transport, which rely upon the PERK/ATF4 transcriptional arm of the integrated stress response (Harding *et al.*, 2003). CDG-I fibroblasts in medium with normal glucose had a microarray profile most similar to that of normal fibroblasts cultured 24 hours in low-glucose (0.25 mM) medium. This is an intriguing observation—as discussed above, chronic ER stress in CDG-Ia cells is most likely caused by a tendency to accumulate truncated LLOs, and incubation in low-glucose medium can cause normal fibroblasts to accumulate truncated LLOs (Shang *et al.*, 2002).

Other useful outcomes of this study (Lecca *et al.*, 2004) include evidence for transcriptional activation of several enzymes of LLO synthesis by TN-induced stress in normal cells (Table 3), though parallel studies with glucose starvation-induced stress were difficult to interpret because no increase was detected for GRP78/BiP mRNA, the hallmark of acute ER stress. The mRNA for the STT3 subunit of OT was constitutively induced (without addition of exogenous stress inducers) in CDG-I cells. However, it should not be assumed that this is a result of chronic ER stress. Rather, the results could indicate that some components of the LLO pathway are under classical transcriptional control depending upon synthesis of its product, $G_3M_9Gn_2$-P-P-Dol, which would be

diminished in all CDG-I cells and by TN treatment. These possibilities could be distinguished in future experiments by evaluating DTT and TG to specifically activate the UPR without directly interfering with LLO synthesis.

UDP-Glucose and ER Stress

The above studies pertain to regulation of steps in LLO synthesis that require GDP-mannose, directly or through the synthesis of MPD. There is also evidence for ER stress regulation of UDP-Glc metabolism. A $Gly^{115}Asp$ mutation in the gene encoding UDP-Glc pyrophosphorylase caused a loss of 65% of the cellular UDP-Glc content in a CHO-derived cell line (Flores-Diaz *et al.*, 1997). This deficit was fully corrected by transfection of a native copy of the cDNA. Compared with the parental and corrected lines, the mutant deficient for UDP-Glc exhibited enhanced expression of a number of UPR-responsive proteins such as GRP78/BiP, GRP94, and calreticulin. Yet, other UPR-responsive proteins (protein disulfide isomerase and UGGT) were not induced, and there was no evidence of activation of the UPR hallmarks of XBP1 splicing, cleavage of ATF6, induction of ATF4, or expression of CHOP (Flores-Diaz *et al.*, 2004). Further, GRP78/BiP promoter activity in the mutant was not affected by co-expression of a dominant-negative ATF6 (although this form of ATF6 was effective against TN-induced promoter activity), or by deletion of the ERSE promoter element that is capable of responding to spliced XBP1 or cleaved ATF6. These data suggest that UDP-Glc deficiency results in a restricted form of ER stress that activates a subset of UPR-responsive genes. Since neither the IRE1/XBP1, ATF6, nor PERK/ATF4 transcriptional programs seemed necessary, this implicated the involvement of a novel UPR response pathway. As noted by the authors, ER stress in the UDP-Glc deficient cell is chronic, and is likely to reflect stress adaptation. This may explain the absence of apparent activation of several of the typical UPR hallmarks. As for why UDG-Glc deficiency induces ER stress, cases can be made for stress caused by aberrant LLO synthesis, inhibition of glycoprotein reglucosylation, or attenuation of a signal from secreted, extracellular UDP-Glc itself (Flores-Diaz *et al.*, 2004). These hypotheses might be resolved by direct analysis of LLO synthesis and reglucosylation in the UDP-glucose pyrophosphorylase-deficient cells, and by applying UDP-Glc extracellularly. The availability of promoter constructs that respond to UDP-Glc deficiency, but do not require the known UPR transcription mechanisms, also suggests the possibility of selections for mutants defective in this UDP-Glc dependent process.

