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Essay

How N-linked Oligosaccharides Affect Glycoprotein Folding in the Endoplasmic Reticulum

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INTRODUCTION

In eukaryotic cells, a large fraction of the proteins synthesized acquire one or more N-linked oligosaccharides. These are added as core oligosaccharide units to specific consensus sequences when the polypeptide chains enter the lumen of the endoplasmic reticulum (ER). In many of these proteins, the first function of the N-linked oligosaccharides involves the process of folding and conformational maturation. Without added oligosaccharides, many of them misfold, aggregate, and get degraded without transport from the ER to the Golgi complex and beyond. Not only is the addition of oligosaccharides important, but also their trimming, which begins in the ER with the removal of glucose and mannose residues.

Although a large body of data shows that the oligosaccharides and their modification are necessary for proper glycoprotein folding and assembly, the molecular basis for the requirement remains unclear. The rational for assembling complex oligosaccharide entities, adding them to polypeptide chains, and then immediately dismantling them saccharide by saccharide remains elusive.

In this review, I address these long-standing problems. Recent findings suggest that the N-linked oligosaccharides may play multiple roles during the conformational maturation of glycoproteins. They are needed to stabilize folded domains and provide solubility-enhancing polar surface groups that prevent aggregation of folding intermediates. In addition, they enable newly synthesized glycopolypeptide chains to interact with a set of resident ER enzymes and chaperones. These unique ER components include a lectin-like protein called calnexin. This chaperone and accessory factors are apparently designed to provide assistance to glycoproteins during folding, oligomerization, ER retention, and quality control. The stepwise trimming of the oligosaccharides is linked to interactions between newly synthesized glycoproteins and the components of this putative folding machinery.

SYNTHESIS AND TRIMMING OF N-LINKED OLIGOSACCHARIDES IN THE ER

The steps in the biosynthesis, trimming, and terminal glycosylation of N-linked oligosaccharides are known in detail and have been extensively reviewed (for reviews see Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985; Rademacher *et al.*, 1988; Elbein, 1991a). Briefly, a core unit of 14 saccharides ($Glc_3Man_9GlcNAc_2$) (see Figure 1) is synthesized as a dolichol pyrophosphate precursor by enzymes located in the ER membrane. It is then transferred to the asparagine side chain of Asn-X-Ser/Thr consensus sequences by a membrane-bound oligosaccharide transferase. For glycoproteins that are cotranslationally translocated, the addition of core oligosaccharides occurs as the nascent polypeptide emerges into the lumen of ER through a translocation complex that forms a channel through the rough ER membrane. The oligosaccharide transferase is a transmembrane enzyme located close to, or forming part of, the translocation complex (Kelleher *et al.*, 1992).

Immediately after addition of the core oligosaccharide, trimming begins with the removal of the three terminal glucose residues (Figure 1). Glucosidase I, a membrane-bound homotetramer, removes the terminal α1,2-linked glucose (Hubbard and Robbins, 1979; Michael and Kornfeld, 1980; Atkinson and Lee, 1984; Hettkamp *et al.*, 1984). Glucosidase II, a homodimer or tetramer peripherally attached to the lumenal surface of the ER membrane (Brada and Dubach, 1984), re-

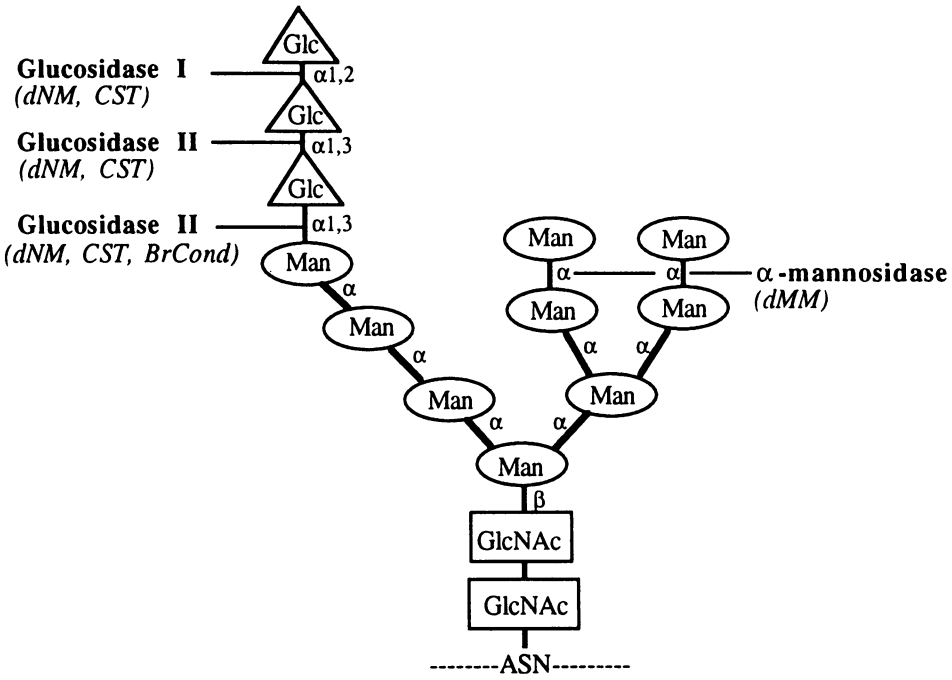

Figure 1. The structure of the N-linked core oligosaccharide and its processing in the ER. Trimming begins cotranslationally by the removal of the terminal α1,2-linked glucose residue followed by the remaining two α1,3-linked glucoses. More slowly, a variable number of mannoses are removed by α-mannosidases. The sites of action of some inhibitors to specific glycosidases are also shown: CST, castanospermine; dNM, 1-deoxynojirimycin; dMM, 1-deoxymannojirimycin.

moves the remaining α1,3-linked glucoses. The first of these are detached cotranslationally, whereas the monoglucosylated form usually persists for periods up to 20 min, and in some cases even longer (Hubbard and Robbins, 1979; Suh *et al.*, 1989).

