STUDIES ON THE STRUCTURAL ORGANIZATION OF GOLGI COMPLEX

by

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(ABSTRACT)

Golgi complex is a multi-compartmental organelle involved in posttranslational modification and sorting of secretory proteins. We have characterized the distribution in Vero cells of three Golgi cisternal membrane proteins, Sialyltransferase (SialylT, tans-Golgi/TGN), galactosyltransferase(GalT, trans-Golgi/TGN), and N-acetylglucosametransferase-1 (GlcNAcT-1, medial-Golgi), during the process of Golgi disassembly and reassembly following the addition or removal of drugs. After 1 h nocodazole (microtubule depolymerizer) treatment, SialylT and GalT were found in scattered punctate structures that increased in number over time as less and less of these proteins were found perinuclearly. Initially these punctate structures were often negative for GlcNAcT-1. Over a 2 to 3-fold slower time course, GlcNAcT-1 co-localized with SialylT and GalT in the scattered punctate structures. Cis-Golgi network marker was found in a separate set of scattered punctate structures from that of cisternae even at 4 h following nocodazole addition. Following nocodazole removal, all the cisternal markers accumulated perinuclearly into a reassembled Golgi at the same rate. After brefeldin A treatment (coat protein inhibitor), all the cisternae markers dispersed to ER with similar kinetics, albeit, in some cases by different tubular extensions of the Golgi. GlcNAcT-1 and GalT showed similar kinetics of Golgi reassembly following BFA removal while SialylT lagged somewhat behind. Our data suggest that CGN, medial-Golgi and trans-Golgi/TGN are distinct subcompartments that can be separated one from the other by drug treatment; any

exchange of components between the Golgi subcompartments must be slow with respect to the observed kinetics of Golgi disassembly.

An epitope tagging approach was used to delineate the importance of the above Golgi protein's cytoplasmic tail domain in Golgi targeting and retention. we found that the cytoplasmic tail could be lengthened considerably (3-4 fold) and SialylT and GalT still accumulated in a perinuclear, Golgi-like distribution with little ER background. One construct, VSV-SialylT, localized essentially exclusively to the Golgi complex. For SialylT, the longest constructs (40-42 amino acids) located relatively well to the Golgi complex while for GalT, the longest constructs (32-34 amino acids) located exclusively to the ER. Surprisingly, the epitope tags of several different GalT constructs was inaccessible to antibody in fixed cells. Any lengthening of the cytoplasmic domain of GlcNAcT-1 resulted in considerable to exclusive accumulation of the chimeric proteins in ER. No cell surface accumulation of any of the chimeric proteins was detected. The specific sequence of the epitope tag was important; the neutral to positively charged VSV epitope tag was preferred over negatively charged myc or FLAG tags. Depending on the exact tail alteration, we found that all three transferases accumulated in the ER with no detectable Golgi or cell surface accumulation. In some cases accumulation in the intermediate compartment or CGN was observed. Most surprisingly, in the one homologous case studied, expression of chimeric human GlcNAcT-1 in HeLa cells, ER accumulation of GlcNAcT-1 led to disruption of pre-existing Golgi. Based on these results, we propose, as the simplest explanation of the data, that alteration of the cytoplasmic tail of Golgi resident proteins can decrease their rate of exit from ER, presumably due to premature oligomerization, and may lead to capture of Golgi proteins in the ER.

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Table of contents

Chapter 1. General Introduction

1.1. Eukaryotic cells are composed of a set of distinct compartments.....1

1.2. Structure and function of Golgi complex.....1

1.3. Transport of proteins between Golgi cisternae..... 6

1.4. Retention and retrieval of resident proteins of the ER and the Golgi complex 11

1.5. Effects of brefeldin A (BFA) on membrane traffic and organelle structure of Golgi 19

1.6. Effect of nocodazole on the organization of the Golgi complex.....22

1.7. References.....24

Chapter 2. Compartmentation of the Golgi Complex: Comparative Behavior of Golgi Subcompartments in Response to Nocodazole and Brefeldin A Challenge

2.1. Abstract.....37

2.2. Introduction.....39

2.3. Materials and methods.....42

2.4. Results.....46

2.5. Discussion.....53

2.6. References.....57

2.7. Figures.....61

Chapter 3. Alterations of cytoplasmic domain, by an epitope tagging approach, and their affect on the subcellular of Golgi resident enzymes