An important question, then, relates to whether UDP-Glc levels are controlled by the UPR. This has not yet been addressed directly, for example by direct analytical measurements of UDP-Glc, but some experiments have implied that UDP-Glc levels might be enhanced by ER stress. In normal fibroblasts cells, relative glucosylation of $Glc_{0-3}M_9Gn_2$-P-P-Dol is the same with or without the addition of ER stress inducers (Doerrler & Lehrman, 1999). In CDG-Ic fibroblasts, however, which are impaired for first LLO glucosyltransferase, glucosylation of M_9Gn_2-P-P-Dol is partially corrected by DTT-induced stress (Shang *et al.*, 2002). Possible mechanisms for enhancing LLO glucosylation include elevation of UDP-glc, enhanced GPD synthesis, or activation of the GPD-requiring glucosyltransferase. Further, normal cells in low-glucose medium labeled more $G_3M_9Gn_2$-P-P-Dol with [^{3}H]mannose after ER stress, but paradoxically labeled less with [^{3}H]galactose (which is converted to UDP-[^{3}H]Glc before incorporation into LLO) after ER stress (Gill *et al.*, 2002). This would be expected if UDP-Glc synthesis increased during the stress response, diluting the intracellular pool of UDP-[^{3}H]Glc.

Figure 4 summarizes the steps in LLO synthesis and N-linked glycosylation suggested to be stimulated by ER stress in the prior sections.

N-LINKED GLYCOSYLATION MODULATED BY CYTOPLASMIC STRESS

Defects in LLO synthesis and N-linked glycosylation are not known to cause cytoplasmic stress, so it is unlikely that cytoplasmic stress responses could be part of a physiological feedback mechanism. However, the following sections discuss various ways in which cytoplasmic stress responses appear to stimulate LLO synthesis and N-linked glycosylation (Figure 1). As exemplified with arsenite, diamide, and disulfiram (Table 2), conditions that cause cytoplasmic stress do not necessarily cause ER stress, so distinct mechanisms are probably involved. By taking advantage of the effects of cytoplasmic stress, it may be possible to devise