The reason why the monoglucosylated form has a longer half-life than the tri- and diglucosylated counterparts is, at least in part, that glucose-free, high-mannose side chains devoid of glucose residues are subject to readdition of single glucose residues while in the ER (Parodi *et al.*, 1984; Suh *et al.*, 1989). It has been estimated that >50% of the N-linked oligosaccharides are posttranslationally reglucosylated by an ER glucosyltransferase after they have lost their original glucose residues (Ganan *et al.*, 1991). Transient reglucosylation is thus a common event and may, as discussed below, play a significant role in retaining glycoproteins in the ER until properly folded.

Some of the mannose residues are also hydrolyzed by α-mannosidases (see Kornfeld and Kornfeld, 1985). As a result, most glycoproteins leave the ER with $Man_{8-7}GlcNAc_2$ side chains. Upon entry into the Golgi complex, the majority of the oligosaccharide chains undergo further trimming followed by terminal glycosylation.

N-LINKED OLIGOSACCHARIDES IN MATURE PROTEINS

In mature folded glycoproteins, the N-linked oligosaccharides are located on the surface and accessible to bulk solution. Whether present in high-mannose or processed terminally glycosylated forms, they cover large areas of a glycoprotein's surface, constituting dominant surface structures. They are generally mobile and essentially unaffected by the polypeptide (Brockbank and Vogel, 1990). The oligosaccharide side chains can often be removed from mature glycoproteins using glycosidases without immediate structural consequences to the protein (Olden *et al.*, 1982; West, 1986; Rademacher *et al.*, 1988; Paulson, 1989; Driscoll *et al.*, 1991). This may suggest that, once folded, most glycoproteins do not depend on their N-linked carbohydrates for overall stability or solubility. There are, however, exceptions. One is human CD2, which, unlike its rat homologue, depends for stability on a critical N-linked oligosaccharide in its adhesion domain (Withka *et al.*, 1993). As more detailed structural information on glycoproteins becomes available, more cases like this will, no doubt, emerge.

Although this review focuses on the role of N-linked oligosaccharides during glycoprotein folding in the ER, it is important to stress that the protein-bound oligosaccharides have many biological functions in cell life. Their role in folding is only one. They serve in the intracellular targeting of glycoproteins, in modulating clearance rates, and in numerous recognition-related processes on the plasma membrane (see Olden *et al.*, 1982; Rademacher *et al.*, 1988; Paulson, 1989; Cumming, 1991; Varki, 1993). They are particularly important for cell–cell interactions during embryogenesis and tissue organization. Many

of the associations involve specific carbohydrate binding proteins, i.e., lectins.

GLYCOSYLATION AND TRIMMING AFFECT FOLDING, SECRETION, AND ER DEGRADATION

Whereas most N-linked oligosaccharides may be structurally dispensable in the mature protein, they are often crucial during glycoprotein folding and oligomerization. When glycosylation is inhibited using tunicamycin (an inhibitor of dolichol oligosaccharide precursor synthesis), or by mutational elimination of consensus sequences, the folding of numerous glycoproteins fails (Leavitt *et al.*, 1977; Gibson *et al.*, 1978; Morrison *et al.*, 1978; Olden *et al.*, 1978; Ronnett *et al.*, 1984; Miyazaki *et al.*, 1986; Dorner *et al.*, 1987; Machamer and Rose, 1988b; Matzuk and Boime, 1988; Taylor and Wall, 1988; Hurtley *et al.*, 1989; Ng *et al.*, 1990; Gallagher *et al.*, 1992). Typically, the proteins aggregate soon after synthesis and associate noncovalently with BiP/GRP78, an abundant ER chaperone known to bind to a variety of misfolded and incompletely folded proteins (Leavitt *et al.*, 1977; Dorner *et al.*, 1987; Hurtley *et al.*, 1989; Machamer *et al.*, 1990; Gallagher *et al.*, 1992). Their ultimate fate is degradation, which takes place in the ER or other pre-Golgi compartments within a few hours of synthesis (Klausner, 1989). The misfolded proteins are often covalently linked to each other by aberrant interchain disulfide bonds (Machamer and Rose, 1988b; Hurtley *et al.*, 1989; Marquardt and Helenius, 1992).

Although most glycoproteins need their N-linked oligosaccharides during folding, the degree of dependence is variable. Some display only partial misfolding in the absence of sugars; a fraction folds correctly and is transported normally out of the ER, whereas the rest misfolds and remains in the ER (Hickman and Kornfeld, 1978; Miyazaki *et al.*, 1986). Quite a few have a temperature-dependent requirement for glycosylation; they misfold at 37°C but fold correctly without added sugars at reduced temperatures (Gibson *et al.*, 1978; Gibson *et al.*, 1979; Gallagher, 1988; Machamer and Rose, 1988b; Roberts *et al.*, 1993). Finally, there are numerous proteins that do not seem to need their N-linked sugars at all during folding (Hickman and Kornfeld, 1978; Olden *et al.*, 1978; Struck *et al.*, 1978; Shackelford and Strominger, 1983; Landolfi *et al.*, 1985).

Why glycoproteins differ in this way is not known. The need for oligosaccharides does not, for instance, correlate directly with the number of N-linked chains (Sidman, 1981). In some cases, very subtle factors determines the outcome. For example, only one of the G proteins of two vesicular stomatitis virus strains needs N-linked oligosaccharides for folding. A single point mutation, however, suffices to make the otherwise oligosaccharide-requiring protein independent of glycosylation (Pitta *et al.*, 1989). The only discernible rule is that glycoproteins that have folding problems with their sugars in place, have even more if the N-linked oligosaccharides are missing or modified. Robust folders are less sensitive to interference.