3.1. Abstract.....78

3.2. Introduction.....80

3.3 Material and Method.....86

3.4. Results.....93

3.5. Discussion.....101

3.6. References.....106

3.7. Table and Figures.....111

Chapter 4. General discussion

4.1. Comparative behavior of Golgi subcompartments to drug challenge.....124

4.2. Modification of cytoplasmic tail of resident Golgi proteins and their effects to protein targeting.....127

List of Abbreviation

ARF, ADP ribosylation factor

BSA, Bovine serum albumin

BFA, brefeldin A

BHK, Baby hamster kidney

CGN, cis-Golgi network

COP I, Coat protein I

ER, endoplasmic reticulum

GalT, galactosyltransferase

GIMP$_c$, Golgi integral protein located at cis-cisternae

GIMP$_t$, Golgi integral protein located at trans-cisternae

GlcNAcT-1, N-acetylglucosaminetransferase-1

FITC, fluorescencin isothiocynate

HEPES, N-2-hydroxylethylpiperazine-N'-2-ethanesulfonic acid

kD, Kilodalton

MEM, minimum essential medium

NSF, N-ethylmaleiaimide sensitive factor

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

PDI, Protein disulfide isomerase

SDS, Sodium dodecyl sulfate

SialylT, sialytransferase

SNAP, Soluble NSF attachment protein

SNARE, SNAP receptor

TGN, trans-Golgi network

VSV, vesicular stomatitis virus

VSV-G, VSV glycoprotein

Chapter 1. General Introduction

1.1. Eukaryotic cells are composed of a set of distinct compartments

Eukaryotic cells contain intracellular membranes that enclose nearly half of the cell's total volume in separate intracellular compartments called organelles. The main types of membrane-bounded organelles present in all eukaryotic cells are the endoplasmic reticulum (ER), Golgi complex, nucleus, mitochondria, lysosomes, endosomes, and peroxisomes. Although each organelle contains a distinct set of proteins that mediates its functions, most organelles are related to each other by a process called membrane or protein traffic. For example, the endoplasmic reticulum and the Golgi complex are functionally related by the process of protein synthesis and modification. Newly synthesized proteins are cotranslationally inserted into rough endoplasmic reticulum (RER) and transported to the Golgi complex for modification before they reach their final destination. One consequence of this multicompartment arrangement and interrelationship is that there must exist a mechanism to deliver the protein components from one compartment to the correct acceptor compartment. The most widely held view is this is achieved though transport vesicles that bud from one organelle and then fuse with the next. (Farquhar et al., 1981)

1.2. Structure and function of Golgi complex

The Golgi complex was named after Camillo Golgi who discovered it in silver stained brain tissue preparations in 1898. He observed a precipitated metallic silver in network surrounding the nuclei. Although originally thought to be a "fixation artifact", the basic cisternal organization of the Golgi complex is now accepted as a certainty, owing to improved methods of sample preparation and the application of phase contrast and electron microscopy to cell biology (Dalton and Felix, 1954, 1956). The Golgi complex appears centrally located in close proximity to the cell nucleus, and in animal cells it is often close to the centrosome, or cell center. Its morphology varies in different cells and with different

preparation methods. Under the electron microscope, it is composed of a collection or stack of flattened sacks termed cisternae along with tubular membrane structures at both sides. The cisternae usually have a flattened, plate-like center and more dilated rims. Each cisternae is bounded by a smooth membrane, is 0.5-1.0 μm in diameter, and is about 20 nm apart from the next cisternae in the stack. Each of these Golgi stacks usually consists of four to multiple cisternae (Rambourg et al., 1990). The number of Golgi stacks per cell varies greatly depending on cell type: most animal cells contain only one large stack, while certain plant cells contain hundreds of small ones (Griffing et al., 1991).

The Golgi complex is organized in a polar fashion. It has two distinct faces: a cis face (or entry face) and a trans (or exit face). At both cis and trans faces are network of interconnected tubular structures. These are the cis Golgi network (CGN) and the trans Golgi network (TGN). Proteins enter the stack at its cis face and depart at the opposite trans face. This was best demonstrated by an immunoelectron microscopy study of a synchronized wave of transported viral membrane protein, which entered the stack at one side, the cis face and exited at trans face (Bergmann and Singer, 1983). Thus cis and trans ends are biologically distinct, differing at the very least in the kinds of proteins responsible for the entry and exit processes. The stacks between the CGN and TGN can be further divided structurally and functionally into cis, medial and trans cisternae. The cis, medial and trans cisternae host a different subsets of glycosidase and glycotransferase enzymes that sequentially process the Asn-linked oligoscharides of proteins as they pass through the compartments as well as other enzymes (Roth, 1987). Differences in lipid composition among Golgi cisternae and CGN/TGN also exist. A gradient of cholesterol is known to exist across the Golgi cisternae (Orci et al., 1981), and similarly, particular lipids are thought to be enriched in either the cis or trans face of this organelle, with the cis part having a lipid composition much like the ER and the trans part having a lipid composition much like the plasma membrane (Van Meer, 1989)

CGN

The CGN which is located at cis side of Golgi functions in receipt of newly synthesized material from ER. Structurally, The cis-most portion of Golgi is typically a continuous array of tubules (Huttner and Tooze, 1989; Hsu et al, 1991; Pelham, 1991). The CGN may also include the poorly understood but possibly interconnected system of tubules between ER and Golgi referred as "salvage or intermediate compartment" (Warren, 1987; Pelham, 1991). In addition to serving as a site for entry into Golgi, recent evidence suggests that the CGN functions in the recycling of protein and lipid components back to the ER, while having a relatively limited role in glycosylation. Using both animal and yeast cells, Pelham and colleagues have found that soluble ER proteins containing the KDEL (or HDEL) ER retrieval signal are delivered to a post-ER site, in which limited "Golgi-like" oligosaccharides processing occurs and from which these protein are returned to the ER by KDEL receptor (Pelham, 1988, 1989; Dean and Pelham, 1990). In animal cells, when the lysosomal enzyme cathepsin D was given a COOH-terminal KDEL tag and expressed in cells, it was found not to receive any terminal sugar modification associated with Golgi-liked glycosylation. However, mannose residues on the enzyme did receive a GlcNAc-phosphate. Thus although the KDEL construct reached a GlcNAc-phosphotransferase-containing compartment, it did not encounter phosphodiesterase, that would remove the N-acetylglucoseamine that masks the mannose-6-phosphate residue. The KDEL receptor can retrieve these proteins before they reach the bulk of the oligosaccharides transferases; thus recycling is a function associated with CGN. In summary, CGN is viewed as a unique Golgi compartment responsible for receiving and sorting components arriving from ER.