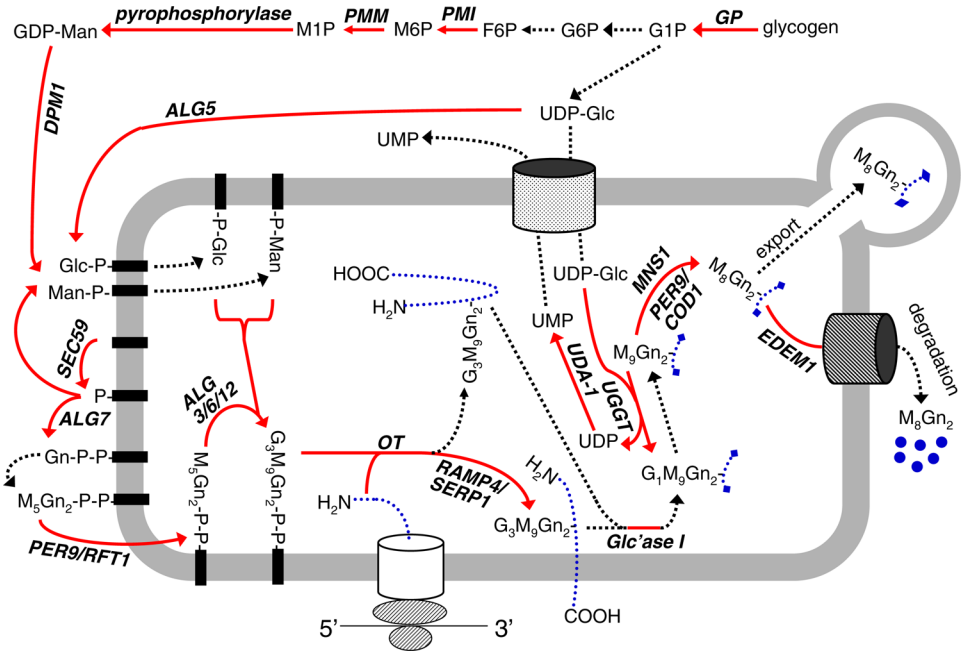

FIGURE 4 **Glycosylation reactions that are responsive to ER stress. Evidence for ER stress-stimulation of the reactions shown with solid arrows (red in Web version), and with italicized labels, is discussed in the text. All other reactions are shown with dashed arrows. Nascent polypeptides are indicated by dotted lines (blue in Web version), the ER membrane by the thick gray line, dolichols by the black rectangles in the ER membrane, and membrane transport activities (but not lipid flippases) by cylinders in the ER membrane. For simplicity, some polypeptides are truncated graphically with diamond symbols, and some enzyme substrates (such as P_i for GP) and by-products (such as GDP for DPM1) are not shown.**

strategies for management of N-linked glycosylation disorders.

$G_3M_9Gn_2$-P-P-Dol as Stress-Responsive Lipid M14

Studies with human A375 melanoma cells reported several-fold increases in the amount of [^{3}H]mannose-labeled LLO after heat shock, or treatments with agents (heat shock, sodium arsenite, heavy metals, and thiol reactants) expected to cause cytoplasmic stress (Niewiarowska *et al.*, 1987). As expected, each of these conditions stimulated synthesis of known stress proteins. Curiously, the [^{3}H]LLO was initially detected as an anomalous species termed "M14" migrating on SDS-polyacrylamide gels as if it weighed 14 kDa. The species was identified as LLO, not protein, based upon the ability of TN to block its synthesis; metabolic labeling with [^{32}P]orthophosphate (but neither [^{35}S]methionine nor [^{3}H]arginine); solubility in chloroform:methanol:water (10:10:3), digestion with endoglycosidase H; sensitivity to hydrolysis with weak acid; and a metabolic half-life of 15 minutes. The [^{3}H]mannose-labeled material released by weak acid eluted from a Biogel P-6 column at the position expected for free $G_3M_9Gn_2$, suggesting that the LLO was $G_3M_9Gn_2$-P-P-Dol. [^{3}H]mannose-labeled M14 was similarly increased by cytoplasmic stress in human carcinoma (Colo 201 and HT-29) and fibroblasts.

The mechanism by which LLO labeling with [^{3}H]-mannose increased remains unknown. Since many [^{3}H]mannose-labeled glycoproteins were detected, but their radioactive signals did not increase after stress treatment, it does not appear that M14 increased as a result of a higher intracellular specific activity of the [^{3}H]mannose. The authors considered that M14 accumulation might have been due to decreased utilization of the LLO after treatment with stress inducers, but discounted this possibility because treatments with sodium arsenite did not result in significant protein synthesis inhibition in their hands. Further, decreased utilization (turnover), as occurs with inhibitors of protein or RNA synthesis, actually lessens incorporation of [^{3}H]mannose into newly synthesized LLO (Gao & Lehrman, 2002b). Neither cycloheximide nor actinomycin D were concluded to inhibit synthesis of M14, but re-inspection of the original data suggests that these agents caused partial inhibition of M14 labeling.

Consequently, the best explanation is that the stressed cells generated greater chemical quantities of LLO, perhaps by increasing availability of Dol-P or UDP-GlcNAc. This hypothesis should be amenable to direct testing by FACE analysis of LLO (Gao & Lehrman, 2002a).

