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Role of Carbohydrate in Glycoprotein Traffic and Secretion

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I. INTRODUCTION

This chapter reviews the evidence that carbohydrate groups may function as markers for intracellular transport and sorting of membrane and secretory glycoproteins in vertebrate cells. The receptor-mediated trafficking to lysosomes of acid hydrolases with oligosaccharide chains containing mannose 6-phosphate residues is the best understood pathway by which proteins translocated into the endoplasmic reticulum are segregated and delivered to their ultimate destination. Evidence for this pathway is presented by Robbins in Chapter 11 of this volume as well as in several recent reviews (Sly, 1982; von Figura and Hasilik, 1986; Sahagian, 1987), and I shall not focus on the mannose 6-phosphate pathway in this chapter. However, there is increasing evidence that carbyhydrate groups

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may also be involved in the intracellular transport and/or sorting of specific plasma membrane and secretory proteins, and I shall review the literature relevant to this topic.

Several experimental approaches have given evidence that protein-bound glycan chains may have important roles in regulating intracellular glycoprotein traffic and will be discussed in this chapter. An exicting recent approach is the use of site-directed mutagenesis of the coding sequence to insert or delete glycosylation sites in recombinant proteins. Studies of mutant cells have shown that glycosylation defects often account for a significant fraction of the mutants that are isolated after selection for absence of cell surface receptors. Numerous studies have used tunicamycin, an inhibitor of synthesis of *N*-asparagine-linked glycan chains, to evaluate the functional role(s) of oligosaccharide chains in glycoprotein traffic in cells. Drugs that inhibit specific steps in the major processing pathway of N-linked glycan chains have recently become available, and these drugs have been used in studies to evaluate the potential involvement of oligosaccharide intermediates in glycoprotein traffic. A number of carbohydrate-binding proteins or lectins have been isolated from vertebrate cells, and I shall also present suggestive evidence that some of these lectins may have a role in regulating glycoprotein traffic.

The interpretation of the results of studies in which carbohydrate-deficient proteins are synthesized by cells owing to either mutations or drug treatment is often difficult because in specific cases unglycosylated proteins have been shown to be more sensitive to proteases, less soluble, or abnormally folded. Therefore, in order to evaluate in the proper context the evidence that carbyhydrate groups have a direct role in regulating the intracellular traffic of specific glycoproteins, a brief overview is presented of the structure and synthesis of protein-linked glycans and the influence of glycan chains on protein solubility, structure, and stability against proteolytic degradation.

II. EVIDENCE FOR INTRACELLULAR TRANSPORT SIGNALS

Secretory proteins are synthesized on ribosomes attached to the rough endoplasmic reticulum (RER), and following vectorial discharge into the lumen of the RER they are transported in vesicles from the RER to the Golgi apparatus and finally in secretory vesicles to the cell surface (Palade, 1975; Tartakoff, 1983a; Farquhar and Palade, 1981; Farquhar, 1985). A simple model for the intracellular transport of secretory proteins is the following: The luminal volume of the RER is continually being packaged into transport vesicles for shipment first to the Golgi and then to the cell

surface. Since plasma membrane proteins are also synthesized on the RER there would be a parallel flow of both secretory and plasma membrane proteins in vesicles to the cell surface. This nonspecific, passive-flow model for protein transport predicts that, since all secretory proteins are following the same pathway, the kinetics of secretion of all proteins in a single cell will be identical. Likewise, the model predicts that the kinetics of transport to the cell surface of all plasma membrane proteins will be identical. Although passive-flow models may explain the intracellular transport of some proteins (Cohen *et al.*, 1979; Cohen and Phillips, 1980), there is increasing evidence that the transport of most secretory and membrane proteins is highly regulated and that structural determinants on these proteins function as markers for intracellular transport.

Strous and Lodish (1980) were the first to report that, in the same cell, different secretory proteins are secreted at markedly different rates. They found, using cultured rat hepatoma cells, that newly synthesized albumin appeared in the medium roughly twice as fast as transferrin. These results were consistent with previous *in vivo* studies that found that albumin is secreted faster by liver than is transferrin (Morgan and Peters, 1971; Schreiber *et al.*, 1979). Asynchronous export of secretory proteins has been demonstrated in human and mouse hepatoma cell lines (Lodish *et al.*, 1983; Ledford and Davis, 1983), cultured rat hepatocytes (Fries *et al.*, 1984), and in exocrine pancreas (Scheele and Tartakoff, 1985). Export of nine secretory proteins by the human hepatoma cells in tissue culture fell into three discrete kinetic classes: (1) a rapidly secreted class with an intracellular retention half-time of 30–40 min (albumin, fibronectin, α-fetoprotein, and α_1-antitrypsin), (2) an intermediate secreted class with a half-time of 75–80 min (ceruloplasmin, α_2-macroglobulin, and plasminogen), and (3) a slowly secreted class with a half-time of 110–120 min (fibrinogen and transferrin) (Parent *et al.*, 1985).

Multiple secretory rates in single cells suggest either that there are multiple secretory pathways or that secretory proteins follow one common pathway through the cell but at different rates. Both mechanisms, however, imply that transport of secretory proteins is a selective rather than a passive-flow process and that specific receptors or carriers regulate intracellular transport of secretory proteins.

Similar results have also been reported for the kinetics of intracellular transport of newly synthesized membrane proteins. Fitting and Kabat (1982) have analyzed the synthesis and intracellular transport of two plasma membrane glycoproteins encoded by murine leukemia virus and they found that, although one viral membrane protein (gp93) was transferred quantitatively into plasma membranes of virus-infected cells within 60 min of [^{35}S]-methionine incorporation, the other glycoprotein (gp70) was

still accumulating in the plasma membrane after 210 min. Williams *et al.* (1985) reported that the rates of transport of two closely related membrane glycoproteins (class 1 histocompatability antigens H-2 and H-2D) to the cell surface were remarkably different (i.e., 1 hr versus 4–5 hr). They found by subcellular fractionation that the slowly transported protein accumulated in the endoplasmic reticulum (ER). These results suggest that intracellular transport of membrane proteins is also a selective rather than a passive-flow process.

Lodish and associates reported that differences in rates of serum protein secretion by hepatoma cells were due to variability in rates of transport from the RER to the Golgi; that is, retention in the RER was primarily responsible for the overall rates of secretion. They postulated that one or more receptor proteins in the RER membrane regulates the selective transport of secretory proteins into transport vesicles en route to the Golgi (Strous and Lodish, 1980; Lodish *et al.*, 1983). Fitting and Kabat (1982) reached similar conclusions in a study of two viral glycoproteins that mature from the RER to the cell surface at different rates. In these investigations the primary assay used to measure the rate of protein transport from the RER to the Golgi depended on measurement of the time required for N-linked glycan chains to become resistant to digestion by endo-β-*N*-acetylglucosaminidase H (endo H). The acquisition of endo H resistance, however, does not monitor the entry of secretory glycoproteins into the Golgi since oligosaccharides become endo H resistant after processing by α-mannosidase II, an event believed to occur in the medial Golgi compartment (Dunphy and Rothman, 1985). Therefore glycoproteins may reside in the cis and medial Golgi compartments for some unknown period before becoming resistant to endo H digestion. K. T. Yeo *et al.* (1985) employed three experimental approaches, i.e., cellular fractionation, acquisition of resistance of glycan chains to endo H digestion, and binding of glycoproteins to immobilized plant lectins, to measure the rates of glycoprotein transport within the RER and Golgi of hepatoma cells. They found that the variable export rates of glycoproteins from hepatoma cells reflect heterogeneous rates of glycoprotein transport within both the RER and the Golgi.

If transport receptors with varying affinities recognize specific "markers" on different membrane proteins, then it should be possible to isolate mutants defective in cell surface expression of specific membrane proteins and not others. Several such mutants have been described. For example, studies of Thy-1 antigen synthesis in mutant mouse lymphoma cells have established that specific oligosaccharide structures are essential for maturation of Thy-1 antigen and a selected group of other glycoproteins to the cell surface (Trowbridge *et al.*, 1978a; Chapman *et al.*,

1980). Fitting and Kabat (1982) immunoselected infected cell mutants with defects in the surface expression of either one or both plasma membrane glycoproteins encoded by murine leukemia virus. In addition, treatment of cells with drugs that inhibit either the synthesis or the normal processing of N-linked glycan chains has been shown to inhibit the appearance at the cell surface of specific membrane proteins, but not others (see Sections VII and VIII).

Two types of secretory routes have been identified in cells i.e., regulative and constitutive pathways (Tartakoff and Vassalli, 1978; Kelly, 1985). In the first, which occurs in many exocrine and endocrine cells, newly synthesized secretory proteins are concentrated and stored in intracellular granules awaiting specific signaling for secretion. In the second, which occurs in most cell types including fibroblasts, muscle cells, and hepatocytes, there is an apparently continuous and nonconcentrative exocytosis of secretory products. Sorting of secretory proteins into different vesicles has been demonstrated in a pituitary tumor cell line, which has both regulative and constitutive pathways (Gumbiner and Kelly, 1982; Moore *et al.*, 1983). However, it is presently unknown if similar sorting mechanisms also function in cells, like hepatocytes, which lack regulative pathways. Albumin and transferrin have been localized in the same intracellular vesicles of hepatoma cells, suggesting a common pathway (Strous *et al.*, 1983). Yokota and Fahimi (1981), however, found that albumin and lipoprotein are separately packaged in the ER and the cis Golgi but are intermixed in vacuoles of the trans Golgi consistent with multiple intracellular transport pathways in hepatocytes.

III. OLIGOSACCHARIDE BIOSYNTHESIS

The carbohydrate chains of glycoproteins may be classified according to the type of linkage to the polypeptide backbone. N-Glycosidic chains are attached to the amide group of asparagine, whereas O-glycosidic chains are linked to the hydroxyl group of such amino acid residues as serine, threonine, and hydroxylysine (for recent reviews, see Montreuil, 1980, 1982, 1987; Kornfeld and Kornfeld, 1980; Kobata, 1984). N-Glycosidic chains are divided into two major classes: the complex-type chains containing *N*-acetylglucosamine (GlcNAc), mannose (Man), galactose (Gal), fucose (Fuc), and sialic acid (SA) and the high-mannose-type chains containing *N*-acetylglucosamine residues and variable amounts of mannose. The O-linked chains commonly contain *N*-acetylgalactosamine, galactose, fucose, and sialic acid.

The number of different oligosaccharide species that have been identi-

fied on vertebrate glycoproteins is large and numbers in the hundreds. Synthesis of this diverse array of structures requires a considerable investment of a cell's total biosynthetic capacity, and at least 9 sialyltransferases, 7 fucosyltransferases, 9 galactosyltransferases, and 14 *N*-acetylglucosaminyltransferases are required to account for biosynthesis of linkages commonly observed in mammalian glycoproteins (Beyer and Hill, 1982). The large diversity of oligosaccharide species found on vertebrate glycoproteins suggests that protein-linked glycans have important roles in physiological processes that require a large information content.

Although individual cells can synthesize many different protein-linked glycan chains, the process is quite specific. It is controlled so that the oligosaccharide chains at an individual glycosylation site on most glycoproteins have one or a small number of closely related structures (Swiedler *et al.*, 1983, 1985). This implies that for many glycoproteins the specific structure of the oligosaccharide chain is important for the normal *in vivo* function of the glycoprotein.

The major pathway for biosynthesis of both complex and high-mannose N-linked glycans proceeds via a common high-mannose lipid-linked intermediate with the structure $Glc_3Man_9GlcNAc_2$-PP-dolichol (Fig. 1). The assembly of this dolichol-linked high-mannose oligosaccharide takes place in a series of reactions referred to as the lipid pathway (for reviews, see Snider, 1984; Krag, 1985; Kornfeld and Kornfeld, 1985). The first step in this pathway is the transfer of GlcNAc-1-P from UDPGlcNAc to dolichol phosphate (Dol-P) to form Dol-PP-GlcNAc. One GlcNAc, nine Man, and three Glc residues are then added by means of the corresponding sugar nucleotides or the glycolipid intermediates Dol-P-Man and Dol-P-Glc, resulting in the formation of the lipid-linked high-mannose oligosaccharide $Glc_3Man_9GlcNAc_2$. After assembly, the $Glc_3Man_9GlcNAc_2$ oligosaccharide is transferred en bloc to the nascent polypeptide chain, which is transported across or inserted into the RER membrane. Glycan transfer is carried out by oligosaccharide transferase which recognizes the acceptor amino acid asparagine (Asn) in the tripeptide sequence Asn-X-Ser (Thr), where X cannot be proline.