The Cisternal Golgi

The cisternal Golgi functions primarily as a glycosylation device. The cisternae of the Golgi stack are not simply apposed replicas of identical units. For example, during prolonged osmication at elevated temperature, one or two cisternae at the cis Golgi face

stain selectively (Friend and Murray, 1965). Uridine diphosphatase, which degrades the UDP produced in many glycosylation reactions, is restricted to cisternae at the opposite trans face (Cheetham et al., 1971). Several investigators (Smith, 1980: Amgermuller and Fahimi, 1984) have localized a distinct phosphatase (NADPase) to the central cisternae. The differential staining of the cisternae by the periodic acid-Schiff reagent (Rambourg and Leblond, 1967) initially suggested that the structural heterogeneity of Golgi cisternae might be related to a regionalization of the numerous glycosylations that occur in this organelle (Neutra and Leblond, 1966). The later elucidation of the elaborate pathway by which N-linked oligosaccharide chains are processed in Golgi (for review, see Kornfeld and Kornfeld, 1985) has led to a detailed description of Golgi compartment function. The construction of N-linked oligosaccharides in the Golgi complex is a three-stage process. The first stage, thought to take place in the cis-cisternae, continues trimming of mannose residues started in ER. The second stage, in medial-cisternae, involves addition of N-acetylglucosamine, the removal of a further two mannose residues and the addition of another N-acetylglucoseamine. Fucose may also be added at this stage. The third stage, in trans-cisternae, involves addition of galactose followed by sialic acid. These activities have been assigned to the different cisternae based on localization of different enzymes and their products (for review, see Dunphy and Rothman, 1985). The first stage enzyme mannosidase I is thought to reside in the cis-cisternae, because it acts on the oligosaccharides attached to the proteins that have just left the ER (Balch et al., 1986) and before the second stage enzyme, N-acetylglucosaminetransferase 1 (GlcNAcT-1), which has been immunolocalized to medial cisternae (Dunphy et al., 1985). Other second stage enzymes including mannosidase II and GlcNAcT-2 cofractionate with GlcNAcT-1 on sucrose gradients. Since they cofractionate and can be partially separated from the third stage enzymes (β-1,4-galactosyltransferase [GalT] and α-2,6- sialytransferase [SialylT]) on the same gradients (Dunphy et al., 1981), they are assumed to occupy the medial-

cisternae. The third stage enzymes have been immunolocalized to the trans-cisternae and TGN (for example, Roth and Berger, 1982; Nilsson et al, 1993). There is some data suggesting that SialylT, unlike GalT, might be concentrated more in TGN than the trans-cisternae (Roth et al., 1985). These data led to the view that enzymes involved in the three stage of N-linked glycosylation in the Golgi are physically separated in cisternae that are sequentially ordered in a stack. Recently by using double immuno-electron microscopy Nilsson and colleagues (Nilsson et al., 1993) found the subcellular localization of GlcNAc transferase 1 and galactosyltransferase are partially overlapped in Hela cells. GalT was found in approximately equal amounts in trans-cisternae and TGN. Unexpectedly, GlcNAcT-1 was found in both in the medial- and trans-cisternae, overlapping the distribution of GalT. About one third of GlcNAcT-1 and half of GalT were found in the trans-cisternae.

TGN

The TGN is viewed as the compartment that mediates the sorting and final exit of the material from the Golgi. Structurally, it is defined as the sacculotubular network located on the trans side of the Golgi (Rambourg and Clermont, 1990). The TGN can be best visualized by electron microscopy after labeling cells with C_6-NBD-ceramide (Pagano et al., 1989), with C_5-DMB-ceramide (Pagano et al., 1991), or after accumulation of vesicular stomatitis virus (VSV) G protein at 20°C in the TGN (Griffith et al., 1985). The TGN may also have a lower pH than the preceding compartments in the pathway (Anderson and Pathak, 1985). The main function of the TGN is to sort proteins and lipids leaving for different post-Golgi destinations (Griffiths and Simons, 1986). The TGN also receives membrane traffic from endocytic the pathway (Kornfeld and Mellman, 1989).

Golgi Intercisternal Matrix

During purification, the Golgi stacks resist unstacking forces (Morre et al., 1970), suggesting that there are links between adjacent cisternae, these links must be numerous

because the width of the intercisternal space is relatively constant (Cluett and Brown, 1992). Components of an intercisternal matrix have been visualized by several workers in a variety of systems and appear either as inter-cisternal elements of electron-dense material running between and parallel to the cisternae or as regularly spaced intercisternal cross-bridges (for references see, Mollenhauer and Morre, 1975). Isolated Golgi stacks contain similar cross-bridges which are sensitive to treatment with a variety of proteases (Cluett and Brown, 1992). Since such treatment also causes cisternae to separate, the cross-bridges are more directly implicated in the stacking mechanism. The isolation of intercisternal matrix from detergent treated rat liver Golgi stacks has been reported by Slusarewicz et al. (Slusarewicz et al., 1994). It is suggested to bind two medial Golgi enzymes (GlcNAcT-1 and mannosidase II) tightly, it may have a role in stacking.

1.3. Transport of proteins between Golgi cisternae

The extensive network of intracellular membrane bound organelles allows the eukaryotic cell to carry out a variety of specialized tasks and increases its surface to volume ratio. The existence of these organelles, however, raises a question of how proteins of each compartment are targeted to their destination. Protein transport is necessary for biogenesis of plasma membrane, lysosomes and endosomes, and uptake of external molecules by endocytosis. Moreover, its specificity is harnessed to generate the distinct apical and basal surface of the polarized cell type in most tissues.

The outline of the secretory pathway was delineated by Palade and his colleagues thirty years ago (Palade, 1975). Nascent proteins are delivered to the lumen of the endoplasmic reticulum cotranslationally, pass through Golgi for post-translational modification, and are either secreted or stored in specialized secretory vesicles which fuse with the cell surface upon receipt of a signal for exocytosis. Movement of proteins between these compartments occurs by the budding and fusion of transport vesicles.