Prompt-Stress N-Linked Glycosylation

A number of studies have reported enhanced glycosylation of specific proteins after treatments consistent with, or suggestive of, cytoplasmic stress. Treatment of Sendai virus-infected monkey kidney cells with prostaglandin Δ^{12}-PGJ_2 was accompanied by heat shock protein synthesis, and induced glycosylation of a 47 kDa cellular protein while most cellular glycoproteins were unaffected (Amici *et al.*, 1992). It was also noted that 45°C shock of CHO cells for brief periods caused the appearance of certain glycoproteins, designated prompt-stress glycoproteins (P-SGs) (Henle *et al.*, 1993, 1995). The major P-SG appeared to weigh 67 kDa, but other species of approximately 47, 60, 64, 100, and 160 kDa were noted. P-SGs were sensitive to TN, digested by N-glycosidase F, and labeled with [^{3}H]mannose. In the case of P-SG67, [^{3}H]mannose label was associated with high mannose oligosaccharides after digestion with endoglycosidase H, and with a mixture that appeared to include both high mannose and complex-type oligosaccharides after N-glycosidase F digestion. P-SGs were also detected with [^{3}H]glucose label. These data are highly consistent with P-SGs acquiring an N-glycan. Importantly, appearance of P-SGs occurred while incorporation of [^{3}H]mannose label into the vast majority of glycoproteins was inhibited (presumably due to heat shock-dependent translation arrest), and was insensitive to cycloheximide. No P-SGs were detected with a [^{35}S]methionine/cysteine mixture. These results suggest that P-SGs were pre-existing proteins that were glycosylated by OT in a post-translational process. Assuming that P-SGs are ER proteins oriented in the lumenal space, the mechanism of this post-translational process might involve stress-induced exposure of cryptic OT acceptor sequons, perhaps due to conformational changes or dissociation of binding partners (Henle *et al.*, 1993).

It was further proposed that, in response to stress, the glycans might modulate interactions of the P-SGs with chaperones or other folding factors (Henle *et al.*, 1993). Interestingly, P-SG67 was subsequently identified as calreticulin (Jethmalani *et al.*, 1994), an ER protein later shown to be a lectin-chaperone with strict specificity for monoglucosylated high mannose glycans such as $G_1M_9Gn_2$ (Spiro *et al.*, 1996). Although only a minor fraction of calreticulin was later determined to be modified by prompt glycosylation (Henle *et al.*, 1997), the ability to post-translationally acquire an N-glycan hints at a novel regulatory function. [2-^{3}H]mannose as used (Henle *et al.*, 1993) limits the labeling to mannosyl residues and precludes the incorporation of label into glucose. Conversely, [^{3}H]glucose is a very inefficient precursor for labeling mannosyl residues of N-glycans in intact cells. However, it is more efficient for direct labeling of glucosyl residues via formation of UDP-[^{3}H]glucose, followed either by conversion to [^{3}H]GPD which serves as a precursor for LLO, or by direct incorporation into monoglycosylated glycans on glycoproteins by UGGT. Interestingly, the best substrates for UGGT are partially folded nascent or pre-existing glycoproteins bearing M_9Gn_2 and having molten-globule structure (Caramelo *et al.*, 2003). Although the principle species reported was M_9GlcNAc, reexamination of the Bio-Gel P-4 profile of [^{3}H]mannose-labeled glycans released from P-SG67 by endoglycosidase H (Henle *et al.*, 1993) suggests that the major peak may be a mixture of M_9GlcNAc and G_1M_9GlcNAc. Taken together, the data are consistent with P-SG67 being formed by partial denaturation of the unglycosylated protein, and then post-translational modification by both OT and UGGT, resulting in mixture of P-SG67 species bearing either M_9Gn_2 or $G_1M_9Gn_2$. P-SG67 bearing $G_1M_9Gn_2$ might then be subject to auto-inhibition of its lectin activity, and allow P-SG67 to act solely through its lectin-independent chaperone activity (Saito *et al.*, 1999). It is worth noting that while heat shock is a strong inducer of cytoplasmic stress, in some cases it has been reported to promote induction of ER stress proteins (Doerrler & Lehrman, 1999). Thus, it would be interesting to determine whether prompt glycosylation may occur during ER stress. It would also be informative to characterize labeling of P-SG67 in cells incubated with [^{3}H]galactose, a superior precursor for UDP-[^{3}H]glucose used by UGGT, and to test the effects of CSN, which should inhibit deglucosylation of $G_3M_9Gn_2$ and prevent the formation of the UGGT substrate glycan.