When the effects of glycosylation are analyzed by site-specific elimination of consensus glycosylation sequences, it is often found that no single N-linked oligosaccharide is essential for folding. Only when more than one consensus site is altered do folding problems arise (Ng *et al.*, 1990; Gallagher *et al.*, 1992; Roberts *et al.*, 1993; Shipley *et al.*, 1993; Williams and Enns, 1993). Nevertheless, some sites often prove more important than others (Dube *et al.*, 1988; Matzuk and Boime, 1988; Ng *et al.*, 1990; Gallagher *et al.*, 1992; Pique *et al.*, 1992; Roberts *et al.*, 1993). Not surprisingly, these tend to be conserved. It has been suggested that such key oligosaccharides may function in a cooperative manner, enhancing the chances for a protein to reach a stable correctly folded conformation (Roberts *et al.*, 1993).

Strikingly, it has also been reported that the creation of consensus glycosylation sites in entirely novel locations in a polypeptide can sometimes replace the need for normal constitutive oligosaccharides (Machamer *et al.*, 1985; Machamer and Rose, 1988a; Williams and Enns, 1993). This illustrates an unexpected degree of flexibility in the acceptable positions of carbohydrates. A related observation by Guan *et al.* (1985) showed that a membrane-anchored nonglycosylated form of growth hormone, otherwise transport-incompetent, was transported to the cell surface when a consensus glycosylation site was engineered into the sequence. In such instances, it seems that it is important to have a sugar moiety present somewhere in the protein, the location may be of secondary importance. The oligosaccharide seems to play more of a "global" than a "local" role in folding.

SACCHARIDE COMPOSITION AND GLUCOSE TRIMMING AFFECT MATURATION OF GLYCOPROTEINS

Not just the presence but also the extent of trimming of N-linked oligosaccharide side chains in the ER make a difference in the folding and transport of glycoproteins. Inhibitors of glucosidases I and II, such as castanospermine, 1-deoxynojirimycin, and bromoconduritol, often prevent or delay the folding and intracellular transport of newly synthesized glycoproteins (Robbins *et al.*, 1977; Datema *et al.*, 1982; Gross *et al.*, 1983; Peyrieras *et al.*, 1983; Lemansky *et al.*, 1984; Lodish and Kong, 1984; Schwartz and Datema, 1984; Duronio *et al.*, 1988; Machamer and Rose, 1988a; Elbein, 1991b).

Again, the effects vary between individual proteins (Pan *et al.*, 1983; Burke *et al.*, 1984; Lodish and Kong, 1984; Schlesinger *et al.*, 1984). Glycoproteins that are

dramatically affected include immunoglobulin (Ig)D (Peyrieras *et al.*, 1983); low-density-lipoproteins receptor (Edwards *et al.*, 1989), α_1-protease inhibitor (Gross *et al.*, 1983; Parent *et al.*, 1986), vesicular stomatitis virus G protein (San Juan strain) (Schlesinger *et al.*, 1984); v-fms oncoprotein (Nichols *et al.*, 1985), env-sea oncoprotein (Crowe and Hayman, 1993), Friend mink cell focus forming murine leukemia virus envelope protein (Pinter *et al.*, 1984), Sindbis virus E2 glycoprotein (McDowell *et al.*, 1987), E2 of mouse hepatitis virus (Repp *et al.*, 1985), and human immunodeficiency virus type 1 (HIV-1) glycoprotein (Tyms *et al.*, 1987; Pal *et al.*, 1989; Taylor *et al.*, 1991).

The same glucosidase inhibitors are also known to delay the processing and secretion of insulin receptor, α_1-antitrypsin and α_1-antichymotrypsin, ceruloplasmin, α_2-macroglobulin, cathepsin D, and β-hexosaminidase (Gross *et al.*, 1983; Lemansky *et al.*, 1984; Lodish and Kong, 1984; Parent *et al.*, 1986; Arakaki *et al.*, 1987; Duronio *et al.*, 1988). The antiviral effect of glucosidase inhibitors in tissue culture cells has led to possible clinical development of castanospermine derivatives as drugs against HIV-1 and other retroviruses (Tyms *et al.*, 1987; Pal *et al.*, 1989; Elbein, 1991b; Taylor *et al.*, 1991). This is feasible because glucosidase inhibitors are generally not very toxic to cells or animals (Nichols *et al.*, 1985; Sunkara *et al.*, 1989).

Bromoconduritol, a glucosidase inhibitor that specifically blocks the removal of the innermost glucose residue, seems to be more potent as an inhibitor of glycoprotein secretion than castanospermine and 1-deoxynojirimycin (Datema *et al.*, 1982). For Fowl Plague influenza virus hemagglutinin (HA), for example, the inhibition of trimming of the outermost glucoses has no effect on virus maturation, whereas inhibiting the trimming of the innermost glucose with bromoconduritol leads to a transport-incompetent glycoprotein (Datema *et al.*, 1982; Romero *et al.*, 1983). This suggests that the innermost glucose is somehow more important in HA maturation than the two outer glucoses.

It is interesting to note that IgD, which is not secreted in the presence of 1-deoxynojirimycin (Peyrieras *et al.*, 1982), is secreted in the presence of tunicamycin (Neuberger and Rajewsky, 1981; Sidman, 1981). This implies that IgD folds more efficiently without any sugars than with untrimmed N-linked oligosaccharides.