Machinery For Vesicular Transport

In the past ten years, the molecular basis for putative vesicular transport between successive Golgi cisternae has been established. A cell free *in vitro* biochemical approach, largely due to Rothman and his colleagues, established the principles and molecular mechanisms underlying protein transport. A genetic approach, largely due to Schekman and his colleagues, has contributed by establishing the *in vivo* relevance and overall generality of the *in vitro* work. Transport between Golgi cisternae was reconstituted by incubation of partially purified Golgi membranes with cytosol and a source of energy (Balch et al., 1984). When a "donor" population of Golgi containing G protein of VSV is incubated with an "acceptor" population of Golgi, containing an enzyme GlcNAcT-1 that adds GlcNAc residue to oligosaccharides side chain, transfer of G protein from donor to acceptor can be measured by addition of radioactive GlcNAc to G protein. Electron microscopy revealed that the transport intermediates are vesicles with a diameter about 70 nm (Balch et al., 1984). Most of the fully formed vesicles, and all of the vesicles still in the process of budding, have a distinct protein coat on the cytoplasmic face, which is now called COP I (for coat protein I). COP I coated vesicles resemble clathrin coated vesicles (Pears and Robinson, 1990). The newly found COP II proteins have a different molecular weight and are proposed to function in ER-Golgi protein transport (Barlowe et al, 1994)

Purification of docked COP I coated vesicles that accumulate following treatment with GTP-γS (Malhotra et al, 1989) shows that the coat contains eight polypeptides. One is ADP-ribosylation factor (ARF) (Kahn et al., 1986), a GTP binding protein found mainly in cytosol. The other seven are associated in an coatomer complex (Waters et al., 1991), which contributes most of the mass to the coat. ARF and coatomer co-assemble on the Golgi surface to form vesicles; their later release constitutes uncoating. The physiological properties of ARF in the coating process suggests that GTP binding and hydrolysis may trigger budding and uncoating. ARF is N-myristylated and its GDP bound form is water-

soluble; the GTP bound form, however is inserted into membrane in a myristic acid-dependent manner (Kahn et al., 1991). A GTP-GDP conformational switch appears in this process to modulate membrane insertion by controlling exposure of covalently attached fatty acid.

An in vitro reconstitution system revealed that ARF can bind to membrane in the absence of coatomer (Donaldson et al., 1992). Its binding to Golgi membrane is saturable and specific. This implies the existence of an ARF receptor at Golgi membrane. Then the coatomer binds to the membrane bound ARF(Donaldson et al., 1992). Coatomer binding and assembly drives the budding, whereas ARF initiates budding by providing the binding site for coatomer. The resulting complexes incases the coated buds, virtually completed vesicles which are not yet pinched off at their base. With addition of long-chain fatty acyl-CoA, fully formed coated vesicles capable of fusion are released from Golgi stacks. The COP I coated vesicles diffuse to their target membrane. After binding to the target membrane, the ARF-bound GTP is hydrolyzed to GDP before the protein coat can dissociated from the vesicle. A clear conceptual outline of the core process of budding has emerged: a coat acts as a mechanical device to shape the membrane into a bud that can pinch off following membrane fusion at its base, and coat is then released, when previous bound GTP is hydrolyzed.

When NEM is added to the in vitro transport assay, uncoated vesicles accumulate on the acceptor Golgi membrane (Malhotra et al., 1989), indicating that NEM-sensitive factor (NSF) must play a part in the next step. As transport can be restored to such reactions by adding back fresh cytosol, this fusion factor must be cytosolic. Restoration of fusion capability to NEM treated membrane preparations provided an assay that led to the purification of NSF (Block et al, 1988). NSF has two homologous domains containing an ATP binding site. Mutation of either site seriously reduces both ATPase activity and fusion. Mutation of both sites eliminates ATPase activity and fusion (Whiteheart et al.,

1994). These results indicate that the intrinsic ATPase activity of NSF is necessary for fusion. Binding of NSF to Golgi membranes requires addition of a cytosolic factor called soluble NSF attachment protein (SNAP) (Clary et al., 1990). Of the three species of SNAP, α–SNAP and γ-SNAP have been found in all cell type examined so far, while β-SNAP is confined to brain. SNAPs bind to Golgi membrane in the absence of NSF, indicating SNAPs bind before NSF. Binding of SNAPs to Golgi membrane is saturable and proteinase sensitive indicating that the SNAP receptor (SNARE) is a membrane protein. SNAP proteins do not interact with NSF in solution, but once bound to SNARE sites mediate saturable and stoichiometric NSF binding to Golgi membrane (Wilson et al., 1992). On binding to SNAP-SNARE complexes, NSF hydrolyzes bound-ATP and releases itself. It can be trapped in its membrane bound form by using non-hydrolyzable ATP analog ATP-γS. A complex of NSF, SNAPs and SNARE can be purified from intact Golgi membrane with non-ionic detergent. It sediments at 20 S. It forms the core of a general "fusion machine" (Wilson et al., 1992). It is proposed that the hydrolysis of ATP may create a "fusion active" conformation which facilitates bilayer fusion. It is not clear, however, whether NSF, SNAPs and SNAREs participate directly in membrane fusion, or whether these proteins alone are sufficient for fusion.