Pharmacological Mimics of Cytoplasmic Stress: Activators of AMP-Activated Protein Kinase

AICAR and Metformin Activate Responses Signaling Energy Deficits

Recent studies from the author's laboratory have shown that LLO synthesis and N-linked glycosylation can be enhanced by metformin and 5-aminoimidazole-4-carboxamide riboside (AICAR). These two reagents are reported to activate the AMP-activated protein kinase (AMPK), a stress-responsive enzyme with a master role in energy metabolism (Hardie, 2003). Unexpectedly, each can also stimulate LLO extension and N-linked glycosylation (as discussed below). AMPK is activated by AMP under conditions that increase the AMP/ATP ratio and thus signal energy deprivation. Interaction at the AMP binding site explains the activation of AMPK in intact cells by treatment with the nucleoside AICAR, an adenosine analog. After uptake via adenosine transporters, AICAR is phosphorylated by adenosine kinase to form the nucleotide AICAR-P, an analog of AMP. In addition to allosteric activation by AMP, AMPK can be activated covalently by an upstream AMPK-kinase complex (AMPKK). Rather than by allosteric activation, the primary mechanism by which AICAR-P activates AMPK is by making it a better substrate for AMPKK (Hawley *et al.*, 2003).

Initial evidence suggested that the AMPKK was a heterotrimeric complex consisting of LKB1-MO25-STRAD (Hawley *et al.*, 2003). The LKB1 subunit of AMPKK appears to be absolutely necessary to mediate activation of AMPK by biguanides (Zhou *et al.*, 2001) such as metformin, widely prescribed for treatment of type 2 diabetes. The clinically useful effects of metformin are reduced hepatic glucose output, due mainly to reduced gluconeogenesis, and increased glucose uptake by skeletal muscle due to elevated surface expression of GLUT4. Recently AMPKKs have been identified that are independent of LKB1 (Zou *et al.*, 2004; Birnbaum, 2005), and consistent with previous studies, none have been reported to respond to biguanides although they respond to other stimuli. The mechanism by which metformin activates the LKB1-containing heterotrimer is complex, but does not involve a direct interaction (Zhou *et al.*, 2001).

Using the fibroblast system discussed above, in which LLO intermediates were made to accumulate as a result of a brief incubation in medium with 0.5 mM glucose, both metformin (Shang & Lehrman, 2004b) and AICAR (Shang & Lehrman, 2004a) were shown to markedly enhance extension of LLO intermediates to $G_3M_9Gn_2$-P-P-Dol and improve glycosylation of protein with $G_3M_9Gn_2$.

Metformin, LLO Synthesis, and N-Linked Glycosylation

Metformin improved LLO extension by increasing uptake of [2-^{3}H]mannose (Shang & Lehrman, 2004b). Curiously, the metformin-stimulated transport did not mediate uptake of the widely used glucose transporter substrates 2-deoxyglucose and 3-O-methylglucose, but was inhibited by them instead. These and other experiments suggested that a novel mannose transport activity was involved. The metformin-stimulated mannose transport (MSMT) was detected in some, but not all mammalian cell cultures. Significantly, MSMT was detected in HeLa cells, which lack LKB1 and do not activate AMPK in response to treatment with biguanide (Hawley *et al.*, 2003). Therefore, HeLa cells have a novel LKB1-independent MSMT, suggesting that the LKB1 pathway may not account for all of the clinical effects of metformin. This is appealing because evidence exists for actions of metformin on insulin receptor tyrosine kinase activity (Cusi & DeFronzo, 1998) and membrane fluidity (Wiernsperger, 1999).