On the basis of such inhibitor effects, several investigators have suggested that glycoprotein transport out of the ER may be regulated by lectins (Fitting and Kabat, 1982; Olden *et al.*, 1982; Lodish and Kong, 1984; Parent *et al.*, 1986). These have been thought to specifically bind to trimmed glycoproteins and facilitate their transport from the ER. This notion is not consistent with the so-called "bulk flow" concept (Pfeffer and Rothman, 1987), which suggests that membrane and soluble proteins move out of the ER without the need for specific receptors. As discussed below, recent data do give reason to believe that lectin-like receptors for glycoproteins exist in the ER (Ou *et al.*, 1993; Hammond and Helenius, 1993; Hammond *et al.*, 1994). Their role seems, however, to be retention rather than active forward-transport.

In contrast to glucosidase inhibitors, inhibitors of ER α-mannosidases do not have detectable effects on the folding and secretion of glycoproteins (Elbein *et al.*, 1984; Bosch *et al.*, 1985; Nichols *et al.*, 1985; Repp *et al.*, 1985; Duronio *et al.*, 1988; Hammond *et al.*, 1994). 1-Deoxymannojirimycin was, however, recently found to block the degradation of a transport-incompetent yeast glycoprotein in the ER of mammalian cells (Su *et al.*, 1993). Two reports have shown that glucosidase inhibitors can accelerate the degradation of unassembled forms of major histocompatibility complex (MHC) class I heavy chains (Peyrieras *et al.*, 1983; Moore and Spiro, 1993). These results suggest that mannose trimming may be somehow linked to ER quality control and degradation.

MUTATIONS IN N-LINKED OLIGOSACCHARIDE SYNTHESIS AND PROCESSING AFFECT GLYCOPROTEIN FOLDING

Numerous cell lines in yeast and mammalian cells have been isolated with defects in synthesis, transfer, and trimming of N-linked core carbohydrates (Reitman *et al.*, 1982; Stanley, 1984; Hearing *et al.*, 1989a). Of these, many show a temperature-sensitive growth phenotype consistent with protein folding defects. Conversely, attempts at isolating mutant mammalian cell lines with pleiotropic glycoprotein transport defects have frequently resulted in the isolation of cells with defects in N-linked sugar synthesis (Tufaro *et al.*, 1987; Hearing *et al.*, 1989a,b).

Hearing and coworkers (1989a,b), for example, selected Chinese hamster ovary (CHO) cell mutants for temperature-sensitive surface expression of influenza HA. The cell lines analyzed were found to add $Man_5GlcNAc_2$ side chains. About half of the chains corresponded to such incomplete N-linked sugars, whereas the rest were normal. The addition of defective chains, which was not temperature conditional by itself, resulted in temperature-dependent misfolding and aggregation of newly synthesized HA. This finding is consistent with the notion that not only is the presence of N-linked oligosaccharide chains critical for the outcome of HA's folding but also the detailed composition of the chain. It is interesting to note that the HA protein of the particular influenza strain used belongs to those that can fold without any sugars (Gallagher *et al.*, 1992). Thus, the presence of a few incompletely assembled oligosaccharide chains constitutes more of a handicap

for this protein than having no N-linked carbohydrates at all.

MISFOLDED GLYCOPROTEINS UNDERGO DE- AND REGLUCOSYLATION IN THE ER

As already mentioned, full removal of glucoses from core oligosaccharides usually occurs within minutes after synthesis. However, when glycoproteins remain misfolded and transport incompetent the oligosaccharides remain in a monoglucosylated state ($Glc_1Man_{9-7}GlcNAc_2$) (Rizzolo and Kornfeld, 1988; Suh *et al.*, 1989). The single glucose residue in these chains is subject to rapid turnover (Suh *et al.*, 1989) due to continuous de- and reglucosylation. Deglucosulation is caused by glucosidase II, reglucosylation by UDP-glucose:glycoprotein glucosyltransferase, an enzyme described by Parodi and coworkers (Parodi *et al.*, 1984; Trombetta and Parodi, 1992).

The glucosyltransferase is a soluble, ubiquitously expressed, resident ER enzyme composed of two identical 150-kDa subunits (Parodi *et al.*, 1984; Ganan *et al.*, 1991; Gotz *et al.*, 1991; Trombetta *et al.*, 1991; Sousa *et al.*, 1992; Trombetta and Parodi, 1992). It catalyzes the posttranslational reglucosylation of protein-linked high-mannose oligosaccharides. The oligosaccharides to which the glucose are added are $Man_{9-7}GlcNAc_2$. $Man_9GlcNAc_2$ is most efficiently glucosylated, with decreasing activity observed as the number of mannoses decreases (Trombetta *et al.*, 1989). When the mannoses are six or fewer, no reglucosylation activity is observed. UDP-glucose, which is needed as a substrate, is supplied from the cytosol via a transporter in the ER membrane (Perez and Hirschberg, 1986).

What is particularly interesting about the UDP-glucose:glycoprotein glucosyltransferase is that it only uses denatured or unfolded glycoproteins as its substrate (Sousa *et al.*, 1992). Mature folded glycoproteins are not glucosylated, although the oligosaccharides are identical and equally exposed. Evidently, it can, like many chaperones, recognize a property (or properties) common to many misfolded proteins. Parodi and coworkers have argued that the transferase may be involved in sensing untrimmed oligosaccharides on unfolded, misfolded, or incompletely folded glycoproteins (Ganan *et al.*, 1991; Sousa *et al.*, 1992; Trombetta and Parodi, 1992). They suggest that it may somehow participate in mechanisms by which glycoproteins fold in the ER and by which misfolded structures are recognized and degraded. As described below, it is, indeed, likely in view of more recent data that this enzyme is part of the quality control system operating in the ER.