Maintaining the identity of membrane bound compartments in the face of massive flux between them requires a fusion mechanism with great specificity. Purification of SNAREs from bovine brain indicates that SNAREs themselves play a critical role in determining docking of the vesicles to the correct target membrane (Sollner et al., 1993). SNAREs are composed of three polypeptides which were originally purified from synaptic nerve endings. Two of these protein (syntaxin and synaptosome-associated protein-25) are localized in presynaptic membrane. The other protein (VAMP) is confined to synaptic vesicles, and was already known to play a role in neurotransmitter release, as it is selectively cleaved by tetanus toxin, causing paralysis by blocking neurotransmission.

Thus the 20 S particle involved in the fusion of synaptic vesicles consists of NSF, SNAPs, VAMP, syntaxin and synaptosome-associated protein-25. That fusion particles contain both a protein derived from synaptic vesicle (VAMP) and two proteins derived from pre-synaptic membrane (syntaxin and synaptosome-associated protein-25) suggests a hypothesis for vesicle targeting in which each transport vesicle bears a unique address marker consisting of one or more v-SNAREs (general VAMP homologues) obtained from its parent membrane during budding, while each target membrane is identified by one or more t-SNAREs (generally syntaxin and synaptosome-associated protein-25 homologues). Targeting specificity would thus be achieved by v-SNARE binding to matching t-SNARE.

Regulation of GTP Binding Proteins in Intracellular Protein Transport

The rab GTP binding proteins also play important roles in mediating correct vesicular targeting (for review, see Pfeffer, 1994). Eukaryotic cells contain many types of rab proteins, each associated with a particular membrane bounded organelle involved in secretory or endocytic pathways. Each organelle has at least one kind of rab protein on its cytoplasmic face. The first rab protein called Sec4 was identified by a mutant that interferes with secretion at post Golgi stage. The Sec4 protein shares 30% amino acid sequence identity with ras. Sec4 was subsequently shown to be a component of secretory vesicles and required for their docking at plasma membrane. The amino acid sequences of rab protein are most dissimilar near their carboxyl-terminal tail, and tail swapping experiments indicate that it is the tail that determines the intracellular location of each family number. Currently, rab proteins are believed to check the fit between a v-SNARE and a t-SNARE. In this view rab proteins become attached to the surface of a budding coated vesicle. When a vesicle encounters the correct target membrane, the binding of v-SNARE and t-SNARE cause the vesicle to bind long enough to allow the rab protein to hydrolyze to its bound GTP locking the vesicle to the target membrane and readying it for subsequent fusion.

1.4. Retention and retrieval of resident proteins of the ER and the Golgi complex

Current opinion favors the idea that the newly synthesized proteins destined for insertion into the plasma membrane or for secretion move though the Golgi apparatus en route from ER to cell surface. These proteins are thought to move through the exocytic pathway by default; thus not requiring signals for transport. This hypothesis has been challenged by recent findings that cargo proteins are concentrated during their exit from the ER and that they interact with transport machinery (Balch et al., 1994; Storrie, et al,. 1994). This default transport, often referred as bulk flow, continues along the exocytic pathway until the protein reaches the plasma membrane. A subset of molecules, however, resist bulk flow. These include ER and Golgi enzymes involved in post-translational modifications such as protein folding, glycosylation, sulfation and phosphorylation. Each of these enzymes needs to be maintained at particular points along the exocytic pathway. At least two types of signals are believed currently to ensure such compartmental localization. The first, termed a retention signal, would permit movement along the secretory pathway until the correct site has been reached. Forward movement would then be prevented by denying the protein access to budding transport vesicles of the anterograde pathway. The second type of signal, termed retrieval signal, will only act when the protein has left the compartment in which it resides. The signal would depend on specific binding to the components involved in retrograde transport. Resident proteins containing only retrieval signals would be expected to cycle between two or more compartments. Most ER resident proteins are found to maintain their residency by a retrieval signal, while the majority of Golgi resident proteins are resident because of a retention signal. Some proteins like TGN 38 may contain both retrieval and retention signals.

Retrieval of ER Resident Proteins

The KDEL retrieval signal

The carboxyl-terminal tetrapeptide KDEL (single-letter code for amino acids) is found in many resident in lumenal ER proteins. When transplanted onto various reporter molecules, it localized them to ER, showing that it is both necessary and sufficient for this process (Pelham, 1989). However the reporter molecules display post-translational modification by enzymes which are located in the Golgi complex, showing that they have left the ER at least once during their lifetime. This finding suggests proteins resident in ER escape it and enter the Golgi, where they are sorted and returned to the ER (Pelham 1988). A retrieval receptor was postulated and later identified both in yeast (Lewis et al., 1990a) and mammals (Lewis et al., 1990b). At steady state, the receptor, termed erd2, localizes to the cis side of the Golgi complex and upon ligand binding redistributes to ER (Lewis et al., 1992). Biochemical characterization of the receptor shows that it specifically binds to the ligand and does so in a pH-dependent manner, with an optimum around pH 5.0 (Wilson et al., 1993). It has been observed that the pH along the exocytic pathway becomes increasingly acidic towards the trans-Golgi network (TGN), and this likely allows erds to bind the ligand with high affinity. The binding would then signal the receptor and its ligand to be returned to the ER, where a neutral pH would release the ligand into the lumen. Erd2 would then return to its original location to await another round of ligand delivery. How erd2 cycle between the ER and Golgi is still unknown.

The K(X)KXX and RR retrieval signals

Several ER resident membrane proteins have been shown to contain signals similar to that of the KDEL motif in their cytoplasmic domain (Schutze et al., 1994). In resident proteins with a type I topology (amino terminus is in the lumen), the signal has been shown to consist of two critical lysine residues, which have to be in a -3 and a -4/5 position relative to the carboxyl terminus [-K(X)KXX, where X is any amino acid]; in type II membrane proteins (carboxyl terminus is in the lumen), the signal consists of two critical arginine (RR), which have to be in the first five amino-terminal residues of the protein.