The $Glc_3Man_9GlcNAc_2$ oligosaccharide is the precursor of both high-mannose and complex-type oligosaccharides. In order for corresponding conversions to take place, a number of trimming reactions are required. α-Glucosidases I and II remove sequentially the outermost α-1,2-linked glucose and the two innermost α-1,3-linked glucose residues. α-Mannosidase activities then remove the four α-1,2-linked mannose residues and, after addition of GlcNAc to the $Man_5GlcNAc_2$ oligosaccaride, the α-1,3- and α-1,6-linked mannose residues to create the "core" $Man_3GlcNAc_2$ oligosaccharide intermediate. Until recently, all of the known oligosaccharide-trimming reactions involved the removal of one sugar residue in a

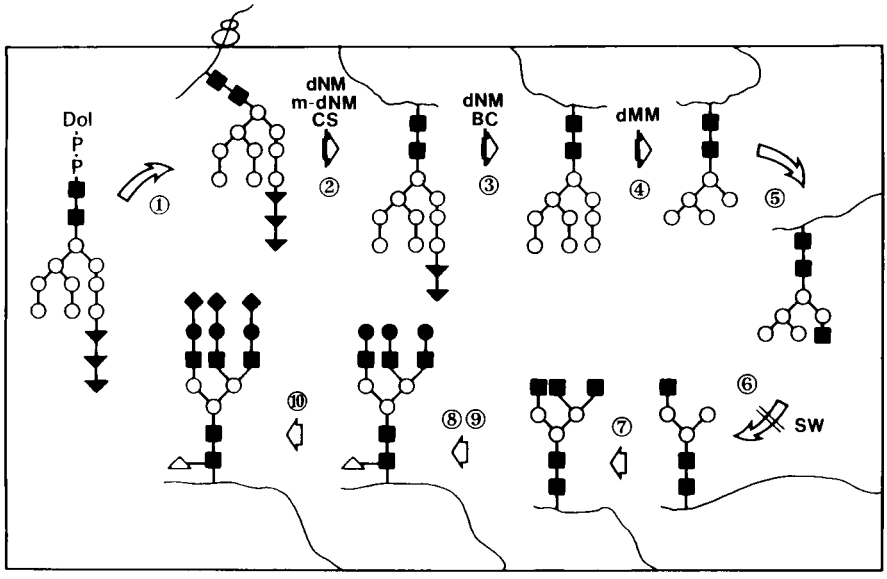

Fig. 1. Proposed sequence for the processing of peptide-bound N-linked glycans. Sites of inhibition by the following drugs are indicated by lines through arrows: dNM, 1-Deoxynorjirimycin; m-dNM, *N*-methyl-1-deoxynorjirimycin; CS; castanospermine; BC, bromoconduritol; dMM, 1-deoxymanojirimycin; SW, swainsonine. The reactions are catalyzed by the following enzymes: (1) oligosaccharyltransferase, (2) RER α-glucosidase I, (3) RER α-glucosidase II, (4) RER α-1,2-mannosidase, Golgi α-mannosidases 1A and 1B, (5) Golgi *N*-acetylglucosaminyltransferase I, (6) Golgi α-mannosidase II, (7) *N*-acetylglucosaminyltransferases II and IV, (8) fucosyltransferase, (9) galactosyltransferase, (10) sialyltransferase. Symbols: ■, *N*-acetylglucosamine; ○, mannose; ▲, glucose; △, fucose; ●, galactose; ▲, sialic acid. Adapted from Kornfeld *et al.* (1978).

single step. However, Lubas and Spiro (1987) reported that rat liver membranes contain a novel enzyme that has the capacity to trim $Glc_1Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ by the removal of the disaccharide Glcα1-3Man. After oligosaccharide trimming, other sugars such as *N*-acetylglucosamine, galactose, sialic acid, and fucose may be added sequentially by the corresponding glycosyltransferases to form a glycan of the complex type.

The first processing reactions, the removal of two or three glucose residues and perhaps also removal of a single α-linked mannose residue from the $Glc_3Man_9GlcNAc_2$ oligosaccharide, occur in the RER. However, the bulk of the oligosaccharide processing reactions occur in the Golgi, and recent studies have shown that processing enzymes are localized in specific subcompartments of the Golgi stack (for recent reviews, see Dunphy and Rothman, 1985; Farquhar, 1985; Hirschberg and Snider, 1987). Using immunocytochemical techniques and thin sections of HeLa cells,

Roth and Berger (1982) showed that galactosyltransferase was localized in only two or three trans cisternae of the Golgi stacks, and Dunphy *et al.* (1985) have localized *N*-acetylglucosaminyltransferase I to the medial Golgi cisternae. It is currently thought that the sequential processing reactions of N-linked oligosaccharides occur during the stepwise transfer of glycoproteins from their site of initial assembly in the RER, through subcompartments of the Golgi apparatus, to their final destinations at the cell surface, extracellular space, or intracellular organelles. Therefore, intermediates in the N-linked oligosaccharide processing pathway are appropriate markers to follow the stepwise transport of glycoproteins through subcellular compartments (Rotundo, 1984; K. T. Yeo *et al.*, 1985; Gabel and Bergmann, 1985).

An unusual feature of the biosynthetic pathway for N-linked oligosaccharides is the specific trimming of nine sugar residues from the precursor oligosaccharide before transfer of additional GlcNAc, galactose, sialic acid, and fucose residues to form complex structures. This appears wasteful unless the oligosaccharide intermediates have important roles within the cell. Recent studies using specific inhibitors of α-glucosidases I and II have demonstrated that the trimming of three glucose residues in the ER is required for efficient transport of some secretory, plasma membrane, and lysosomal enzymes from the ER to the Golgi apparatus (Gross *et al.*, 1983a; Lodish and Kong, 1984; Lemansky *et al.*, 1984; Repp *et al.*, 1985; Parent *et al.*, 1986).

Although $Glc_3Man_9GlcNAc_2$ appears to be the major oligosaccharide transferred from lipid to protein, there is also evidence for alternative pathways for N-linked glycosylation (for a complete discussion, see Krag, 1985). This is an important point because if all N-linked oligosaccharide chains are processed along an identical pathway in the ER and cis Golgi then it is unlikely that early oligosaccharide intermediates in the pathway would have any role in routing glycoproteins to different cellular locations. However, under normal culture conditions, Chinese hamster ovary (CHO) cells synthesize low levels of lipid-linked $Glc_3Man_5GlcNAc_2$ in addition to the predominant $Glc_3Man_9GlcNAc_2$ species which raises the possibility that these cells have a second glycosylation pathway (Chapman *et al.*, 1979). Two mutant cell lines, class E Thy-1$^-$ lymphoma cells (Chapman *et al.*, 1980) and B4-2-1 CHO cells (Stoll *et al.*, 1982,) are known to transfer $Glc_3Man_5GlcNAc_2$ to protein and subsequently process this species to form complex-type oligosaccharides. Similar results are obtained in glucose-deprived CHO cells (Rearick *et al.*, 1981) and in cells treated with an inhibitor of oxidative phosphorylation (Datema and Schwarz, 1981). Yamashita *et al.* (1983) have presented evidence that the sugar chains of ovomucoid and ovalbumin are synthesized by using differ-

ent lipid intermediates, and they suggest that lipid-linked $Glc_3Man_5GlcNAc_2$ may be the oligosaccharide donor for ovomucoid whereas lipid-linked $Glc_3Man_9GlcNAc_2$ is the donor for ovalbumin. Since both ovalbumin and ovomucoid are synthesized in the chicken oviduct these results imply that glycan chains on different proteins in the same cells may be processed by different pathways.

Until recently it was generally accepted that the presence of glucose in the oligosaccharide precursor is required for transfer in mammalian cells. However, Romero and Herscovics (1986) have demonstrated the transfer of a nonglucosylated oligosaccharide ($Man_7GlcNAc_2$) from dolichol pyrophosphate to protein in F9 cells, a mammalian cell line. Parodi *et al.* (1983a) have shown that glucose-free oligosaccharides are transferred in the N-glycosylation of proteins in the protozoa Trypanosoma cruzi, and this mechanism has also been described for yeast mutants which are blocked in the biosynthesis of dolichol pyrophosphate oligosaccharides (Huffaker and Robbins, 1983; Runge *et al.*, 1984).

An enzyme has been found in Triton-treated rat liver Golgi membranes which trims $Glc_1Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ with the releases of Glcα1-3Man (Lubas and Spiro, 1987). By removing a glucosylmannose disaccharide this endo-α-D-mannosidase provides a processing route alternative to the sequential actions of α-glucosidase 11 and α-mannosidase 1. These authors suggest that removal of this dissaccharide from N-linked glycans in the Golgi could serve as a signal in directing the intracellular movement and sorting of certain proteins.

Trimming of the four α-1,2-linked mannose residues from $Man_9GlcNAc_2$ oligosaccharides can be accomplished by two Golgi enzymes, α-mannosidases 1A and 1B (Tabas and Kornfeld, 1978; Tulsiani *et al.*, 1982). These two enzymes are present in comparable activities in membranes enriched in Golgi enzymes and are quite similar in substrate specificity and response to inhibitors. However, they are clearly distinguishable on the basis of behavior on cellulose phosphate chromatography, thermolability, and activity toward $Man_5GlcNAc$ and *p*-nitrophenylmannoside. As Tulsiani *et al.* (1982) have pointed out, the existence of two distinct Golgi enzymes, both of which can trim α-1,2-linked mannose residues from oligosaccharide chains, raises the possibility that different processing routes occur within the Golgi apparatus, leading to different classes of glycoproteins and/or different subsequent routing. Interestingly, mannose trimming of oligosaccharides destined to become high-mannose-type structures on mature μ chains of immunoglobulin M (IgM) was found to be much more rapid than mannose trimming on oligosaccharides destined to become complex-type structures (Brown and Hickman, 1986).

Additional evidence for multiple pathways are the findings that glucose can be directly transferred from UDPglucose to N-linked glycan chains in thyroid and liver tissue samples and that the reaction occurs in liver RER membrane fractions (Ronin and Caseti, 1981; Parodi *et al.*, 1983,a,b, 1984). Parodi *et al.* (1983a,b) have suggested that transient glucosylation of N-linked carbohydrates might be responsible for routing glycoproteins to specific cellular locations.

What factors specify the structure of the glycan at specific sites on a protein? Unlike the case of template-directed synthesis of proteins and nucleic acids, N-linked glycan chains are synthesized by the sequential activities of specific glycosyl transferases and glycosidases. Since single cells can synthesize a diverse array of glycans, the information which determines the oligosaccharide structure at an individual glycosylation site on a glycoprotein must be specified, in large part, by the primary structure of the protein. This implies that even if oligosaccharide chains function as transport markers for glycoprotein traffic in cells, perhaps by interacting with specific carbohydrate-binding receptors or carrier proteins, the information which specifies the transport marker must ultimately reside in the protein and gene which encodes the protein.

This argument is supported by recent studies on the specific recognition of lysosomal enzymes by UDP-*N*-acetylglucosamine–lysosomal-enzyme *N*-acetylglucosaminephosphotransferase (GlcNAc-P-transferase). This enzyme is responsible for the transfer of GlcNAc-1-P to selected Man residues on lysosomal enzymes. This reaction is the first in the two-step synthesis of the Man-6-P recognition marker for segregation of acid hydrolases into lysosomes (Kornfeld and Kornfeld, 1985; von Figura and Hasilik, 1986). It has been shown that GlcNAc-P-transferase selectively phosphorylates lysosomal enzymes, being able to recognize nonlysosomal glycoproteins only at very high concentrations (Reitman and Kornfeld, 1981a,b; Waheed *et al.*, 1982). Kornfeld and co-workers found that the determinant that allows the selective recognition of lysosomal enzymes by this enzyme was on the protein rather than the oligosaccharide portion of the lysosomal enzyme molecule, because deglycosylated enzymes (i.e., endo H treated) proved to be specific inhibitors of phosphorylation of intact enzymes (Lang *et al.*, 1984). Moreover, the intact protein may be required because even large proteolytic fragments did not retain the ability to be recognized.

Evidence that the primary structure of proteins has an important role in specifying the processing of N-linked chains to complex or high-mannose forms was obtained by analyzing the types of oligosaccharides present on closely related viral glycoproteins isolated from the same host cells. Since the virus utilizes the processing enzymes of the host cell, any difference in

oligosaccharide structure should reflect differences in the viral glycoproteins. Differences in the processing of N-linked glycan chains on viral glycoproteins to complex or high-mannose forms were observed when different strains of either influenza virus or vesicular stomatitis virus (VSV) were grown in the same host cell (Nakamura and Compans, 1979; Schwarz and Klenk, 1981; Hunt *et al.*, 1983) and when closely related murine leukemia viruses were grown in a number of hosts under various growth conditions (Rosner *et al.*, 1980).