GLYCOPROTEIN FOLDING IN VIVO

The information about the role of N-linked oligosaccharides reviewed above is complex and poorly amenable to simple generalizations. It is clear that N-linked oligosaccharides do play a central role in the conformational maturation of most glycoproteins, but individual glycoproteins depend on their oligosaccharide side chains to varying degrees and in different ways. Many seem to need them for structure and stability only during folding, a few continue to rely on them throughout their existence. For the folding of a significant number of glycoproteins, glucose trimming is essential.

Before attempting to discuss unifying molecular concepts, a few words are needed about glycoprotein folding in vivo. Soluble glycoproteins and the ectodomains of membrane glycoproteins fold in the ER lumen. Folding generally begins cotranslationally (and cotranslocationally) and continues for variable times after chain termination. For most glycoproteins, the folding process involves the formation disulfides. The folding of individual polypeptide chains is followed, in many cases, by oligomeric assembly, which generally occurs before transport to the Golgi complex (see Hurtley and Helenius, 1989; Klausner, 1989; Helenius *et al.*, 1992).

To control outgoing traffic, the ER possesses an efficient "quality control" system that ensures that properly folded and assembled proteins are selectively transported to the Golgi complex and beyond (Rose and Doms, 1988; Hurtley and Helenius, 1989; Klausner, 1989). This type of molecular sorting does not rely on specific signal sequences but on some common structural features. So far, the definition of the structural criteria has not yet been possible except in very general terms, and the cellular machinery involved is still largely unknown.

Glycoprotein folding in the ER is dependent on metabolic energy (Braakman *et al.*, 1992) and relies on cellular factors (see Gething and Sambrook, 1992; Hartl *et al.*, 1992; Helenius *et al.*, 1992). The lumenal space contains a high concentration of soluble chaperones such as BiP/GRP78 and GRP94 that have homologues in other compartments of the cell. The milieu is oxidizing (Hwang *et al.*, 1992), and there are several redox enzymes, including protein disulfide isomerase, responsible for efficient and correct disulfide oxidation of newly synthesized proteins (see Freedman, 1989; Noiva and Lennarz, 1992). Many of the soluble folding factors and enzymes are known to interact transiently with glycoproteins. As in other compartments of the cell, the chaperones and folding enzymes do not impart folding information to incoming polypeptides but prevent aggregation and other nonproductive side pathways during the folding process.

CALNEXIN

Of ER chaperones, calnexin (also called p88, IP90), an abundant *trans*-membrane protein, is particularly interesting as it seems to associate selectively with nu-

merous newly synthesized glycoproteins (Degen and Williams, 1991; Ahluwalia *et al.*, 1992; Degen *et al.*, 1992; Galvin *et al.*, 1992; Hochstenbach *et al.*, 1992; David *et al.*, 1993; Ou *et al.*, 1993; Hammond *et al.*, 1994). Calnexin is a nonglycosylated type I membrane protein (574 amino acids, 65 kDa) with lumenal calcium binding sites and a large cytosolic domain containing several phosphorylation sites (Wada *et al.*, 1991). It is present in all species tested and in the ER of all cell types (Galvin *et al.*, 1992; David *et al.*, 1993). Its sequence is highly conserved (Wada *et al.*, 1991; Galvin *et al.*, 1992; David *et al.*, 1993; De Virgilio *et al.*, 1993). The mammalian calnexins have C-terminal RKxRRx sequences that are likely to serve as ER retention or retrieval sequences (Nilsson *et al.*, 1989; David *et al.*, 1993). Calnexin was originally isolated as a complex together with three smaller transmembrane glycoproteins of the ER membrane—TRAPα, TRAPβ, and gp25L (Wada *et al.*, 1991)—but these may not be true subunits of a larger calnexin complex (Ou *et al.*, 1993; Hammond and Helenius, unpublished observations).

In addition to calnexin, the ER contains an abundant soluble 46-kDa homologue called calreticulin, which has a KDEL retention sequence at its C-terminus (for review, see Michalak *et al.*, 1992). Both calnexin and calreticulin share tandem repeat sequence motifs (including characteristic KPEDWD sequences) in their central domain. The repeat sequences are also found in surface proteins of *Schistosoma* and *Onchocerca volvulus* (Unnasch *et al.*, 1988; Hawn *et al.*, 1993). Calnexin thus belongs to a family of proteins that include soluble and membrane-bound members in various cellular locations.

The function of calnexin is not known in detail, but it is generally considered a chaperone because it interacts transiently with a large number of newly synthesized proteins during their folding and oligomeric assembly (Degen and Williams, 1991; Degen *et al.*, 1992; Galvin *et al.*, 1992; Hochstenbach *et al.*, 1992; David *et al.*, 1993; Ou *et al.*, 1993; Hammond *et al.*, 1994). For the majority of these proteins, calnexin-binding seems to be restricted to a period of 0–60 min immediately after chain termination, during which time individual proteins acquire disulfide bonds and assemble into oligomeric complexes. Judging by coimmunoprecipitation with anti-calnexin antibodies, the efficiency of binding can reach values in excess of 80% for a specific folding or assembly intermediate (Hammond *et al.*, 1994).

Several oligomeric proteins have been shown to assemble from individual subunits while attached to calnexin. These include MHC class I and II antigens (Degen and Williams, 1991; Degen *et al.*, 1992; Anderson and Cresswell, 1994), the T-cell receptor (David *et al.*, 1993), and, possibly, vesicular stomatitis virus G protein and influenza virus HA trimers (Hammond *et al.*, 1994). Soluble monomeric glycoproteins such as transferrin and α_1-antitrypsin also bind to calnexin (Ou *et al.*, 1993).

Dissociation of glycoproteins from calnexin seems, in many cases, to coincide roughly with the beginning of their transport out of the ER (Degen and Williams, 1991; Ou *et al.*, 1993). Prolonged association is only observed when proteins are unable to oligomerize due to lack of complementary subunits or if the proteins remain misfolded (Degen *et al.*, 1992; David *et al.*, 1993; Ou *et al.*, 1993; Hammond *et al.*, 1994). The reason for misfolding can be the intrinsically low folding efficiency of a protein, the presence of mutations, or incorporation of amino acid analogues.