When transplanted to the reporter molecules, the motifs are both necessary and sufficient for ER localization, yet allowed the reporter molecules to acquire Golgi modification (Jackson et al., 1993). They are, therefore, similar to the KDEL motif in that they act as retrieval signals, returning lost ER proteins from as far as TGN to ER. It is not clear, though, whether these motifs are recognized by receptors similar to the one identified for the KDEL motif or whether they interact directly with components of retrograde transport machinery. Microtubules play an intrinsic role in retrograde transport, and K(X)KXX motif can drive polymerization of microtubules in vitro (Dahllöf et al., 1991). This motif has recently shown to bind specifically to coatmer (Cosson and Letourner, 1994). Whether or not binding to coatmer and microtubules negates the need for a receptor remains to be seen.

It is becoming increasingly clear that the sole determinants for correct localization cannot be the double lysine or the double arginine, nor can it be the KDEL retrieval motif, as this motif can be found in proteins that are not residents of ER. For example, ER GIC 53, a type I membrane protein, and p63 a type II membrane protein, contain the double lysine and double arginine motif, respectively. But both of these molecules localize to the intermediate compartment, as does the soluble KDEL-containing CaBP1 (Calcium binding protein 1). Furthermore, removal of the K(X)KXX from the endogenous ER enzyme, UDP glucuronyltransferase (UDP-GT), does not result in loss of ER retention. Oligosaccharide analysis of some endogenous ER proteins has failed to detect any Golgi modifications, suggesting that these endogenous ER proteins may never leave this organelle. Therefore, retention signals, of unknown nature, must exist in endogenous ER proteins and operate independently of ER retrieval signals.

1) CGN proteins

CGN proteins include KDEL receptor, erd2, and several non-characterized antigens such as p53 (Schweizer et al, 1988) and p58 (Saraste et al, 1987). Some virus encoded

membrane proteins, such as avian coronavirus, infectious bronchitis virus (IBV), M protein (Machamer et al., 1991) are specifically targeted to the CGN. The intermediate compartment proteins described above should also ascribed as CGN.

2) Cisternal proteins

In recent years the genes encoding a number of Golgi enzymes involved in carbohydrate modification have been isolated and all encode membrane proteins with a type II orientation (Paulson and Colley, 1989; Moreman and Robins, 1991; Shaper and Shaper, 1992). They all have a short NH_2-terminal cytoplasmic tail (up to 30 amino acids), a 16-20 amino acid signal-anchor domain, and a extended stem region which is followed by a large COOH-terminal catalytic domain. The signal-anchor domain acts as both a noncleavable signal peptide and the transmembrane domain which orients the catalytic domain of these glycotransylferases within the lumen of Golgi apparatus. Some viral type I proteins including rubella virus E2E1 glycoproteins are targeted to cisternal Golgi (Hobman et al, 1993)

3) TGN proteins

Several prohormone-processing proteases that probably reside in the TGN have been cloned. In yeast, Kex 1p and Kex 2p have a type I membrane topology. Dipeptidyl aminopeptidase A has a type II membrane topology (Fuller et al, 1988). These enzymes are believed to reside in the last Golgi subcompartment, but this classification is tentative due to the difficulty in distinguishing yeast Golgi subcompartments morphologically. The mammalian furin/PACE protease shares homology with Kex 2p and has the same membrane topology (Barr, 1991). TGN 38, a protein of unknown function, has a type I topology as well (Luzio et al, 1990).

Targeting signals of resident Golgi proteins

1) CGN proteins

The first Golgi localization signal was identified on a viral membrane protein. The avian coronavirus infectious bronchitis virus (IBV) M protein is targeted to CGN of Golgi complex when expressed from cDNA in mammalian cells (Machamer et al., 1991). The M1 protein spans the membrane three times. Deletion analysis suggests that the information for retention in the CGN is contained in the first membrane spanning domain. Work by Swift et al (1991) has shown that this transmembrane domain does indeed contain a CGN retention signal which is sufficient to retain two proteins that are normally transported to plasma membrane in CGN/cis-Golgi when the transmembrane domain of these proteins are replaced by the first transmembrane domain of M protein. One of these proteins is Gm1, a type I membrane protein in which the transmembrane domain of VSV-G (a cell surface membrane protein) protein is substituted by the first transmembrane segment of M protein. Uncharged polar residues that line the one face of a predicted a-helix constitute the important feature of the retention signal. When any of these key residues was mutated, the reporter protein was transported to the cell surface (Swift et al., 1991). In contrast to the avian IBV M protein, a related protein from a murine coronavirus is targeted to the trans cisternae/ TGN when expressed from cDNA (Locker et al, 1992). Unlike the avian IBV M protein, the first transmembrane domain of murine IBV fails to retain a reporter protein in the Golgi stacks or TGN (Armstrong et al., 1991). The Golgi localization of this protein requires the carboxyl-terminal 18 amino acids; although this sequence is not sufficient for retention of a reporter protein. The targeting signal of two viral Golgi proteins thought to be similar has been shown to reside in different domains of the molecules.

2) Cisternal Golgi proteins

Several groups have recently examined the targeting signal of endogenous Golgi glycosyltransferases (Shaper and Shaper, 1992: Machamer, 1993). Studies with GlcNAcT-1, GalT, and α-2,6 sialyltransferase (SialylT) have presented a general picture that transmembrane domain in a proper sequence context is critical for the Golgi retention. Sequences flanking the transmembrane domain are frequently necessary context for efficient Golgi retention.