Evidence that the primary structure of proteins can also influence the finer aspects of N-linked glycan structure comes from the work of Swiedler *et al.* (1985), using a murine B cell lymphoma. This cell line simultaneously synthesizes two classes of major histocompatability antigens that, within each class, share a high degree of amino acid sequence homology and possess N-linked glycosylation sites at invariant positions. They found that the specific patterns of sialylation and branching at individual glycosylation sites of these structurally related glycoproteins were unique, suggesting that subtle differences in peptide structure have an important influence on both branching and sialylation of N-linked oligosaccharides. Shears and Robbins (1986) studied the expression and glycosylation of the chicken ovalbumin gene in a heterologous cell, the mouse L cell. Mature chicken ovalbumin is heterogeneous with respect to its oligosaccharide chains with approximately equal amounts of high-mannose and hybrid forms. Hybrid chains have more than three α-linked mannose residues in addition to terminal GlcNAc, Gal, and sialic acid residues, and ovalbumin represents a rare class of glycoprotein containing large amounts of hybrid chains. Interestingly, chicken ovalbumin secreted by mouse L cells is processed predominantly to hybrid structures, suggesting that it is the polypeptide chain of ovalbumin which is responsible for processing of a substantial fraction of the oligosaccharide side chains to hybrid structures.

Although much work has focused on the glycosylation of both secretory and cell surface glycoproteins, comparatively little is known about the glycosylation of resident glycoconjugates in subcellular organelles. Recently two groups have described a novel protein–saccharide linkage, O-linked GlcNAc (Torres and Hart, 1984; Schindler *et al.*, 1987). Most of these structures are on a 62-64,000 kDa protein, which is a major component of the nuclear pore complex (Davis and Blobel, 1986; Schindler *et al.*, 1987). Schindler and Hogan (1984) have postulated that glycosylation with O-linked GlcNAc monosaccharide residues could serve to direct the transport of proteins to the nucleus in a manner analogous to the targeting of lysosomal enzymes to lysosomes via the attachment of mannose 6-phosphate residues. However, the finding that O-linked GlcNAc moieties

are also found in other subcellular compartments may argue against this model (Holt and Hart, 1986).

IV. ROLE OF CARBOHYDRATE IN PROTEIN SOLUBILITY, STRUCTURE, AND STABILITY

The presence of glycan chains can have a great effect on the solubility properties of glycoproteins (Tarentino *et al.*, 1974). Many proteins function in aqueous environments, and this environment tends to orient hydrophobic residues in the interior of the protein and hydrophilic residues on the exterior. Oligosaccharide chains can substantially contribute to the net hydrophilicity on the exterior surface of proteins, and this influences protein solubility. The most hydrophilic sugar residues are those with a net ionic charge at physiological pH. Each sialic acid carries one net negative charge, and therefore the presence of N-linked or O-linked oligosaccharide chains containing sialic acid can substantially affect the net charge on a glycoprotein (Schauer, 1985). The effect of covalently attached carbohydrate on the solubility of a number of proteins was examined by Lawson *et al.* (1983), using polyethylene glycol precipitation. They found both increases and decreases in protein solubility depending on the state of glycosylation, the type of protein, and temperature. For example, ribonuclease A (nonglycosylated) was less soluble than ribonuclease B (one *N*-glycan chain containing $Man_6GlcNAc_2$) at temperatures between 0° and 30°C, but at higher temperatures the B form was slightly less soluble.

Protein conformation may be altered by the presence of oligosaccharide chains. However, the relative importance of carbohydrate chains on the conformation and folding of glycoproteins is unclear. Enzymatic removal of carbohydrate from glycoprotein enzymes usually has little or no effect on catalytic efficiency of the enzyme, and this has been demonstrated for numerous enzymes, including RNase B, DNase I, invertase, carboxypeptidase Y, and mung bean nuclease (Tarentino *et al.*, 1974; Trimble and Maley, 1977).

Since N-linked glycosylation of glycoproteins is an early event and often occurs on nascent chains in the RER (Bergman and Kuehl, 1982), it is possible that N-linked oligosaccharide side chains may influence proper folding of nascent proteins. This would explain why removal of oligosaccharide units from the mature protein, which has already achieved its correct conformation, might have few deleterious effects. In recent years, however, a number of eukaryotic glycoproteins have been produced in *Escherichia coli,* using recombinant DNA technology, and many of these

proteins are fully active despite the absence of glycosylation, which suggests that glycan chains are not required for proper folding of these proteins. Examples include fibroblast interferon (Derynck *et al.*, 1980), immune interferon (Grey *et al.*, 1982; Grey and Goeddel, 1983), plasminogen activator (Pennica *et al.*, 1983), α_1-antitrypsin (Courtney *et al.*, 1984), urokinase (Gunnzler *et al.*, 1984, and interleukin 2 (Svedersky *et al.*, 1984).

The influence of carbohydrate on the conformation and refolding of pancreatic ribonuclease has been extensively studied. Using various spectral properties (circular dichroism, optical rotatory dispersion, and UV difference spectroscopy), Puett (1973) concluded that the secondary and tertiary structures of glycosylated and nonglycosylated bovine RNase are identical. Of the known ribonucleases, the procine enzyme has the highest reported content of carbohydrate, around 35%. In a study comparing glycosylated and nonglycosylated porcine RNase, Wang and Hirs (1977) found that carbohydrate was without influence on the rate-limiting steps in the refolding of the protein from the denatured state or on the overall configurational stability of the molecule, but did influence the environment around two tyrosine side chains to effect a stabilization of the surface structure. The effects observed were relatively small, however, and two recent studies have also reported that the mechanism of folding of pancreatic ribonuclease is independent of covalently linked carbohydrate (Krebs *et al.*, 1983; Grafl *et al.*, 1987). Apparently the information for the folding of ribonuclease and perhaps most other glycoproteins is contained exclusively in the protein moiety, i.e., in the amino acid sequence.

Chu *et al.* (1978) found that the presence of carbohydrate on yeast invertase stabilized the enzyme to denaturation by mild acid, heat, or repeated cycles of freezing and thawing. Removal of the oligosaccharides of carboxypeptidase Y by digestion with endo H doubled the rate of inactivation by treatment with sodium dodecyl sulfate as compared to the glycosylated enzyme (Chu and Maley, 1982). Assuming both forms bound the same amount of detergent, this result suggests the carbohydrate may help maintain the active conformation of this enzyme.

The effects of depleting an envelope glycoprotein of carbohydrate has been extensively studied with the G protein of vesicular stomatitis virus (VSV) (Gibson *et al.*, 1979, 1980, 1981; Leavitt *et al.*, 1977). The nonglycosylated G protein of VSV (San Juan) is not transported to the cell surface and aggregates in the ER when virus is grown in cells treated with tunicamycin (TM) either at the normal temperature (38°C) or at a lower temperature (30°C). Nonglycosylated G protein from another VSV strain (Orsay), however, was partially transported to the cell surface at the

lower temperature. A similar strain difference was found using *in vitro* assays designed to measure the solubility on nonionic detergent and the aggregation of nonglycosylated G proteins. The aggregation was most dramatic for the G protein (San Juan), but was also evident for the G protein (Orsay) at the higher temperature (Gibson *et al.*, 1979). This strain difference was also noted in assays for aggregation of G proteins isolated from VSV-infected mutant cell lines in which a truncated core oligosaccharide was transferred to the polypeptide chain (Gibson *et al.*, 1981). The G proteins of the San Juan and Orsay virus strains are closely related, but they contain 10 amino acid differences (Gallione and Rose, 1983). These amino acid differences must be responsible for the variation in the carbohydrate requirement for solubility.

The results of these studies using two VSV strains have been interpreted to indicate that carbohydrate has a significant influence on the folding and conformation of G proteins (Gibson *et al.*, 1980). It should be pointed out, however, that alterations in the folding and conformation of G proteins were not directly demonstrated in these studies. Rather, the influence of carbohydrate on the aggregation and solubility properties of G proteins from the two VSV strains was demonstrated. Since carbohydrate chains per se can have a large effect on the temperature-dependent solubility properties of glycoproteins, it is not clear from the results of these studies if carbohydrate significantly alters the conformation of G proteins.

Perhaps one reason why glycan chains do not appear to have a major effect on the conformation of many glycoproteins is that most glycan chains are located in or near β turns or other turn or loop structures in the protein. Beeley (1977) applied methods for predicting peptide chain conformation to amino acid sequences adjacent to the carbohydrate attachment sites of glycoproteins containing N-linked oligosaccharide chains. Of 31 glycosylated residues examined, 30 occur in sequences favoring turn or loop structures. Twenty-two of the glycosylated asparagine residues occur in tetrapeptides predicted to have the β-turn conformation. Similar conclusions were drawn in another study of 9 O-glycosidic linkages and 28 N-glycosidic linkages (Aubert *et al.*, 1976). For the O-glycosidic linkages, the serine or threonine residues that were involved in the linkages were predicted to be in a β turn. Nineteen of 28 N-glycosidic linkages occur in sequences favoring β turns. The N-glycosidic linkages that were not located in β turns were situated in a random region near a β turn, probably exposed to the solvent or near the carboxy-terminal end.

There are numerous studies that suggest that the presence of oligosaccharide chains on a protein may decrease its susceptibility to proteases (for a recent review, see Olden *et al.*, 1982). These studies include both comparisons of loss of activity during treatments with proteases *in vitro*

and comparisons of turnover rates *in vivo* after treatment of cells with inhibitors of glycosylation. The reason for the increased susceptibility of nonglycosylated protein probably varies with the protein. One reason, however, may be the decreased protection from proteases of bends (β turns) in the polypeptide backbone believed to occur at glycosylation sites in glycoproteins (Bause, 1983). In some cases the rapid degradation of nonglycosylated secretory proteins in TM-treated cells may be due to the improper compartmentalization of lysosomal proteases with secretory proteins rather than to the inherent sensitivity of nonglycosylated proteins to proteolytic attack. TM treatment of cells interferes with the Man-6-P-directed transport of acid hydrolases to lysosomes, and we found that chicken embryo fibroblasts treated with TM secrete lysosomal cathepsin B-like activity (Parent *et al.*, 1982). Examples of proteins known to have an increased susceptibility to proteases in their nonglycosylated form include hemagglutinin precursor of influenza virus (Schwarz *et al.*, 1976), fibronectin in chick embryo fibroblasts (Bernard *et al.*, 1982; Hynes and Yamada, 1982), adrenocorticotropin–endorphin precursor in toad pituitaries (Loh and Gainer, 1978, 1979, 1980), alkaline phosphatase in murine cells (Firestone and Heath, 1981), and carboxypeptidase Y of yeast cells (Chu and Maley, 1982).

V. EVIDENCE FOR CARBOHYDRATE TRANSPORT SIGNALS USING SITE-DIRECTED MUTAGENESIS

The laboratory of John Rose has recently developed a novel experimental approach to study the role of carbohydrate in glycoprotein traffic by the use of site-directed mutagenesis of the coding sequence either to insert or to delete glycosylation sites in cloned proteins. Using these techniques, Machamer *et al.* (1985) investigated the role of glycosylation in intracellular transport and cell surface expression of the VSV G protein in cells expressing G protein from cloned cDNA. The individual contributions of the two N-linked oligosaccharide chains of G protein to the cell surface expression were assessed by eliminating one or the other or both of the glycosylation sites in cloned proteins. Interestingly, they found that one oligosaccharide chain at either position was sufficient for cell surface expression of G protein in transfected cells, and the apparent rates of intracellular transport of these proteins were similar to the rate observed in wild-type protein. However, the nonglycosylated G protein synthesized when both glycosylation sites were eliminated did not reach the cell surface. This protein did appear to reach a Golgi-like region, as determined by immunofluorescence microscopy, and was not subject to increased intracellular proteolytic degradation. These results suggest that

carbohydrate is a direct signal for intracellular transport of G protein from the Golgi to the cell surface.

An alternative model is that carbohydrate is required for maintaining a polypeptide conformation essential for intracellular transport. This model is less appealing, however, because glycosylation at either site promotes efficient transport of G protein, and it seems unlikely that glycosylation at two different positions in the molecule would induce the same conformational changes. In addition, Machamer and Rose have introduced glycosylation sites at several new positions in G protein lacking the two normal sites. Their initial results show that at least one new glycosylation site does promote transport of G protein to the cell surface (Machamer *et al.*, 1985).