CALNEXIN HAS LECTIN-LIKE PROPERTIES

Recently, Bergeron and coworkers observed that of the newly synthesized proteins in HepG2 cells only glycoproteins underwent binding to calnexin, and tunicamycin abolished their binding (Ou *et al.*, 1993). Using viral spike proteins, we confirmed this apparent requirement for N-linked oligosaccharides (Hammond *et al.*, 1994). We found that binding to calnexin was not only prevented by tunicamycin but also by glucosidase inhibitors castanospermine and 1-deoxynojirimycin. Inhibitors of ER α-mannosidases did not have such an effect. Thus, calnexin seems to bind glycoproteins only, with specificity for those that had undergone partial or full glucose trimming. The same was later observed for endogenous glycoproteins of CHO cells (Hammond and Helenius, unpublished results).

Further analysis indicated that it is, most likely, the monoglucosylated form of the oligosaccharide side chain that supports optimal attachment of glycoproteins to calnexin (Hammond *et al.*, 1994). Misfolded glycoproteins such as ts045 G protein, whose oligosaccharide side chains are known to remain monoglucosylated for long periods of time (Suh *et al.*, 1989), fail to be released from calnexin (Hammond *et al.*, 1994). Misfolded HA, either without N-linked sugars or with untrimmed oligosaccharides, do not bind to calnexin, suggesting that incomplete folding per se is not enough to support association (Hammond *et al.*, 1994). Conversely, proper folding does not prevent binding of HA when the oligosaccharides remain partially trimmed. These results suggest that calnexin binding is not directly linked to the conformation of the polypeptide moiety but rather to the composition of the N-linked oligosaccharides.

The simplest interpretation of these results is that calnexin is a lectin that binds to partially trimmed N-linked sugar moieties on glycoproteins. It may, for instance, recognize a single glucose and one or more of the adjoining mannoses. Its preferential binding to newly synthesized glycopolypeptides would be explained by the transient presence of monoglucosylated trimming intermediates during the early stages of glycoprotein maturation. Formal biochemical proof for such lectin activity is, however, lacking. Because there is no obvious

sequence homology between calnexin and known lectins, it may represent a new lectin family.

Other modes of binding between calnexin and its substrates, such as direct interaction between the polypeptide chains, cannot be excluded nor the possibility that there are more than one type of binding sites that could accommodate both oligosaccharides and peptide elements. Recent chemical cross-linking studies with the heavy chain of MHC class I antigens (and various truncated constructs thereof) have suggested that molecular contacts with calnexin occur at the level of the transmembrane domain (Margolese *et al.*, 1993). This implies that calnexin can associate with its substrates within or close to the bilayer and that the oligosaccharide of the heavy chain are not involved. Such a location for a binding site seems unlikely for soluble glycoproteins but is possible for membrane proteins. It is, on the other hand, conceivable that the MHC class I heavy chain is primarily bound via its oligosaccharide to a site in the ectodomain of calnexin, but the chemical cross-link between the polypeptide chains, used to detect the complex, can only form close to the membrane.

Whether a lectin or not, it is likely that calnexin plays a chaperone-like role. It may prevent premature transport of glycoproteins from the ER, their aggregation, and it may directly facilitate their conformational maturation. This would explain why glycoproteins tend to have folding problems in the presence of glucosidase inhibitors and tunicamycin. Although calnexin associated, the newly synthesized chains are probably free to interact with other chaperones. Vesicular stomatitis virus G protein provides an example of a protein that interacts both with BiP/GRP78 and with calnexin (Machamer *et al.*, 1990; Hammond *et al.*, 1994). In the case of oligomeric proteins, calnexin may support productive interactions between subunits during the formation of correctly assembled oligomers (Degen and Williams, 1991; Degen *et al.*, 1992; Hochstenbach *et al.*, 1992; Ou *et al.*, 1993; Anderson and Cresswell, 1994; Hammond *et al.*, 1994).

If calnexin binds primarily to the oligosaccharide moieties, it differs significantly from other chaperones that interact directly with the polypeptide moiety. Predestined to remain on the surface of the maturing protein, the oligosaccharides may provide convenient external appendages for chaperone attachment. As an external scaffold attached to these appendages, calnexin may simply help to keep the protein fixed in place with minimal interference in the folding process. More active functions in folding can also be envisaged. If calnexin has multiple binding sites for N-linked sugars, it may, for instance, help bring N-linked groups together, thereby facilitating specific contacts between peptide domains. This may explain why certain N-linked sugar moieties are more important for folding than others. By attaching to predestined surface groups, calnexin may also help to orient subunits of oligomeric proteins so that surfaces devoid of oligosaccharides not only remain exposed but optimally aligned for productive quaternary interactions.

To what extent the system depends on reversible binding between calnexin and the substrate proteins is unclear. Although the majority of folding intermediates of influenza HA at any given time are bound (Hammond *et al.*, 1994), it is possible that key trimming, folding, and oligomerization steps occur when the protein or individual oligosaccharides are temporarily dissociated. It is also intriguing to speculate that calreticulin—the abundant soluble homologue of calnexin in the ER—also participates as a lectin-like chaperone in glycoprotein maturation. Together with other proteins, calnexin and calreticulin may provide a matrix inside the lumen of the ER not unlike that in a lectin column.