AICAR, LLO Synthesis, and N-Linked Glycosylation

AICAR's effects on LLO synthesis and protein glycosylation in the fibroblast system were more robust than for metformin, and could not be explained by enhanced uptake of mannose (Shang & Lehrman, 2004a), suggesting that AICAR acted through a separate mechanism. AICAR-P is capable of activating glycogen phosphorylase (GP) by allosteric action at the enzyme's AMP-binding site (Longnus *et al.*, 2003). Consistent with this, the GP activity in fibroblast extracts was activated by AICAR-P, and blocked by an indole-2-carboxamide inhibitor of GP (Shang & Lehrman, 2004a). These results suggested that the action of AICAR with fibroblasts may have involved direct activation of glycogenolysis by GP. However, the potential contribution of phosphorylation (inactivation) of glycogen synthase by AICAR-activated AMPK (Carling & Hardie, 1989; Hardie, 2003) was not determined. As

discussed earlier, glycogenolysis has been implicated in ER stress-enhanced LLO synthesis, and as reviewed in the next section glycogen is an important source of hexose for glycosylation.

GLYCOGEN AS A SOURCE OF HEXOSE FOR N-LINKED GLYCOSYLATION

The preceding sections reviewed evidence linking glycogenolysis to LLO synthesis during stress responses. Complementary approaches with cultured cells and animals have implicated glycogen as a source of hexose for glycosylation, independent of cellular stress.

Response of GLUT1 Glycosylation to Glucose Starvation

GLUT1 transcription is stimulated by hydrogen peroxide exposure of L6 myotubes in a manner consistent with oxidative stress in the cytoplasmic compartment (Kozlovsky *et al.*, 1997). In contrast, studies with GLUT1 in 3T3-L1 adipocytes (Kitzman *et al.*, 1996) showed that prolonged (~18 hours) glucose deprivation increased transcription of GRP78/BiP, but not GLUT1, suggesting that GLUT1 was not responsive to ER stress. Curiously, glucose deprivation (18 to 24 hours) caused the appearance of a lower molecular weight (approx. 37 kDa) endoglycosidase-H resistant glycoform of GLUT1 (McMahon & Frost, 1996; Kitzman *et al.*, 1996). This hypoglycosylated form of GLUT1 was also observed in CHO cells, except that effects of glucose deprivation were faster, with the glycoform appearing after about 3 hours (McMahon & Frost, 1996). These findings were consistent with earlier work in mammalian cells, showing that glucose deprivation can inhibit the synthesis of $G_3M_9Gn_2$-P-P-Dol with concomitant accumulation of smaller underglucosylated and undermannosylated LLOs (Turco, 1980; Rearick *et al.*, 1981; Gershman & Robbins, 1981), which are then used to glycosylate proteins. The effects of prolonged glucose deprivation on GLUT1 were used to evaluate the potential role of glycogen as a source of hexose units for protein glycosylation (McMahon & Frost, 1996). These authors concluded that glycogen could be used as a source of hexose for protein glycosylation during glucose deprivation and suggested that the relatively low glycogen stores in CHO cells (compared with 3T3-L1) accounted for the more rapid effects of glucose deprivation on GLUT1 glycosylation. In glucose-deprived 3T3-L1 cells, glycogen was depleted by half after 6 hours, by 80% after 12 hours, and by more than 90% by 24 hours. CHO cells contained much less glycogen, having 1% and 10% of the glycogen of 3T3-L1 cells before and after glucose deprivation, respectively. Addition of 25 mM fructose during glucose deprivation prevented the appearance of the 37 kDa GLUT1 glycoform in 3T3-L1 cells, but glycogen depletion was still extensive. The authors suggested this was perhaps due to preferential metabolism of fructose for protein glycosylation, or differential control of glycogenolysis by glycogen phosphorylase. Another possibility stems from the fact that in glucose-free medium supplemented with fructose, glycogen levels stabilized at about 20% of normal. Since the GLUT1 glycoform did not appear until about 18 hours of glucose depletion (in the absence of fructose, when glycogen contents likely fell below 20% of normal), 20% of normal glycogen stores might be adequate to maintain normal rates of LLO synthesis and protein glycosylation in 3T3-L1 cells.