Additional evidence that carbohydrate can be a signal for intracellular transport has come from studies of a hybrid membrane protein. Guan and Rose (1984) constructed a hybrid gene encoding a membrane-anchored form of rat growth hormone. This protein is anchored in cellular membranes by a carboxy-terminal extension on the hormone composed of the transmembrane and cytoplasmic domains of the VSV G protein. The protein is transported efficiently to the Golgi but not to the cell surface. In order to determine if N-linked carbohydrate promotes surface expression of this hybrid protein, Guan *et al.* (1985) generated mutant proteins using *in vitro* mutagenesis in which single amino acids at two sites in anchored growth hormone were changed to generate consensus sequences required for addition of N-linked oligosaccharides. These mutant proteins, and a protein with both glycosylation sites, were both glycosylated and transported to the cell surface. Kalderon *et al.* (1985) have suggested that one criterion of a transport signal is that it be able to function at multiple sites in the polypeptide chain. Therefore, the findings that glycosylation at multiple sites in the G protein as well as in the growth hormone–hybrid membrane protein promotes efficient transport to the cell surface is strong evidence that the carbohydrates function as a transport signal on these proteins.

VI. MUTATIONS THAT ALTER GLYCOSYLATION AND GLYCOPROTEIN TRAFFIC

It is frequently found that mutants defective in surface appearance of membrane proteins are defective in carbohydrate processing (Stanley, 1984, 1985; Tartakoff, 1983b). Perhaps the most extensive studies have been concerned with the surface expression of the Thy-1 surface antigen by mouse lymphoma cells (Trowbridge *et al.*, 1978a,b; Trowbridge and

Hyman, 1979). The Thy-1 glycoprotein is about 30% carbohydrate by weight (Barclay, 1976), and the molecule from rat brain contains three N-linked oligosaccharides (Campbell, 1981). Thy-1 is found on the neurons and T lymphocytes of many animal species, and it is the most abundant surface protein of the mouse T cell. Mutant mouse lymphoma cells which bore less than 0.1% as much surface Thy-1 as did the wild-type cells were selected. Several clones were identified that continued to synthesize Thy-1 and apparently were defective in intracellular transport. The mutations proved to be recessive, and five complementation classes of mutant cells were characterized by somatic genetic analysis. Defects in the oligosaccharide units of Thy-1 glycoprotein were demonstrated in four of the five classes of mutants.

The specific defect in most of these mutant classes remains unknown; however, in the case of mutants belonging to complementation group E the analysis has been taken much further. The class E Thy-1$^-$ mutation has been shown to be due to a mutation in a gene that acts to transfer mannose from GDPmannose to dolichol phosphate (Chapman *et al.*, 1980). In consequence, class E Thy-1$^-$ mutant cells are unable to synthesize a normal lipid-linked $Glc_3Man_9GlcNAc_2$ oligosaccharide but instead synthesize a smaller $Glc_3Man_5GlcNAc_2$ moiety that is transferred to the nascent polypeptide. Consequently, the cell surface glycoproteins of class E Thy-1$^-$ mutant cells show a pleiotropic alteration in their mannose-containing N-linked oligosaccharides. Interestingly, lipid-linked $Glc_3Man_5GlcNAc_2$ has also been identified as a minor species present in normal CHO cells (Chapman *et al.*, 1979) and may be the oligosaccharide donor lipid for specific proteins in normal cells (Yamashita *et al.*, 1983).

Many cell surface glycoproteins are expressed normally on the class E Thy-1$^-$ mutant cell. However, in addition to the Thy-1 glycoprotein, molecules bearing the Ly-6.2 and H9/25 determinants are not expressed on the cell surface of class E Thy-1$^-$ mutant cells (Horton and Hyman, 1983). It is probably due to the selectivity of the defect that the mutant cells are viable. Immunocytochemical studies show that wild-type cells have Thy-1 glycoprotein predominantly localized on the cell surface with only minimal amounts distributed in the cytoplasm. In class E mutant cells, Thy-1 glycoprotein is not expressed on the cell surface but is detectable in the ER, Golgi region, and lysosomes (Bourguignon *et al.*, 1982).

It is not clear why the lesion in normal oligosaccharide processing characteristic of class E mutant cells prevents transport of Thy-1 and other glycoproteins to the cell surface. However, specific changes in oligosaccharide structure are necessary to block transport of Thy-1 glycoprotein. Lectin-resistant mutants with specific defects in glycosylation have been selected from mouse lymphoma cells (Trowbridge *et al.*,

1978b). Some glycosylation defects that confer resistance to the cytotoxic effects of concanavalin A block the expression of Thy-1 glycoprotein on the cell surface. However, glycosylation defects in other lectin-resistant mutants have no effect on the transport of Thy-1. The solubility properties in detergent of Thy-1 molecules isolated from Thy-1$^-$ cells suggests that the block in transport is not due to aggregation and precipitation of abnormally glycosylated molecules within the cell (Trowbridge *et al.*, 1978b). Apparently, transfer of the normal oligosaccharide precursor to protein is required for transport of Thy-1 glycoprotein and a selected group of other glycoproteins to the cell surface in mouse lymphoma cells. It is not known, however, if altered oligosaccharide processing is the primary defect that prevents Thy-1 transport in class E mutants. Alternatively, the activity of another protein that regulates intracellular transport may be affected in these pleiotropic mutants.

A CHO mutant (B4-2-1) that was isolated because of decreased levels of Man-6-P receptor activity (Robbins *et al.*, 1981) has been shown to have a general defect in synthesis of high-mannose N-linked carbohydrate chains. This defect results from the inability of these cells to synthesize mannosylphosphoryldolichol and is very similar to the class E Thy-1$^-$ mutation (Stoll *et al.*, 1982). Lysosomal enzymes in these CHO mutants, which synthesize a truncated oligosaccharide–dolichol precursor, appear to acquire the Man-6-P recognition marker (Stoll *et al.*, 1982; Gabel and Kornfeld, 1982). However, they are not compartmentalized correctly in the mutant cells because of an altered Man-6-P receptor (Robbins and Myerowitz, 1981).

A large number of mutant CHO cells that do not express active cell surface low density lipoprotein (LDL) receptors have been isolated, and they have been shown to fall into four complementation groups (ldlA–ldlD) by somatic genetic analysis (for a recent review, see Krieger *et al.*, 1985). The mutants in three of the complementation groups (ldlB–ldlD) have defects in processing of N-linked, O-linked, and lipid-linked carbohydrate chains (Kingsley *et al.*, 1986b). LDL receptors synthesized by cultured human cells contain one N-linked glycan chain and six to nine O-linked chains (Cummings *et al.*, 1983). The glycosylation defects that dramatically affect LDL receptor function are specific since 17 different types of previously isolated CHO glycosylation mutants have nearly normal levels of LDL receptor function. ldlB and ldlD mutants have significant levels of cell surface Man-6-P receptor activity; therefore, only the expression of specific surface proteins is affected by the glycosylation defects (Leichter and Krieger, 1984). Enhanced degradation of LDL receptors in ldlB, ldlC, and ldlD mutants can largely account for their LDL

receptor-deficient phenotype although defective transport of mutant receptor to the cell surface may also be involved.

The biochemical lesions that cause the glycosylation defects and LDL receptor-deficient phenotype in ldlB and ldlC mutants are unknown. The structural changes in ldlD cells, however, can be accounted for by a general defect in addition of Gal and GalNAc residues to glycoconjugates caused by a severe deficiency in UDPGal/UDPGalNAc 4-epimerase activity (Kingsley *et al.*, 1986a). Without this enzyme, ldlD cells are unable to synthesize normal amounts of UDPGal and UDPGalNAc from their corresponding Glc precursors. Addition of Gal and GalNAc to the culture medium allows the ldlD mutant to synthesize these UDP-sugars via salvage pathways and fully corrects all of the structural defects in the cells. Gal alone fully corrects N-linked but not O-linked processing defects and does not induce LDL receptor activity. In contrast, GalNAc alone partially corrects O-linked but not N-linked processing defects and substantially increases LDL receptor activity. It thus appears that defects in O-linked processing may be the primary cause of the LDL receptor-deficient phenotype in the ldlD mutant. Seventy to 85% of the O-linked chains of the LDL receptor are clustered in a single serine/threonine-rich domain of the protein. Because this domain can be deleted without affecting either the function or the stability of LDL receptors (Davis *et al.*, 1986), O-linked chains elsewhere on the LDL receptor may be particularly important for LDL receptor function. Alternatively, addition of GalNAc to the culture medium of ldlD mutants may alter other cell components that normally interact with the LDL receptor protein.

In the examples of mutant cells discussed until now, the mutation alters the ability of the cell to accomplish normal glycosylation of glycoproteins or alters the machinery of glycoprotein transport itself. This often results in a pleiotropic defect in the expression of many glycoproteins in mutant cells. The close associations between glycosylation defects and altered expression of the Thy-1 antigen, the Man-6-P receptor, and the LDL receptor emphasize the importance of carbohydrate chains for the expression and function of some cell surface glycoproteins.

In theory, mutations could also alter the primary structure of the protein undergoing transport and thereby prevent normal attachment and processing of carbohydrate transport markers. A possible example in this category of transport mutations may be found in the human disease α_1-antitrypsin deficiency (for a recent review, see Carrell *et al.*, 1984). α_1-Antitrypsin, the principal protease inhibitor in serum, is a single polypeptide glycoprotein, and the normal form in serum has three complex, N-linked oligosaccharides (Hodges *et al.*, 1979; Mega *et al.*, 1980). The *Z*

variant allele encodes a mutant protein with a single substitution of glutamic acid by lysine at position 342 (Yoshida *et al.*, 1976; Jeppsson, 1976). Since homozygotes for the *Z* allele have an 85% reduction in circulating levels of α_1-antitrypsin and substantial deposits of α_1-antitrypsin accumulate within the RER of hypatocytes, there appears to be a defect in secretion of the *Z* variant. The *Z* form of α_1-antitrypsin isolated from RER inclusions has normal protease inhibitory activity and essentially normal thermal stability (Bathurst *et al.*, 1984). Thus, the *Z* variant of α_1-antitrypsin must be in its native form with the reactive center in the intact strained conformation (Lobermann *et al.*, 1984), and it is unlikely that accumulation in RER is due to a gross misfolding of the polypeptide.

Interestingly, RER inclusions recovered from the liver of *ZZ* homozygotes contained α_1-antitrypsin bearing high-mannose N-linked glycan chains with attached glucose residues (Hercz *et al.*, 1978; Hercz and Harpaz, 1980; Bathurst *et al.*, 1984). Removal of glucose from the oligosaccharide precursor on glycoproteins usually occurs very rapidly after transfer to protein (Hubbard and Robbins, 1979; Atkinson and Lee, 1984). Treatment of rat hepatocytes and human hepatoma cells with deoxynorjirimycin, an inhibitor of processing ER glucosidases, has been shown to inhibit secretion and transport of α_1-antitrypsin from the RER to the Golgi, and it has been postulated by Lodish and Kong that oligosaccharide processing intermediates may function as part of a tag for rapid transport of α_1-antitrypsin from the RER to the Golgi (Gross *et al.*, 1983a; Lodish and Kong, 1984; Parent *et al.*, 1986). A plausible hypothesis is that the *Z* form of α_1-antitrypsin accumulates in the liver of *ZZ* homozygotes because the single amino acid substitution in the primary sequence alters the conformation of the molecule, thereby preventing the trimming of glucose residues from N-linked glycan chains on α_1-antitrypsin. In consequence, the rapid, receptor-mediated transport of α_1-antitrypsin from the RER to the Golgi is blocked. In this context, it is interesting that in a class of cellular transport mutants identified among immunoselected cells infected with Friend erythroleukemia virus, viral glycoprotein gp55 is not transported to the cell surface and the intracellular gp55 in these mutants contains immature forms of high-mannose oligosaccharides containing glucose (Ruta *et al.*, 1982).

VII. TUNICAMYCIN AND GLYCOPROTEIN TRAFFIC

One of the most widely used inhibitors to evaluate the role of N-linked oligosaccharides in the transport and expression of secretory and membrane glycoproteins is tunicamycin (TM), a nucleoside analog which was isolated from *Streptomyces lysosuperificus* by Takatsuki *et al.* (1971).

This antibiotic inhibits the transfer of GlcNAc-1-P from UDPGlcNAc to dol-P (Tkacz and Lampen, 1975, Takatsuki *et al.*, 1975). Since this step is first in the lipid-linked pathway, TM treatment blocks N-linked glycosylation of protein (for reviews, see Schwarz and Datema, 1982a,b; Olden *et al.*, 1982; Elbein, 1984).