THE DE- AND REGLUCOSYLATION CYCLE AND THE ROLE OF CALNEXIN

To explain how the release of glycoproteins from calnexin is adjusted so that it occurs only when complete folding has taken place, we have recently proposed a model that is shown in Figure 2 (Hammond and Helenius, 1993; Hammond *et al.*, 1994). We believe, that UDP-glucose:glycoprotein glucosyltransferase is a crucial factor in regulating the release process. We propose that the de- and reglucosylation cycle, driven by glucosidase II and the glucosyltransferase, represents a way for the cell to impose quality control. The capacity of the glucosyltransferase to recognize and specifically reglucosylate conformationally defective proteins secures, according to the model, continued calnexin association and ER retention of incompletely folded glycoproteins. Escape from the cycle occurs when a glycoprotein reaches a folded conformation and is no longer a substrate for the glucosyltransferase. For some proteins, this may require assembly into oligomers; for others it is enough to reach some specified degree of folding, which may only take a few minutes.

There may well be alternative ways for a glycoprotein to escape the de- and reglucosylation cycle. The trimming of mannoses by ER α-mannosidases may, for example, result in the removal of terminally misfolded proteins from the cycle. As already discussed, progressive loss of mannoses makes the N-linked oligosaccharide moieties of misfolded proteins less and less efficient as a substrate for the glucosyltransferase (Sousa *et al.*, 1992). Because ER mannosidases work slowly, proteins would be given plenty of time to take advantage of the folding machinery in the ER before final dissociation from calnexin and exposure to the ER degradation system.

In summary, it seems established that the ER contains a unique folding and quality control machinery for gly-

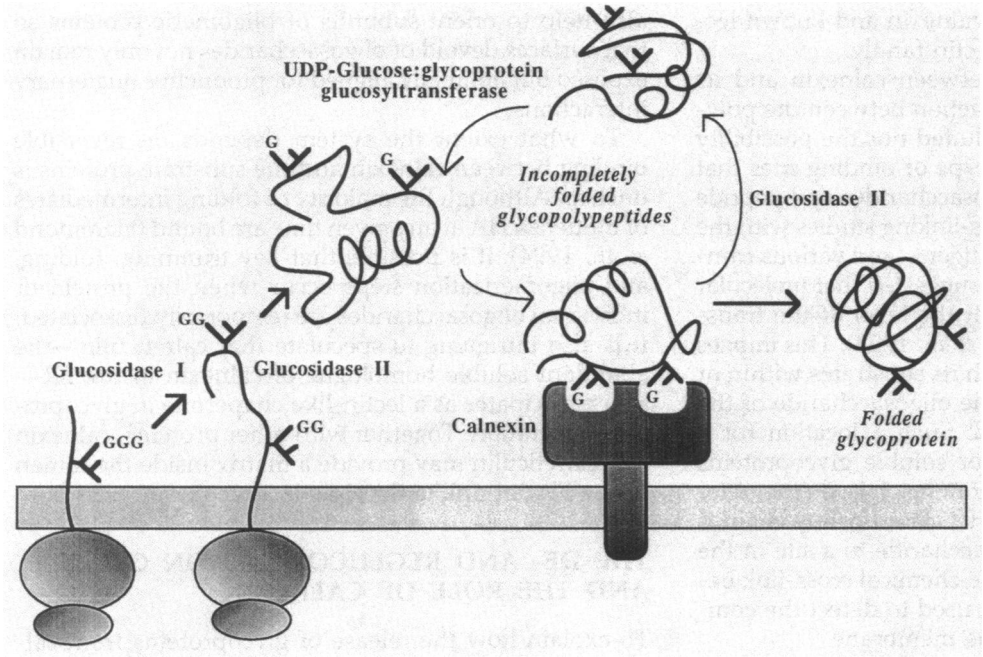

Figure 2. The suggested role of calnexin as a lectin-like ER chaperon. Calnexin binds to the monoglucosylated N-linked oligosaccharides of a soluble glycoprotein (G stands for a glucose residue). The binding to calnexin is transient and depends on the presence of a single glucose in the partially trimmed N-linked oligosaccharide side chains. Also shown is the hypothetical role that UDP-glucose:glycoprotein glucosyltransferase may be playing in the de- and reglucosylation cycle, in folding, and in quality control. Together with glucosidase II it drives a cycle of glycoprotein binding and release from calnexin. Because the glucosidase only uses incompletely folded proteins as its substrate, the cycle results in the retention of glycoproteins in the rough ER until they have folded and/or assembled properly.

coproteins. Calnexin serves as a retention factor and a chaperone. Glucosidase I is a signal activator that removes the terminal glucose from the protein-bound core oligosaccharide, thus converting it to a substrate for glucosidase II. Glucosidase II, in turn, serves as a signal modulator regulating binding to calnexin. We hypothesize, in addition, that UDP-glucose:glycoprotein glucosyltransferase serves as a quality control factor.

These functions, taken together, may explain why oligosaccharide trimming takes place in the ER. The controlled removal and readdition of individual glucose and mannose residues provides a way for the cell to sort and monitor glycoproteins at different stages of folding and assembly. The model also explains why glucosidase inhibitors affect folding and secretion of many proteins (see above) and why cell mutants with defects in glycosylation and trimming have problems in glycoprotein folding. Many of the observations described in the first part of this review, such as the drastic effect of bromoconduritol and the folding-enhancing effect of novel nonconstitutive glycosylation sites in proteins devoid of N-linked sugars, are also understandable in the light of these findings.

Because many proteins are able to fold and mature without oligosaccharide addition and because cells tolerate the presence of glucosidase inhibitors, it is clear that cells are not entirely dependent on the calnexin pathway. They can apparently handle the folding of most of their glycoproteins by alternative pathways. The redundancy of different folding factors in the ER lumen with overlapping capabilities is likely to be so great that the loss of calnexin binding does not lead to a block in central ER functions, at least in tissue culture. Although most glycoproteins have evolved not to rely entirely on calnexin during their folding, the calnexin pathway clearly increases the rate and efficiency by which they mature and get secreted.