Colley et al (1989), first showed that SialylT, a transferase enriched in trans Golgi and TGN in most cell types, was efficiently secreted from the transfected cells when a cleavable signal sequence replaced the normal N-terminus (including cytoplasmic tail, transmembrane domain and part of stem region). This localized the retention signal to subregion of the protein which included the cytoplasmic tail, transmembrane domain and a short portion of the lumenal part called stem. Later Munro (1991) found that only portion of SialylT required to retain a type II plasma membrane protein in the Golgi complex was its membrane spanning domain; although leaking to the cell surface was prevented when a portion of stem was also included. At the same time Wang et al.(1992) showed Golgi distribution of a chimeric protein in which the 17-amino acid SialylT transmembrane region replaced the transmembrane region of a plasma membrane protein, dipeptidyl peptidase IV (Wang et al., 1991). More recent work by Dahdal et al (1993) challenges the notion that transmembrane domain of ST is the dominant targeting feature for Golgi localization. Chimeric proteins consisting of SialylT cytoplasmic tail and transmembrane domain fused to extracellular domain of two cell surface proteins (transferrin receptor and influenza neuraminidase) demonstrated leaky Golgi retention. Their results suggest that appropriately spaced short, a few amino acids, cytoplasmic and lumenal flanking sequences are important elements of SialylT Golgi retention signal. Both the work of Munro (1991) and Dahdal and

Colley (1993) indicates the actual sequence of the SialylT transmembrane domain is of little importance in its retention in the Golgi..

The relative role of the transmembrane domain together with flanking sequences in Golgi retention of both type I and type II Golgi proteins has been investigated in detail for GlcNAcT-1, GalT, and viral cisternal proteins (type I). Tang et al. (1992) demonstrated that the transmembrane domain of GlcNAcT-1 contained a Golgi retention signal that could retain a reporter molecule in the Golgi. Further work by Burke et al. (1994) suggested that besides the transmembrane domain of GlcNAcT- 1 the cytoplasmic tail and lumenal domain also contribute significantly to preventing the proteins leaking to the plasma membrane. In the study, specific domain domains were swapped between GlcNAcT-1 and human transferrin receptor which has cytoplasmic tail with seven amino acids. Russo et al. (1992) showed that the cytoplasmic tail and transmembrane domain of GalT could retain the marker protein pyruvate kinase in trans Golgi. Nilsson et al. (1991) showed that for a chimera of GalT and a type II protein normally found at plasma membrane and in endosomes, as few as 11 amino acid residues from lumenal side of the membrane spanning domain of GalT were sufficient to retain the chimera in the Golgi. The Golgi targeting signals of two viral cisternal proteins, rubella virus E2E1 glycoprotein (Hobman et al, 1995) and bunyavirus G1 glycoprotein (Matsuoka et al, 1994), have also been narrowed down to the transmembrane and short, a few amino acids, proximal flanking sequences.

Thus retention signals for medial, trans-cisternae/TGN, and viral cisternal proteins are all found within a small portion of the proteins, the transmembrane domain and proximal flanking sequences.

Retention mechanism of Golgi resident proteins

Retention of Golgi membrane proteins (for review see Gleeson et al., 1994) appears to operate by a non-satuable mechanism. Overexpression of Golgi membrane proteins from their cDNAs does not lead to accumulation at the cell surface, rather the

overexpressed proteins accumulate in early compartments of the secretory pathway, such as ER (for review see Nilsson and Warren, 1994). This can not be easily explained by a receptor mediated retention mechanism. Receptors are by definition satuable; overexpression leading to receptor saturation should produce cell surface expression of the protein by default membrane flow.

Three different models have been proposed to account for retention of membrane proteins in the Golgi stacks. 1) Formation of large insoluble aggregates or lattices: according to this model, resident Golgi proteins form large insoluble complexes that are unable to enter transport vesicles (Machamer, 1991; Machamer 1993). It is proposed primarily as a way to explain the importance of the transmembrane domain in Golgi retention of proteins. Consistent with this proposal, the Golgi retained chimeric protein Gm1 (VSV-G protein in which transmembrane domain is replaced with the first transmembrane domain from infectious bronchitis virus M protein) forms large, sodium dodecyl sulfate (SDS) resistant oligomers when expressed in COS cells (Swift and Machamer, 1991; Weisz et al, 1993). However, this behavior has not been observed for the IBV M protein. 2) Kin recognition: this model proposes that Golgi enzymes are homodimers (stabilized by lumenal domain interactions) which are retained by interactions between the transmembrane domains of adjacent "kin" homodimers in the same cisternae, thus forming long heterooligomers (Nilsson et al., 1993, 1994). Retention is believed to be enhanced by interaction of cytoplasmic domains of Golgi proteins with an intercisternal matrix (Slusarewicz et al., 1994). Experiments suggest that Golgi membrane proteins are dimers (Fleischer et al, 1993) 3) Bilayer-mediated sorting: this model is based on the observation that the transmembrane domains of endogenous Golgi enzymes are shorter (17 residues on average vesus 21 for plasma membrane proteins). It holds that Golgi membrane proteins are inherently less stable in thicker, cholesterol and sphingolipid-rich

membranes (e.g., plasma membrane) and as a result, partition to the thinner cholesterol/sphingolipid-poor bilayers of the Golgi (Bretscher and Munro, 1993).

All these models predict overexpressed Golgi proteins will accumulated in early compartments of the secretory pathway rather than the cell surface. For aggregation and kin recognition models the overexpressed Golgi proteins are at an abnormally high protein concentration during their synthesis in the ER and hence pre-Golgi associations and oligomerization are promoted. The aggregated protein hence does not exist the ER and is degraded slowly in situ. For the bilayer-mediated modal, the overexpressed protein can not partition to the Golgi membrane; the amount of Golgi lipid is fixed in amount and hence the capacity of the Golgi is limited.