I believe there has been some confusion in the interpretation of results using this drug because it is sometimes assumed that, if N-linked oligosaccharides have an important role in the intracellular transport of secretory and membrane glycoproteins, then TM treatment should totally block secretion or surface expression of these proteins. Therefore, the appearance at the cell surface of TM-treated cells of even greatly reduced levels of secretory or membrane proteins has been used as evidence in some studies that carbohydrate does not have an important role in glycoprotein transport to the cell surface. However, this assumption is misleading because recent studies mostly of the Man-6-P-dependent transport of acid hydrolases to lysosomes have clearly demonstrated that cells can export proteins by a nonspecific pathway when the receptor-mediated pathway is inoperative. Cells defective in the Man-6-P pathway, either because they are deficient in functional Man-6-P receptors (Robbins and Myerowitz, 1981, Gonzalez-Noriega *et al.*, 1980) or lack enzymes for synthesis of the Man-6-P transport marker (Kornfeld and Kornfeld, 1985; von Figura and Hasilik, 1986), have low levels of activities of acid hydrolases in lysosomes and secrete large amounts of acid hydrolases. There is also evidence for a nonspecific pathway for protein export in yeast. Normally carboxypeptidase Y, and vacuolar enzyme in yeast analogous to a lysosomal enzyme in mammals, is efficiently localized to the vacuole. Plasmid-directed overproduction, however, results in secretion of a large fraction of the protein, apparently because the carboxypeptidase Y-sorting receptor is saturable (Stevens *et al.*, 1986).

The nonspecific pathway for protein export which operates when receptor-mediated pathways are inoperative may be analogous to passive flow of protein in vesicles from the RER to the Golgi and then to the cell surface. Therefore, it is not logical to expect that TM treatment would totally prevent the surface appearance of secretory and membrane glycoproteins even if carbohydrate does have an important functional role in the normal transport of these proteins. TM treatment, however, may substantially alter both the kinetics of intracellular transport of secretory and membrane proteins as well as the precise pathway taken by these proteins from their sites of synthesis in the RER to the cell surface.

The effect of TM on the secretion of immunoglogulins has been extensively studied. Different classes of immunoglobulins have different carbohydrate requirements for secretion. Thus, secretion of IgM and IgE was

almost totally inhibited by TM, secretion of IgA was partially inhibited, and secretion of IgG was essentially unaffected (Hickman *et al.*, 1977, Hickman and Kornfeld, 1978). IgA accumulated in the RER, and no increase in intracellular degradation of nonglycosylated immunoglobulins could be demonstrated. The extent of inhibition of immunoglobulin secretion by TM in these studies correlates with the carbohydrate content of the various immunoglobulin classes, and Hickman and Kornfeld (1978) suggested that those molecules that are highly glycosylated might be most affected by inhibition of glycosylation. However, Sidman (1981) found that secretion of IgD, which is as heavily glycosylated as IgM, is not inhibited by TM, and a survey of published studies using this drug does not suggest a correlation between the extent of glycosylation of secretory proteins and the sensitivity of secretion to TM.

The level of differentiation of cells may be important in determining the sensitivity to TM. Sibley and Wagner (1981) found that secretion of IgM by a mouse B cell lymphoma (W279) was not affected by TM treatment, in contrast to the profound inhibition of secretion observed by Hickman and Kornfeld (1978) when plasmacytoma cell (104E) was treated with TM. Although both W279 and 104E cells secrete IgM, the two are models for cells at very different points along the B cell differentiation pathway and the plasmacytoma cell synthesizes about 50 times as much IgM as the B cell lymphoma. Interestingly, Ciccimarra *et al.* (1976) have described a defect in agammaglobulinemia in which the IgG secretory block was associated with a failure to glycosylate the IgG heavy chains while the incorporation of [^{3}H]mannose and [^{3}H]glucosamine into other cellular proteins was normal.

In single cells, TM inhibits secretion of specific glycoproteins only, and export of other glycoproteins is unaffected. TM treatment of human hepatoma cells significantly delayed secretion of α_2-macroglobulin, ceruloplasmin, and α_1-antitrypsin, but secretion of transferrin, fibrinogen, α-fetoprotein, fibronectin, and plasminogen was not affected by the drug (Bauer *et al.*, 1985). The most dramatic effect of TM treatment was on the secretion of α_2-macroglobulin, with more than 90% of the protein still present in the cell fraction in an undegraded form 7 hr after synthesis, while in control cells 50% of newly synthesized α_2-macroglobulin is secreted by 75–80 min. TM treatment of human hepatoma cells also markedly affected thyroxine-binding globulin (TBG) secretion, almost doubling the time required for secretion of 50% of the protein (Bartalena and Robbins, 1984).

Secretion of a major glycoprotein constituent of the mouse egg's extracellular coat (ZP2) was inhibited by TM, and nonglycosylated ZP2 accumulated intracellularly (Roller and Wassarman, 1983). However, TM did not inhibit secretion of ZP3, another mouse egg glycoprotein. TM inhibits

secretion and proteolytic processing of prepro-α-factor in yeast (Julius *et al.*, 1984). The effect appears to be on transport since intracellular forms accumulate. Inhibition of glycosylation of the secreted acid phosphatase of yeast by TM resulted in synthesis of nonglycosylated, membrane-associated forms of the enzyme (Mizunaga and Noguchi, 1982). Treatment of human umbilical vein endothelial cells with TM inhibited secretion of von Willebrand factor (vWf) (Wagner *et al.*, 1986). Nonglycosylated pro-vWf monomers failed to dimerize, were not processed further, and accumulated in the RER. There was no apparent effect of TM on degradation. TM inhibition of vWf transport and processing is specific to human endothelial cells, since precursor subunits were cleaved and secreted by bovine cells in the presence of TM (Lynch *et al.*, 1983). TM inhibited both the intracellular movement and secretion of procollagen from human fibroblasts (Housley *et al.*, 1980), but the secretion of procollagen was only minimally affected by TM in chick fibroblasts (Duksin and Bornstein, 1977; Olden *et al.*, 1978). TM markedly inhibited the secretion of surfactant-associated glycoprotein A from lung epithelial cells (Whitsett *et al.*, 1985) and the secretion of thyroglobulin cells (Björkman and Ekholm, 1982; Eggo and Burrow, 1983).

TM treatment did not significantly affect ovalbumin secretion by chick oviduct (Keller and Swank, 1978), very low density lipoprotein assembly and secretion by hepatocytes (Siuta-Mangano *et al.*, 1982), processing and secretion of haptoglobin by rat hepatocytes (Hanley *et al.*, 1983), nor processing and secretion of proopiomelanocortin peptides by mouse pituitary cells (Budarf and Herbert, 1982).

Glycosylation is important for the structural and functional maturation of the insulin receptor and for its translocation to the cell membrane (Reed *et al.*, 1981; Ronnett and Lane, 1981; Ronnett *et al.*, 1984). Treatment of cells with TM rapidly depletes cell surface insulin-binding capacity and leads to the production of a 180 kDa polypeptide, precipitated by antibodies to the insulin receptor, which is not proteolytically cleaved to yield the α and β subunits, does not bind insulin, and does not reach the cell surface. Ronnett and Lane (1981) have shown by the heavy isotope density shift method that blocking glycosylation with TM caused the intracellular accumulation of the inactive, nonglycosylated receptor. Subsequent withdrawal of TM and a shift of the cells to heavy medium (^{15}N-, ^{13}C-, and ^{2}H-labeled amino acids) led to the "activation" of previously synthesized "light" receptor. Similar levels of light receptor accrued even when protein synthesis was blocked with cycloheximide. Apparently, the inactive nonglycosylated receptor accumulated during TM treatment and then reentered the glycosylation pathway on removal of the inhibitor. Since transfer of oligosaccharide from the oligosaccharide–lipid

donor to protein occurs in the ER, these results suggest that N-linked oligosaccharide chains are required for transport of the receptor precursor out of the RER.

Inhibitors of N-linked glycosylation (TM and glucosamine) also prevent maturation and translocation to the cell surface of active epidermal growth factor (EGF) receptors (Soderquist and Carpenter, 1984; Slieker and Lane, 1985). The transferrin receptor, which has both high-mannose and complex-type oligosaccharide side chains, is transported to the cell surface in the presence of TM, but the levels of surface receptor are significantly reduced compared to untreated cells (Omary and Trowbridge, 1981). LDL receptor expression was reduced at the cell surface following TM treatment (Chatterjee *et al.*, 1979, 1981; Filipovic and von Figura, 1980).

Cell surface acetylcholine receptor expression is greatly decreased when muscle cells are treated with TM. Merlie *et al*, (1982) found that TM treatment of a mouse muscle cell line decreases the amount of functional acetylcholine receptor by blocking assembly of subunit precursors to mature functional molecules. They suggest that TM may prevent transport of subunits to the Golgi where assembly may occur. Prives and Bar-Sagi (1983) suggest that enhanced proteolytic degradation of nonglycosylated acetylcholine receptor in TM-treated chick muscle cells may explain the reduced receptor levels since receptor loss can be prevented, in part, by the protease inhibitor leupeptin. Rat basophilic leukemia cells grown in the presence of TM expressed fewer IgE receptors at the cell surface, as judged both by ligand binding studies and external labeling procedures (Pecoud *et al.*, 1981). TM reduced the expression of surface Na^+ channels in neuroblastoma cells (Waechter *et al.*, 1983) and in chicken muscle cells (Bar-Sagi and Prives, 1983) as measured by [^{3}H]saxitoxin binding and neurotoxin-activated ^{22}Na influx. This effect was partially reversed (30%) by leupeptin in muscle cells. TM completely blocks β-adrenergic receptors in growing human astrocytoma cells (Doss *et al.*, 1985), but the inhibitor had no influence on the steady-state level of β-receptors in S49 cells (George *et al.*, 1986).

In the presence of TM, mouse mammary tumor virus (MTV) glycoproteins synthesized by virus-infected hepatoma cells accumulate within the cells, proteolytic processing of the precursor polyprotein does not occur, and transport of MTV polypeptides to the cell surface and extracellular fractions is inhibited (Firestone, 1983). TM treatment also inhibits mumps virus particle formation (Herrler and Compans, 1983). Since mumps virus polypeptides are synthesized in the presence of the drug and assembly of nucleocapsids is observed, it is likely that TM blocks the surface expression of the viral glycoproteins.

The N-linked oligosaccharides of the H-2D^k mouse histocompatability protein heavy chain may influence its transport and cellular distribution (Le and Doyle, 1985). In mouse macrophages the H-2D^k glycoproteins exist as two distinct forms: an intracellular form with high mannose type chains and a cell surface form with complex type chains. H-2D^k glycoproteins destined for the cell surface are rapidly transported to this localization after synthesis in the RER, whereas most intracellular H-2D^k glycoproteins remain associated with intracellular membranes for prolonged periods in an undegraded form. To determine if the oligosaccharide structure on H-2D^k glycoproteins contains information important in intracellular distribution, macrophage cultures were treated with TM under conditions in which the inhibition of glycosylation is incomplete. In TM-treated cell cultures the newly synthesized nonglycosylated H-2D^k polypeptides were rapidly transported to the cell surface with no significant intracellular pool. By contast, in the same inhibitor-treated cultures, H-2D^k polypeptides containing one, two, or three oligosaccharide side chains of the high mannose type were not transported to the cell surface. These authors suggest that cell surface expression of H-2D^k polypeptides requires either processing of the oligosaccharide side chains from the high mannose form to the complex type or the absence of oligosaccharide chains.

The major human red cell glycoprotein, glycophorin A, contains an N-linked glycan chain and 15 O-linked glycan chains. The synthesis of the N-linked chain is inhibited by TM, while the O-linked chains are not affected. Incomplete glycophorin A, lacking the N-glycosidic oligosaccharide, is incorporated into the surface membrane, but the total amount of glycophorin A is decreased 2- or 3-fold compared to untreated cells (Gahmberg *et al.*, 1980). TM treatment of hepatoma cells delayed the appearance of γ-glutamyltransferase at the cell surface (Barouki *et al.*, 1984). TM also inhibited transport to the cell surface of specific yeast membrane proteins, suggesting that glycosylation is necessary for the correct processing and intracellular transport of some, but not all, yeast membrane proteins (Novick and Schekman, 1983). TM treatment of frog retinas blocks the incorporation of opsin into retinal rod outer segment (ROS) membranes (Fliesler and Basinger, 1985; Fliesler *et al.*, 1985). This effect of TM may be specific to amphibian retinas, however, since Plantner *et al.* (1980) reported that inhibition of opsin glycosylation by TM in the bovine retina does not prevent incorporation of the visual pigment apoprotein into ROS membranes.

Epithelial cells express cell surface polarity with different glycoproteins present on apical and basolateral surfaces (for a recent review, see Simmons and Fuller, 1985). Roth *et al.* (1979) and Green *et al.* (1981) have studied viral protein expression in Madin–Darby canine kidney (MDCK)

cells in the presence of TM and saw no marked effect on polarity. Similar results were obtained with mutant MDCK cells that were defective in glycosylation (Green *et al.*, 1981; Meiss *et al.*, 1982). Although N-linked glycosylation does not appear to have an important role in the polarized expression of viral proteins, it is unknown whether carbohydrate chains function as transport markers for endogenous epithelial apical and basolateral proteins.