OTHER EFFECTS OF N-LINKED OLIGOSACCHARIDES ON FOLDING

It is important to stress, in this context, that the calnexin pathway and the model described for assisted glycoprotein folding is not sufficient to fully explain the effect of N-linked saccharides on folding of many glycoproteins. This is illustrated by influenza HA of the X31 strain, a glycoprotein that normally folds in conjunction with calnexin (Hammond *et al.*, 1994). Castanospermine experiments show that it can fold without calnexin. However, when synthesized in the presence of tunicamycin, it misfolds, aggregates, and gets degraded (Hurtley *et al.*, 1989). This indicates that the oligosaccharides must have functions in folding beyond calnexin binding.

It has been suggested that, being polar, the core oligosaccharides increase the solubility of folding intermediates, thus helping proteins to avoid irreversible aggregation (Jaenicke, 1991; Marquardt and Helenius, 1992). In vitro refolding experiments have shown that the solubility of polypeptides is, indeed, at a low point during the folding process when hydrophobic groups and surfaces are still accessible to solvent. Aggregation due to the limited solubility and stability of folding intermediates is, in fact, the most common practical problem encountered during in vitro refolding experiments (for review, see Jaenicke, 1987).

A thorough in vitro refolding study has recently shown that the presence of core oligosaccharides improves the refolding and reactivation efficiency of yeast invertase by inhibiting aggregate formation (Kern *et al.*, 1992, 1993). The nine core oligosaccharide units present on the secreted form of this protein allow refolding at a 10-fold higher concentration of protein than without them. The aggregation of vesicular stomatitis virus G protein during refolding is also suppressed by the N-linked oligosaccharides during refolding (Crimmins and Schlesinger, 1982). In contrast, RNase A and human tissue plasminogen activator refold equally well with and without oligosaccharide chains (Parent *et al.*, 1986; Grafl *et al.*, 1987; for review, see Jaenicke, 1991).

A tendency of folding intermediates to aggregate is also frequently observed in living cells (Hurtley *et al.*, 1989; Mitraki and King, 1989; Tooze *et al.*, 1989; Valetti *et al.*, 1991). It is encountered as a side reaction in the ER and in other folding compartments. It occurs during overexpression of secretory proteins, and it causes the formation of inclusion bodies in *Escherichia coli* after transfection with foreign genes (Mitraki and King, 1989). If N-linked glycosylation is inhibited, most of the proteins made in the ER end up in aggregates.

In conclusion, it is clear that the N-linked oligosaccharides can have a direct solubilizing and perhaps also stabilizing effect on polypeptides during folding. In view of the high concentration of protein in the ER, this is an important additional reason for addition of N-linked sugars to polypeptide chains.

CONCLUSIONS

To support N-linked glycosylation in the ER, eukaryotic cells have evolved a complex biosynthetic machinery. The enzymes involved are distinct from those operating in the Golgi complex. One may ask why cells need parallel glycosylation systems in two organelles of the same secretory pathway. Why not add all the oligosaccharides to proteins in the Golgi complex, a compartment specializing in the assembly of complex polysaccharides and capable of effective selective addition of oligosaccharide chains to folded proteins and oligomers?

The concepts discussed in this review provide a partial answer to this question. Glycosylation in the ER occurs because oligosaccharides are needed to facilitate efficient folding, oligomerization, and quality control of newly synthesized proteins. To be helpful in these processes, they must not only be added in the ER, but addition must take place before the polypeptides begin to fold, i.e., already on the nascent chains. Once added, the core oligosaccharides allow attachment of the folding glycopolypeptide to calnexin and possibly other lectin-like chaperones in the lumen or membrane of the ER. In their differentially trimmed forms, the oligosaccharides serve as indicators of the folding status of the glycoprotein. In addition, they render the folding intermediates more soluble and stable, thus preventing aggregation and misfolding.

These functions are probably the primary reason why N-linked glycosylation originally evolved in eukaryotic cells. Being virtually invariant in biosynthesis, structure, and early processing from yeast to humans, the core oligosaccharide chains represent the most primitive form of N-linked oligosaccharides (Kornfeld, 1982). In all eukaryotic organisms, their functions are likely to be linked to conformational maturation of proteins in the ER. However, particularly in higher multicellular organisms, the same oligosaccharide groups have acquired numerous additional functions that rely on the extensive posttranslational processing and terminal glycosylation that takes place in the Golgi complex (see Varki, 1993). These additional functions are highly varied and have usually nothing to do with the original role that the oligosaccharides play in the ER. This is possible because, after leaving the ER, the N-linked oligosaccharides are no longer needed in their original role. They can be expropriated by the cell for novel tasks. The extent to which cells take advantage of this opportunity varies between cell type and organism.

Although superficially similar, the trimming and reglycosylation events that occur in the ER have a different function than those in the Golgi. In the ER they are intimately connected with folding and assembly processes, and their trimming reflects the folding status of the protein. In the Golgi their role is to prepare the N-linked oligosaccharide for new functions, which may range from intracellular targeting to cell–cell recognition. Most N-linked oligosaccharides, therefore, play distinct roles early and late in the life of a glycoprotein.

Folding and conformational maturation of newly synthesized proteins are intrinsically a difficult and error-prone processes. The presence of chaperones, folding enzymes, retention factors, and degradation systems is essential. The machinery present in the ER for assisting the folding process is probably the most elaborate and most concentrated in the eukaryotic cell. It allows production of huge quantities of secretory products while, at the same time, supporting the synthesis of elaborate multidomain proteins of large size and extreme complexity. The ER of eukaryotic cells performs its functions with astounding fidelity, expediency, and control. N-linked glycosylation and the calnexin pathway is clearly an integral part of the system that makes this possible.

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