Glycogen as a Source of Circulatory Mannose

The normal diet is probably not a significant source of circulatory mannose, which is usually 20 to 80 μM in adult humans. Consistent with a role in supplying hexose units for N-glycan assembly, hepatic glycogenolysis in rats has been shown to be involved in the production of circulatory mannose (Taguchi *et al.*, 2005). When glucose was administered to 12-hour fasted rats, a condition expected to stimulate hepatic glycogen synthesis (thus inhibit glycogenolysis), circulatory glucose increased but mannose diminished by about 25% in less than an 1 hour. Administration of epinephrine to fed rats to activate hepatic GP and hence glycogenolysis, elevated circulatory mannose by about 20% as well as circulatory glucose. However, neither increased with coadministration of 1,4-dideoxy-1,4-imino-D-arabinitol (a GP inhibitor), or when rats were fasted for 48 hours (expected to deplete hepatic glycogen stores). Together, conditions that favor hepatic glycogen synthesis lessen the amount of mannose delivered to the circulation, while conditions that favor hepatic glycogenolysis elevate circulatory mannose. This relationship was supported by experiments in which perfused liver was treated with epinephrine, which elevated output of both glucose and mannose, and increased hepatic G6P, F6P, and M6P, all of which derive from glycogenolysis.

The increased output of glucose and mannose was blocked if chlorogenic acid, an inhibitor of glucose-6-phosphatase, was included in the perfusate. Mannosyl phosphates, but not free mannose, result from conventional metabolism of G6P and F6P. This suggests either that M6P was not entirely excluded from the lumen of the hepatic ER, which contains glucose-6-phosphatase (as proposed by the authors), or that M6P is fastidiously excluded from the ER (as is widely thought) but a separate enzyme sensitive to chlorogenic acid was responsible for hydrolysing a mannosyl phosphate to produce free mannose. Administration of lactate (a gluconeogenic precursor) to animals, or with perfused liver, elevated output of glucose but not mannose. This was surprising because G6P is the common intermediate of both glycogenolysis and gluconeogenesis leading to elevated glucose output, and G6P is the likely source of mannosyl phosphates discussed above. Since mannose output increased only with glycogenolysis, as proposed by the authors (Taguchi *et al.*, 2005) there may be two pools of hepatic G6P resulting from these two glucogenic processes, with the gluconeogenic pool being unavailable for mannose output.

PERSPECTIVES

To the author's knowledge, this is the first comprehensive review of mechanisms used by metazoan cellular stress responses to stimulate N-linked glycosylation. Most information deals with ER stress responses, which would be expected to have a biological basis for modulating N-linked glycosylation. It seems that ER stress responses affect many steps in LLO synthesis and N-linked glycosylation (Figure 4), which in aggregate should result in a robust effect in concert with stimulatory effects on other ER components. However, one important distinction is the relative rapidity by which ER stresses seem to act upon glycosylation, suggestive of one or more novel ER stress sensors. The very existence of these corrective and compensatory mechanisms implies that reductions in the precursors or enzymes needed to produce LLOs, or to transfer glycans to nascent proteins, can be physiological or pathological causes of ER stress.

The effects of stress responses on glycosylation suggest a number of avenues for clinical intervention in glycosylation disorders. One possibility, relevant to all types of CDG-I, would be the application of ER stress inducers in concentrations mild enough to stimulate LLO synthesis but not apoptosis. Another route might employ adenosine analogs like AICAR (which stimulate GP and AMPK) or biguanides like metformin (which stimulate mannose transport and AMPK) to promote glycan synthesis. In CDG-Ia, it had been thought that increasing circulatory mannose by dietary means might alleviate the glycosylation defect. Notably, clinical administration of mannose has had no beneficial effect for CDG-Ia patients, and recent work from the author's laboratory has identified a potential anti-metabolic effect of M6P on the LLO pool. Although counterintuitive, it might therefore be beneficial in CDG-Ia to decrease circulatory mannose, perhaps with inhibitors of GP to limit glycogenolysis.

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