TM does not alter expression at the cell surface of nonglycosylated HLA-A and HLA-B antigens on two lymphoblastoid cell lines (Owen *et al.*, 1980; Ploegh *et al.*, 1981) nor initial cell surface expression of human complement receptor type 2 (CR2) on human B lymphoblastoid cells (Weis and Fearon 1985). It also does not alter the intracellular routing, turnover, or function of the asialoglycoprotein receptor in a human hepatoma cell line (Breitfeld *et al.*, 1984).

VIII. INHIBITORS OF OLIGOSACCHARIDE PROCESSING AND GLYCOPROTEIN TRAFFIC

Drugs that inhibit specific steps in the processing pathway have become available, and it is now possible to study the role of oligosaccharide processing intermediates in intracellular transport of glycoproteins (Fig. 1) (for reviews, see Lalegerie *et al.*, 1982; Schwarz and Datema, 1984; Elbein, 1984; Fuhrmann *et al.*, 1985). 1-Deoxynojirimycin, *N*-methyldeoxynojirimycin and castanospermine inhibit RER α-glucosidases I and II, but not Golgi endo-α-D-mannosidase and these drugs prevent removal in the RER of three glucose residues from $Glc_3Man_9GlcNAc_2$ (Saunier *et al.*, 1982; Hettkamp *et al.*, 1982; Pan *et al.*, 1983; Palamarczyk and Elbein, 1985; Lubas and Spiro, 1987); bromoconduritol prevents trimming of glucose from $GlcMan_9GlcNAc_2$ by inhibiting α-glucosidase II (Datema *et al.*, 1982). 1-Deoxymannojirimycin inhibits Golgi α-mannosidases 1A and 1B (Fuhrmann *et al.*, 1984; Burke *et al.*, 1984; Elbein *et al.*, 1984) but not the RER α-mannosidase (Bischoff and Kornfeld, 1984). Swainsonine inhibits Golgi α-mannosidase II (Tulsiani *et al.*, 1982). Inhibition of the processing of glycoproteins by swainsonine (Elbein *et al.*, 1981, 1982; Tulsiani *et al.*, 1982) results in the synthesis of glycoproteins with hybrid-type oligosaccharides, in which one branch contains sugars commonly found on complex oligosaccharides (GlcNAc, Gal, NeuAc) and the other mannose-containing branch remains unsubstituted (Gross *et al.*, 1983b; Tulsiani and Touster, 1983; Arumugham and Tanzer, 1983).

An important advantage in the use of these oligosaccharide processing inhibitors over the use of TM is that glycoproteins synthesized by treated

cells do not lack N-linked glycan chains but rather retain intermediates in the normal oligosaccharide processing pathway (1-deoxynojirimycin, *N*-methyldeoxynorjirimycin, castanospermine, bromoconduritol, 1-deoxymannojirimycin) or hybrid oligosaccharides (swainsonine). Therefore, it is unlikely that treatment with these inhibitors will induce glycoprotein aggregation, alter protein folding, or increase sensitivity to proteolysis, and this simplifies the interpretation of studies using these drugs.

Studies using inhibitors of RER α-glucosidases have demonstrated that trimming of glucose residues from the major N-linked oligosaccharide precursor is required for the rapid maturation of specific secretory, membrane, and lysosomal enzymes from the RER to the Golgi apparatus. Gross *et al.* (1983a) were first to report that treatment of rat hepatocytes with 1-deoxynorjirimycin resulted in a long delay in the secretion of α_1-antitrypsin and that the cells accumulated α_1-antitrypsin with carbohydrate chains of the high-mannose type containing glucose, the largest species being $Glc_3Man_9GlcNAc_2$. Secretion of the nonglycoprotein albumin was unaffected by the drug. 1-Deoxynojirimycin did not completely block oligosaccharide processing, and only α_1-antitrypsin molecules processed to the complex type in one or two of their oligosaccharide chains were secreted.

Removal of three glucose residues from the $Glc_3Man_9GlcNAc_2$ precursor is believed to occur rapidly after translocation of the glycoprotein into the RER (Hubbard and Robbins, 1979) and may occur on nascent chains (Atkinson and Lee, 1984). Lodish and Kong (1984) found that glucose removal from N-linked oligosaccharides is required for efficient passage of some secretory glycoproteins from the RER to the Golgi complex. 1-Deoxynojirimycin treatment inhibited the secretion of α_1-antitrypsin and antichymotrypsin by human hepatoma cells, but it had little effect on secretion of glycoproteins C3 and transferrin or albumin. Since, after removal of 1-deoxynojirimycin, α_1-antitrypsin was secreted normally, its inhibitory effect is reversible. From cellular fractionation studies using sucrose density gradients, it was found that drug treatment caused α_1-antitrypsin and antichymotrypsin to accumulate in the RER. In untreated cells α_1-antitrypsin is rapidly transported from the RER whereas transferrin leaves the RER slowly. Lodish and Kong (1984) postulated that rapid transport of α_1-antitrypsin and antichymotrypsin from the RER to the Golgi requires that the N-linked oligosaccharide chains be processed to at least the $Man_9GlcNAc_2$ form and that this oligosaccharide forms parts of the recognition site of a transport receptor for these proteins. The slow transport of transferrin or α_1-antitrypsin synthesized in the presence of 1-deoxynojirimycin would not involve binding to a receptor, but rather bulk-phase fluid movement from the ER to the Golgi. Interestingly, in the

human disease α_1-antitrypsin deficiency the *Z* variant of α_1-antitrypsin is poorly secreted by the liver, accumulates in the RER, and retains glucose residues (Carrell *et al.*, 1984).

In hybridoma cells producing IgD and IgM, Peyrieras *et al.* (1983) found that secretion of IgD but not IgM was blocked by 1-deoxynojirimycin. From experiments using the drug, Lemansky *et al.* (1984) found that the transport of the lysosomal enzymes cathepsin D and β-hexosaminidase from the ER to the Golgi apparatus depends on the removal of glucose residues from the carbohydrate side chains. 1-Deoxynojirimycin had no effect on surface expression of VSV G protein, influenza virus hemagglutinin, and human class I histocompatability antigens (Schlesinger *et al.*, 1984; Burke *et al.*, 1984). Glucosidase inhibitors also did not have any effect on transport of incorrectly processed *erbB* oncogene glycoprotein to the cell surface (Schmidt *et al.*, 1985).

The effect of processing inhibitors on the intracellular migration of glycoprotein E2 of mouse hepatitis virus was studied by Repp *et al.* (1985). In the presence of *N*-methyldeoxynojirimycin or castanospermine, glycoprotein E2 was synthesized in normal amounts but accumulated intracellularly, with transport to the cell surface being greatly delayed. Glycoprotein E2 isolated from virus particles from cells grown in the presence of *N*-methyldeoxynojirimycin carried predominantly $Glc_3Man_9GlcNAc_2$ oligosaccharide side chains, and the remaining chains showed trimming of one or two mannose residues. TM treatment also prevented surface expression of glycoprotein E2, but inhibitors of trimming α-mannosidases did not affect transport. Apparently, efficient transport of glycoprotein E2 to the plasma membrane requires removal of glucose but not trimming of mannose.

Treatment of Sindbis virus-infected cells with either 1-deoxynorjirimycin or castanospermine inhibited proteolytic cleavage of the glycoprotein polyprotein precursor to glycoproteins E1 and E2 but did not prevent the migration of the precursor to the cell surface (Schlesinger *et al.*, 1985). However, treatment of Sindbus virus-infected cells with bromoconduritol, which prevents removal of the innermost glucose residue but not the outer two glucose residues, did not inhibit proteolytic cleavage of the polyprotein precursor (Datema *et al.*, 1984). Fowl plague virus shows the interesting characteristic that release of infectious virus is inhibited by bromoconduritol but not by *N*-methyldeoxynorjirimycin (Datema *et al.*, 1982; Romero *et al.*, 1983). Unfortunately, bromoconduritol is quite unstable and has a half-life in water of only 16 min at 37°C and pH 7.3. This, and the fact that millimolar concentrations are required for inhibition, complicates interpretation of studies using this drug.

We have found that 1-deoxynorjirimycin treatment of human hepatoma

cells inhibits the maturation of N-linked glycan chains on only a selected group of secretory glycoproteins to forms resistant to digestion by endo H. (Parent *et al.*, 1986). Also, drug treatment inhibits transport from the RER to the Golgi of the same subgroup of glycoproteins, including α_1-antitrypsin, ceruloplasmin, and α_2-macroglobulin but not transferrin, α-fetoprotein, or fibronectin. Interestingly, TM treatment had a similar differential effect on secretion of these glycoproteins (Bauer *et al.*, 1985). Since trimming of three glucose residues from the α-1,3 branch is required for processing of N-linked chains to endo H-resistant forms in the major N-linked glycan processing pathway (Harpaz and Schachter, 1980), our findings that 1-deoxynorjirimycin treatment inhibits the maturation of N-linked glycans on some, but not all, secretory glycoproteins was unexpected. One possible explanation for these results is that glycan chains on some secretory glycoproteins are processed by α-glycosidases resistant to the drug. Alternatively, there may be another pathway for processing N-linked glycan side chains on some secretory glycoproteins to endo H-resistant forms that does not involve glycosylated intermediates.

Both of these explanations are consistent with previous reports in the literature. Reitman *et al.* (1982) examined the glycopeptides produced in a mouse lymphoma cell line that is severely deficient (less than 0.3% of parent) in RER α-glucosidase II. The major oligosaccharides produced in this mutant were characterized as $Glc_2Man_9GlcNAc_2$ and $Glc_2Man_8GlcNAc_2$. However, this mutant still contained 25% of the complex chains of the parent line. The authors suggest that the mutant may still have enough α-glucosidase II to allow some processing to complex chains, or there may be an alternative pathway.

Parodi *et al.* 1983a) have shown that glucose-free oligosaccharides are transferred in the N-glycosylation of proteins in the protozoa *Trypanosoma cruzi* and Romero and Herscovics (1986) have demonstrated transfer of glucose-free oligosaccharides from lipid to protein in a mammalian cell line. Krag (1979) has described a CHO cell mutant, B211, selected for increased resistance to concanavalin A, that is defective in the glucosylation of lipid-linked oligosaccharides. Interestingly, incorporation of mannose into glycoproteins occurred at near normal rates in B211, and these mutant cells also synthesized high levels of complex-type oligosaccharides (Krag and Robbins, 1982; S. Krag, personal communication). Similarly, in other studies, using several different glucosidase inhibitors (Datema *et al.*, 1982; Pan *et al.*, 1983; Gross *et al.*, 1983a; Romero *et al.*, 1985), the inhibition of synthesis of complex-type glycan chains was not complete even at high concentrations of inhibitors, suggesting either that some processing α-glucosidase activity is resistant to the inhibitors or that there are alternative pathways.

Lubas and Spiro (1987) have described a novel enzyme present in Golgi membranes which is capable of converting the $Glc_1Man_9GlcNAc_2$ unit to $Man_8GlcNAc_2$ with the release of Glcα1-3Man. This endomannosidase is fully active in the presence of 1-deoxynojirimycin. The authors point out that this alternative pathway for the removal of glucose and mannose as a disaccharide rather than the sequential release of the monosaccharides by α-glucosidase 11 and α-mannosidase 1 could provide an explanation for the incomplete block in oligosaccharide processing which is observed in cells with inhibited or deficient α-glucosidase 11. This alternative pathway may also explain why treatment of hepatoma cells with 1-deoxynojirimycin has differential effects on both the glycan maturation and intracellular transport of specific secretory glycoproteins (Parent *et al.*, 1986).

At least four distinct exo-α-mannosidases are responsible for the trimming of six mannose residues in the conversion of $Glc_3Man_9GlcNAc_2$ oligosaccharides to complex forms in the major processing pathway of N-linked glycans (Fig. 1), and inhibitors of three of the trimming mannosidases are known. As yet no inhibitor has been reported for the α-mannosidase activity localized in the RER (Bischoff and Kornfeld, 1983). Inhibitors of trimming α-mannosidases, unlike those of the α-glycosidases, do not appear to have dramatic effects on the transport of secretory and membrane glycoproteins. 1-Deoxymannojirimycin inhibits Golgi mannosidases 1A and 1B, blocking the conversion of high-mannose oligosaccharides to complex-type oligosaccharides. 1-Deoxymannojirimycin did not prevent the secretion of either IgD or IgM (Fuhrmann *et al.*, 1984), in contrast to 1-deoxynorjirimycin, which blocked the secretion of IgD. Gross *et al.* (1985) found that high-mannose-type α_1-antitrypsin and α_1-acid glycoprotein were secreted by 1-deoxymannojirimycin-treated hepatocytes at the same rate as the normal glycoproteins.

Using another α-mannosidase inhibitor, α-mannopyranosylmethyl-*p*-nitrophenyltriazene, Docherty *et al.* (1986) found that the drug treatment did not alter the secretion of α_1-acid glycoprotein although the secreted form contained $Man_{(7-9)}GlcNAc_2$ chains rather than usual complex-type chains. 1-Deoxymannojirimycin was also without effect on the surface expression of several membrane glycoproteins tested, such as VSV G protein, influenza virus hemagglutinin, and human class I histocompatability antigens (Burke *et al.*, 1984; Elbein *et al.*, 1984).

1-Deoxymannojirimycin, which inhibits Golgi α-mannosidase 1 but not ER α-mannosidase, has been used to determine the role of the ER α-mannosidase in the processing of the N-linked oligosaccharides on glycoproteins in intact cells (Bischoff *et al.*, 1986). Analysis of the total [^{3}H]-mannose labeled N-linked oligosaccharides synthesized by control and 1-deoxymannojirimycin-treated rat hepatocytes indicates that about one-third of the oligosaccharides on secretory glycoproteins and about one-

half of the oligosaccharides on cellular glycoproteins in rat hepatocyte cultures are processed by the 1-deoxymannojirimycin-resistant ER α-mannosidase. Results from similar studies using the UT-1 cell line indicate that the ER α-mannosidase is responsible for the conversion of $Man_9GlcNAc_2$ oligosaccharides to $Man_8GlcNAc_2$ units on a resident ER glycoprotein, 3-hydroxy-3-methylglutaryl-CoA reductase (Bischoff *et al.*, 1986). The finding that only specific glycoproteins are substrates for the 1-deoxymannojirimycin-resistant ER α-mannosidase is additional evidence for multiple oligosaccharide processing pathways in cells.

Swainsonine is an inhibitor of Golgi α-mannosidase II, and T. K. Yeo *et al.* (1985) found that swainsonine treatment of human hepatoma cells accelerated the secretion of a number of glycoproteins (transferrin, ceruloplasmin, α_2-macroglobulin, and α_1-antitrypsin) by decreasing the lag period before secretion by 10–15 min relative to untreated cultures. Since conversion of the high-mannose precursor on α_1-antitrypsin to the hybrid form in swainsonine-treated cells occurred more rapidly (by about 10 min) than the conversion to the complex form in control cells, these results suggest that swainsonine-modified glycoproteins in hepatoma cells traverse the Golgi more rapidly than their normal counterparts. The magnitude of this effect on the rate of protein secretion is small, however, and in other studies it is reported that swainsonine treatment had little effect on the secretion of a number of proteins, including fibronectin by skin fibroblasts (Arumugham and Tanzer, 1983), α_1-antitrypsin by rat hepatocytes (Gross *et al.*, 1983b), aminopeptidase N by organ-cultured intestinal mucosal explants (Danielsen *et al.*, 1983), five glycoproteins by human hepatoma cells (Lodish and Kong, 1984), and surfactant-associated glycoprotein A by lung epithelial cells (Whitsett *et al.*, 1985). Treatment of thyroid cells with both swainsonine and 1-deoxynojirimycin increased the half-time of maximal secretion of thyroglobulin from 90 to 120 min (Frank *et al.*, 1986).

In addition, swainsonine treatment had little effect on the intracellular routing, turnover, or function of the asialoglycoprotein receptor in human hepatoma cell (Breitfeld *et al.*, 1984). Soderquist and Carpenter (1984) demonstrated that drug treatment of A431 cells resulted in the production of incompletely processed EGF receptors which were still able to function normally. Duronio *et al.* (1986) found that swainsonine treatment did not significantly affect the movement of insulin and insulin-like growth factor 1 receptors through the cells, their proteolytic processing, hormone binding, or hormone-stimulated autophosphorylation. The number and affinity of β-adrenergic receptors in swainsonine-treated S49 cells were normal (George *et al.*, 1986). Influenza particles isolated from swainsonine-treated infected cells had the same infectivity and hemagglutination titer as virus particles from control cells (Elbein *et al.*, 1982).

IX. VERTEBRATE LECTINS AND GLYCOPROTEIN TRAFFIC

The receptor-mediated trafficking to lysosomes of acid hydrolases with oligosaccharides containing phosphorylated mannose is the best understood pathway by which proteins translated into the RER are segregated and delivered to their ultimate destination (for reviews, see Sly, 1982; von Figura and Hasilik, 1986; Robbins, Chapter 11, this volume). Currently it is thought that newly synthesized acid hydrolases bearing the Man-6-P transport marker bind to specific receptors for Man-6-P located on Golgi membranes, to be transported from these Golgi cisternae by a receptor-mediated process, similar to that which occurs at the cell surface for other receptor–ligand complexes, and to be delivered in vesicles to lysosomes where low pH causes dissociation from receptor.

Two receptors with a binding specificity for Man-6-P have been identified. The first to be isolated was a 215 kDa integral membrane protein which is found in many different cell types (Sahagian *et al.*, 1981). Recently a second Man-6-P receptor has been isolated from murine P388D1 macrophage and bovine liver that differs from the 215 kDa receptor in its subunit molecular weight (46 kDa), oligosaccharide-binding specificity, and by the requirement for divalent cations for binding (Hoflack and Kornfeld, 1985a,b). The new receptor has been named the cation-dependent Man-6-P receptor (CD Man-6-P receptor) to distinguish it from the 215 kDa or cation-independent Man-6-P receptor (CI Man-6-P receptor). In preliminary experiments, Hoflack and Kornfeld (1985b) have identified the CD Man-6-P receptor in a number of cell types, including those that contain the CI Man-6-P receptor. They suggest that the two Man-6-P receptors may be involved in targeting acid hydrolases to different populations of lysosomes in the same cells.

In addition to the two Man-6-P receptors, a number of carbohydrate-binding proteins or lectins have been identified in vertebrate cells (Table I), and, although their physiological function is generally unclear, some may have roles in the intracellular transport or targeting of glycoproteins (for reviews, see Ashwell and Harford, 1982; Barondes, 1981, 1984; Kobiler, 1987). Vertebrate lectins can be classified roughly into two groups. The first comprises integral membrane proteins generally of high molecular weight that can be solubilized by detergents (lectins 1–6, Table I). The second comprises a group of soluble or peripheral membrane proteins, low in molecular weight and often developmentally regulated (lectins 7–12, Table I).

Many of the carbohydrate-binding membrane receptors are known to bind and internalize glycoproteins, and it is often assumed that they function on the cell surface. However, one important lesson that comes from

the work on Man-6-P receptors and lysosomal localization is that identification of an efficient cell surface, receptor-mediated glycoprotein uptake system does not necessarily mean that the primary *in vivo* function of the receptor is associated with the plasma membrane. Identification of the CI Man-6-P receptor resulted from earlier studies in which it was found that fibroblasts from patients with various inherited lyosomal storage diseases could take up lysosomal enzymes secreted by normal fibroblasts, and that this process was specific and saturable (Neufeld *et al.*, 1975). An early model for the delivery of acid hydrolases to lysosomes suggested by Neufeld *et al.* (1977) emphasized the role of cell surface receptor activity; they postulated that the main route for incorporation of these enzymes into lysosomes required their prior secretion and recapture by the cells. Subsequent studies have not supported this model (von Figura and Weber, 1978; Sly and Stahl, 1978; Vladutiu and Ratazzi, 1979). The demonstration that only a small fraction of the Man-6-P receptor activity is localized on the cell surface directed attention to the physiological role of intracellular receptors, and it is now generally accepted that the major pathway for transport of hydrolases to lysosomes is mediated by receptors on Golgi membranes.

Like the CI Man-6-P receptor, several other carbohydrate-binding proteins are also concentrated on intracellular membranes. Pricer and Ashwell (1976) demonstrated the binding of [^{3}H]-asialoorosomucoid to Golgi and lysosomal membranes isolated from rat liver, and these observations were confirmed by studies on solubilized hepatocytes (Steer and Ashwell, 1980). Rat hepatocytes were found to contain a total of 860,000 asialoglycoprotein receptors of which only 80,000 reside in the plasma membrane. Similar results have been provided by Weigel and Oka (1983), using hepatocytes permeabilized with digitonin. Only 20–30% of the Man/GlcNAc-binding activity of alveolar macrophages could be inhibited by mild trypsinization, suggesting that 70–80% of the receptor may be inside the cell inaccessible to trypsin (Stahl *et al.*, 1980). This result was subsequently confirmed by direct binding assays on cells made permeable by the presence of saponin (Wileman *et al.*, 1984). Human fibroblasts contain a large intracellular pool of binding sites specific for Man-6-P; this pool contains 80% of the total receptors found in the cell (Fisher *et al.*, 1980). In the presence of permeabilizing agents (saponin and digitonin), macrophage Man-6-P receptors showed a distribution of 15–20% on the surface and 80–85% inside (Shepherd *et al.*, 1984). Receptor distribution within cells has also been studied by electron microscopy. Antibody bound to the asialoglycoprotein receptor can be visualized when frozen sections are incubated with colloidal gold absorbed to Protein A. A quantitative analysis of gold bead distribution in sections of hepatocytes shows that

TABLE I

Vertebrate Lectins

Designation, carbohydrate-binding specificity[a]	Physical characteristics	Cellular localization	Major sources	Other characteristics
1. Cation-dependent Man-6-P receptor,[1] Man-6-P	Integral membrane glycoprotein, subunit M_r 215,000	80–90% on internal membranes (endoplasmic reticulum, Golgi, lysosomes), 10–20% on plasma membrane	Many mammalian tissues including testis, brain, spleen, liver, and lung	No divalent cation requirement for binding; receptor-mediated endocytosis
2. Cation-independent Man-6-P receptor,[2,3] Man-6-P	Integral membrane glycoprotein, subunit M_r 46,000	Plasma membranes and internal membranes	Murine P388D1 macrophages, bovine liver, and other cell types	Requires divalent cations for binding; oligomer (3 subunits)
3. Asialoglycoprotein receptor,[4,5] GalNAc > Gal, Glc	Integral membrane glycoprotein, subunit M_r 40,000–65,000	80–90% on internal membranes (endoplasmic reticulum, Golgi, lysosomes), 10–20% on plasma membrane	Mammalian liver	Requires Ca^{2+} for binding; receptor-mediated endocytosis
4. Avian N-acetylglucosamine receptor,[6,7] GlcNAc, Man, Glc	Integral membrane glycoprotein, subunit M_r 26,000	Unknown	Avian liver	Requires Ca^{2+} for binding
5. Mannose/*N*-acetylglucosamine receptor,[8–11] D-Man, L-Fuc > D-GlcNAc > Glc	Integral membrane glycoprotein M_r 175,000–180,000	80% on internal membranes, 20% on surface membranes	Alveolar macrophage, Kupffer cells, hepatic endothelial cells, spleen and skeletal muscle	Requires Ca^{2+} for binding; receptor-mediated endocytosis
6. Fucose receptor,[12,13] D-Fuc, D-GalNAc > D-Gal > L-Fuc ≫ D-GlcNAc	Integral membrane glycoprotein, subunit M_r 88,000	Plasma membranes and perhaps also internal membranes	Kupffer cells	Requires Ca^{2+} for binding; receptor-mediated endocytosis

7. Galactose-particle receptor,[19-21] GalNAc > Gal > Fuc	Peripheral membrane protein, subunit M_r 30,000	Plasma membranes and internal membranes	Kupffer cells	Receptor-mediated endocytosis of particulate structures with exposed Gal
8. Mannose/*N*-acetylglucosamine core-specific lectin,[14-18] Man, Fuc > GlcNAc > Glc	Soluble or peripheral membrane protein, subunit M_r 24,000–32,000	80% in microsomal fraction, primarily in rough microsomes	Mammalian and avian hepatocytes	Slowly secreted; requires Ca^{2+} for binding, contains collagenlike domains
9. Ligatin,[22,23] Glc-1-P and Man-6-P	Peripheral membrane protein, M_r 10,000–20,000	Cell surface	Suckling rat ileum, brain macrophages, and embryonic chick neural retina	Forms filaments 3nm in diameter in the presence of Ca^{2}
10. Lactose-binding lectin (Class I),[24,25] Gal (β-1,4) GlcNAc > Gal (β-1,4) Glc, Gal (β-1,3) GalNAc	Soluble dimeric protein, subunit M_r 13,000–16,500	Intracellular and on the cell surface	Many mammalian and avian tissues including heart, lung, spleen, muscle, and liver	Developmentally regulated; requires thiol-reducing groups for activity
11. Lactose-binding lectin (Class II),[26,27] Gal (β-1,4) Glc	Monomeric soluble protein, M_r 13,000–14,000	Secretory granules of intestinal goblet cells	Rabbit bone marrow, chicken intestine and kidney	
12. Heparin-binding lectin,[28] heparin, GalNAc	Soluble protein	Unknown	Rat lung, chicken muscle and liver	Developmentally regulated; secreted by embryonic muscle

[a] Key to references: 1. von Figura and Hasilik (1986); 2. Hoflack and Kornfeld (1985a); 3. Hoflack and Kornfeld (1985b); 4. Ashwell and Harford (1982); 5. Schwartz (1984); 6. Kawasaki and Ashwell (1977); 7. Drickamer (1981); 8. Maynard and Baenziger (1981); 9. Stahl *et al.* (1980); 10. Haltiwanger et al. (1986c); 11. Lennartz et al. (1987); 12. Lehrman et al. (1986a,b,c); 13. Haltiwanger et al. (1986a); 14. Maynard and Baenziger (1982); 15. Mori *et al.* (1983); 16. Brownell *et al.* (1984); 17. Mori *et al.* (1984); 18. Colley and Baenziger (1987a,b); 19. Kolb-Bachofen et al. (1982); 20. Roos et al. (1985); 21. Schlepper-Schofer et al. (1986); 22. Jakoi *et al.* (1981); 23. Marchase *et al.* (1984); 24. Barondes (1984); 25. Barondes (1981); 26. Beyer *et al.* (1980); 27. Harrison and Chesterton (1980); 28. Ceri *et al.* (1981).

35% of the receptors are confined to the plasma membrane; the rest are found near Golgi, smooth endoplasmic reticulum, and endosomal membranes (Gueze *et al.*, 1982, 1983).

The asialoglycoprotein receptor on mammalian hepatocytes has been extensively studied as a model system for receptor-mediated endocytosis (for reviews, see Harford and Ashwell, 1982; Schwartz, 1984; Weigel, 1987). The *in vivo* function of this receptor, however, is uncertain. Numerous investigators have shown in many systems that desialylated glycoproteins, when injected into mammals, are rapidly removed from serum by this receptor in liver. However, four laboratories have shown that under normal circumstances serum glycoproteins are not desialylated *in vivo* and are not cleared from the circulation by this receptor (Wong *et al.*, 1974; Clarenburg, 1983; Kuranda and Aronson, 1983; Lefort *et al.*, 1984).

Vertebrate lectins have binding specificities for sugars that are commonly found in membrane and secretory glycoproteins. Several are known to have binding specificities for the sugars that occur at the nonreducing termini of oligosaccharide chains (Sarkar *et al.*, 1979; Briles *et al.*, 1979; Kawasaki and Ashwell, 1977). For example, liver cells contain a surface receptor that binds glycan chains containing terminal fucose in α-1,3 linkages; glycan chains containing terminal fucose in α-1,2 or α-1,6 linkages appear not to be recognized by this lectin (Prieels *et al.*, 1978). The asialoglycoprotein receptor of mammalian liver binds Glc-terminated oligosaccharides in addition to Gal- and GalNAc-terminated oligosaccharides (Stowell and Lee, 1978; Baenziger and Maynard, 1980).

Purified human hepatic asialoglycoprotein receptor has a much higher affinity of binding for complex oligosaccharides bearing three terminal Gal residues than those with two residues (Baenziger and Maynard, 1980). Triantennary complex oligosaccharides with three terminal Gal residues are rapidly endocytosed by hepatocytes, but complex oligosaccharides with one or two terminal Gal residues are not endocytosed although they are bound by the hepatocytes (Baenziger and Fiete, 1980). Maynard and Baenziger (1981) have studied the specificity of the Man/GlcNAc receptor for binding N-linked glycan chains and found that the minimum structure required for binding and endocytosis by rat liver reticuloendothelial cells is the six residue oligosaccharide GlcNAcβ-1,6 or Manα-1,6, Manα-1,6Manα-1,3Manβ-1,4GlcNAcβ-1,4GlcNAcβAsn. Attachment of additional sugar residues to this hexasaccharide can prevent binding and endocytosis by liver reticuloendothelial cells. This lectin must have an extended binding site which accommodates several sugars and a highly restricted binding specificity.

A Man/GlcNAc-specific lectin has been isolated from rabbit and rat

liver by affinity chromatography on mannan–Sepharose (Kawasaki *et al.*, 1978; Mizuno *et al.*, 1981; Townsend and Stahl, 1981). Detailed characterization of this lectin's carbohydrate specificity by Maynard and Baenziger (1982) indicated that the structural requirements for recognition of N-linked glycans include primarily the core region of oligosaccharide chains plus at least some peptide. This lectin is mostly associated with the rough microsomal fractions of liver (Mori *et al.*, 1984) and is either a soluble or peripheral membrane protein rather than an integral membrane protein since it can be extracted without detergent (Maynard and Baenziger, 1982; Mori *et al.*, 1983).

Mori *et al.* (1984) found that there was a gradient of lectin occupancy of endogenous glycoproteins from the RER to the Golgi. Most of the lectin binding sites in the rough microsomes (85%) and about one-third of the binding sites in the smooth microsomes (36%) and Golgi membranes (35%) were occupied by specifically bound (i.e., mannan-displaceable) glycoproteins. In this respect, the Man/GlcNAc lectin resembles the CI Man-6-P receptor that is also found in association with endogenous ligands (Fisher *et al.*, 1980). The Man/GlcNAc lectin is also found in the plasma of a number of mammals (Kozutsumi *et al.*, 1980, 1981). Brownell *et al.* (1984) reported that the lectin is secreted by hepatocytes and hepatoma cells; however, the kinetics of secretion are unusually slow (~50% of the lectin is secreted in 7 hr).

Although the *in vivo* function of the liver Man/GlcNAc lectin is currently unclear, its binding specificity, localization in the RER, and association with endogenous ligands have led to the suggestion that the lectin may serve as a carrier protein for the transport of biosynthetic intermediates of glycoproteins with high-mannose-type oligosaccharides from the RER to the Gogi apparatus (Mori *et al.*, 1984; Kawasaki *et al.*, 1987). Alternatively, this lectin may serve to anchor glycoproteins within the cisternal space of the ER, preventing them from moving to the Golgi apparatus as has been described by Le and Doyle (1985). The presence of high-mannose-type oligosaccharide chains on microsomal enzymes supports this latter hypothesis (Mizuochi *et al.*, 1981; Liscum *et al.*, 1983).

Soluble lectins with a binding specificity for lactose have been implicated in secretion of mucin by chicken intestinal mucosa. Both chicken lactose-lectin I (the minor component) and chicken lactose-lectin II have been localized in secretory vesicles of the goblet cells of the intestinal mucosa (Beyer *et al.*, 1979; Beyer and Barondes, 1982). Purified chicken intestinal mucin, which is also concentrated in these vesicles, can interact with these lectins since it is a potent inhibitor of their specific hemagglutination activities. These results suggest that the lectins are somehow in-

volved in the organization and/or secretion of mucin from goblet cells into the intestinal lumen.

Marchase *et al.* (1982) have identified a distinct subclass of peripheral membrane glycoproteins whose N-linked glycan chains, unlike those on lysosomes, contained phosphodiester-linked glucose. A lectin, ligatin, has been isolated with a binding site for such a phosphooligosaccharide and seems to utilize this binding site to secure the phosphoglucose-containing glycoproteins to the external surfaces of cells. Ligatin was first identified as the filamentous baseplate for a morphologically distinctive array of β-*N*-acetylhexosaminidase molecules in suckling rat ileum (Jakoi *et al.*, 1976). It has since been found to bind hydrolases in rat macrophages (Jakoi *et al.*, 1981) and brain (Gaston *et al.*, 1982). In addition, it has been found in embryonic chicken neural retina (Jakoi and Marchase, 1979), where it inhibits the intercellular adhesion of dissociated retinal cells (Marchase *et al.*, 1981), and thus has been implicated in intercellular recognition.

Although not identical, ligatin preparations from all of the above sources are closely related and share the following properties: they are solubilized from membranes by high Ca^{2+} concentrations and are depolymerized by ethylenebis (oxyethylenenitrilo) tetraacetic acid; they form filaments 3 nm in diameter in the presence of Ca^{2+}; they have apparent monomeric molecular weights of 10,000–20,000 and they possess amino acid compositions characterized by relatively high levels of acidic residues and low levels of hydrophobic residues. Additionally, in affinity studies, they bind proteins that cosolubilize with them and release these proteins on treatment with specific phosphorylated sugars.

The synthetic origin of the phosphate present in the ligatin-associated oligosaccharide has been studied (Koro and Marchase, 1982; Hiller *et al.*, 1987). Similar to the results found by Reitman and Kornfeld (1981a) and Hasilik *et al.* (1980) in their studies of lysosomal enzymes, the presence of phosphate in the ligatin-associated oligosaccharides seems to arise from the transfer of sugar 1-phosphate by a phosphoglycosyltransferase to terminal mannose residues on high-mannose N-linked oligosaccharides. The reaction for UDPGlc:glycoprotein glucose-1-phosphotransferase (Glc-P-transferase) in the retinal system is UDPGlc + oligosaccharide $\rightarrow$ oligosaccharide-P-Glc + UMP. Unlike synthesis of the lysosomal transport marker, the phosphodiester linkage described for ligatin-associated retinal cell surface proteins does not seem to undergo cleavage *in vivo* to expose Man-6-P, since phosphodiester-linked glucose is present in cell surface preparations and apparently is required for binding to ligatin.

The location of ligatin and its associated phosphoglycoproteins on cell surfaces has led to the suggestion that ligatin may function in intracellular

localization and that phosphodiester-linked glucose may be a tag for targeting specific glycoproteins to the surface membrane in much the same way that Man-6-P targets acid hydrolases to lysosomes (Jakoi *et al.*, 1981; Marchase *et al.*, 1982, 1984, 1987). Newly synthesized glycoprotiens destined for the cell surface may share a common feature of their peptide structure that would result in their recognition by Glc-P-transferase. The phosphodiester-linked glucose would then be recognized by a receptor distinct from the Man-6-P receptor utilized in localization of lysosomal hydrolases. This receptor could be ligatin or another protein of similar binding specificity. This recognition would ultimately lead to the segregation of these proteins in vesicles transported to the cell surface. At the cell surface these protiens could establish a stable presence through binding with cell surface baseplate, ligatin. Additional studies are needed, however, to confirm this interesting model.

X. CONCLUDING REMARKS

The stepwise transport of newly synthesized secretory and membrane glycoprotiens through the subcompartments of the RER and the Golgi apparatus to the cell surface is highly regulated, and presumably signaling and sorting mechanisms are essential at every step for proper traffic regulation. The evidence that carbohydrate groups have important functional roles in the intracellular transport and/or sorting of specific secretory and membrane glycoproteins has increased substantially in the last 5 years. The elegant studies from the laboratory of John Rose, using site-directed mutagenesis techniques, have clearly demonstrated that N-linked glycan chains promote the efficient transport of specific membrane proteins to the cell surface. The close associations between glycosylation defects and altered expression of the Thy-1 antigen, the Man-6-P receptor, and the LDL receptor emphasize the importance of carbohydrate chains for the expression and function of some cell surface glycoproteins. Many, but not all, glycoproteins fail to be secreted or to be expressed on the cell surface if normal glycosylation is inhibited with TM. In addition, studies mostly using 1-deoxynorjirimycin have demonstrated that the trimming of glucose residues from N-linked glycan chains on specific secretory, lysosomal, and membrane proteins is required for efficient transport of these glycoproteins from the RER to the Golgi.

Although these studies demonstrate that carbohydrate groups are important for the traffic of many, but not all, secretory and membrane glycoproteins, the precise role of carbohydrate in intracellular transport is

unclear. In analogy with the Man-6-P pathway for transport of acid hydrolases to lysosomes, it is tempting to postulate that specific carbohydrate-binding receptors mediate the intracellular transport of specific nonlysosomal glycoproteins. This has not been clearly established, as yet, however, and efforts to identify and iolate carbohydrate-binding transport receptors distinct form the Man-6-P receptors are clearly indicated. In future studies in this area, it will also be very important to determine completely the structure of the oligosaccharide chains attached to glycoproteins being investigated. There is considerable evidence for alternative pathways for the processing of N-linked glycans, and detailed structural information will probably be required to make significant progress in understanding the presumably complex roles of carbohydrate in glycoprotein traffic.

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