1.5. Effects of brefeldin A (BFA) on membrane traffic and organelle structure of Golgi

BFA is a macrocyclic lactone synthesized from palmitate (C16) by a variety of fungi. It was originally isolated and characterized as an antiviral antibiotics. BFA's utility for cell biologist began with recognition that in BFA-treated cells, protein secretion is blocked at an early step in the secretory pathway (Oda et al., 1987). That the block occurred in a pre-Golgi compartment was supported by immunofluorescence and electron microscopy observations demonstrating that secretory and membrane proteins were retained in the ER of BFA-treated cells (Lippincott-Schwartz et al., 1989). Although newly synthesized proteins appear to be retained in the ER of BFA-treated cells, further biochemical characterization of these proteins reveal the surprising result that these proteins become processed by Golgi enzymes (Lippincott-Schwartz et al., 1989). Moreover, ER resident glycoproteins themselves show evidence of Golgi processing, becoming endoglycosidase H resistant in BFA-treated cells (Lippincott-Schwartz et al., 1989). The explanation for these paradoxical results is that within 1 hr of BFA treatment, the Golgi marker enzymes mannosidase II and thiamine pyrophosphatase appear within both reticular

and cisternal elements of the ER including the nuclear envelope. Indeed no recognizable Golgi stacks are observed in BFA-treated cells. Other markers of the Golgi complex including galactosyltransferase, NBD-ceramide (a Golgi lipid marker), and newly synthesized VSV-G passing through the Golgi complex all redistribute into ER in the presence of BFA (Lippincott-Schwartz et al., 1990). In contrast, some components of the TGN do not redistribute into the ER (Lippincott-Schwartz et al., 1991).

Within minutes of adding BFA to the cells at 37°C, Golgi markers are no longer localized by immunofluorescence microscopy to a compact, perinuclear cisternal structure. Golgi cisternae appear first to swell and then to extend long tubular processes out to the cell periphery. Tubules budding from swollen Golgi cisternae during BFA treatment are uniformly 90 nm in diameter with no apparent cytoplasmic "coat" material associated with them. The Golgi derived tubules extended along the microtubules. After about 30 min of BFA treatment Golgi tubules are no longer visible and Golgi marker instead show a punctated-reticular distribution representing their steady-state mixing with ER. The tubules thus appear to be intermediates in the movement of Golgi membrane into the ER. Reduced temperature and ATP-depletion inhibit tubule formation and retrograde transport of Golgi membrane into ER in BFA treated-cells. These effects are rapidly and completely reversed by removing the drug (Lippincott-Schwartz et al., 1989).

One of the most clear-cut effects of adding BFA to cells is a tight block of membrane traffic out of ER. While BFA inhibits anterograde movement of membrane beyond the mixed ER/Golgi system, it appears to enhance a second pathway, movement of Golgi membrane into ER. A growing body of evidence supports the notion that there is a normal retrograde pathway from Golgi back to the ER (Lippincott-Schwartz et al., 1990). Thus, it is possible that transport into ER induced by BFA represents enhanced trafficking though this normal retrograde pathway as a result of absorption of Golgi protein and membrane components into this pathway, or BFA may selectively inhibit the anterograde

transport from the ER to Golgi. To further characterize proximal changes in Golgi apparatus induced by BFA, Donaldson et al. (1990) tested by immunofluorescence whether any cytoplasmic proteins which are specifically associated with Golgi apparatus are released upon addition of BFA. One protein of 110-kD protein, now known as β-COP I, was found to redistribute from a Golgi-like pattern to a diffuse, cytosolic pattern upon BFA treatment. Upon removal of BFA, β–COP rapidly reassociates with Golgi membrane. Conditions that inhibit Golgi membrane into ER during BFA treatment, namely lowered temperature and microtubule depolymerization, do not inhibit the rapid cytoplasmic redistribution of β-COP I.

The effects of guanine nucleotides on BFA-induced b-COP I redistribution were examined in filter perforated , semi-intact cells (Donaldson et al., 1991b). As in intact cells, treatment of semi-intact cells with BFA alone causes the rapid release of β-COP I and movement of Golgi membrane into the ER. Pretreatment of semi-intact cells with GTP-γS inhibit these effects. The ability of GTP-γS to inhibit the BFA action can be abrogated by adding GTP. A model was proposed for regulated cycling of β-COP I between the membrane and cytosol whereby β-COP I rapidly associates/dissociates with Golgi membrane, requiring GTP to bind membrane and GTP hydrolysis to be released from membrane. The addition of BFA to cells results in rapid redistribution of ARF to a cytoplasmic pattern, which can be reversed by removal of drug. Using the in vitro binding assay (Donaldson et al., 1991a), binding of ARF to the Golgi membranes is enhanced in the presence of GTPγS, inhibited by BFA. It is suggested that BFA may inhibit the exchange of ARF bound GDP to GTP to prevent it and COP Is to assemble at Golgi membrane.

The ability of BFA to inhibit the assembly of cytosolic coat proteins onto then Golgi membranes has been examined in the in vitro intra-Golgi transport assay developed by Rothman and colleagues. In the absence of BFA, the ability of transfer membrane between

two distinct sets of Golgi requires ATP and cytosol and is inhibited by GTPγS. In the presence of BFA, intra-Golgi membrane transport occurs but no longer inhibited by subsequent addition of GTPγS, while addition of two drugs in opposite order abrogates this transport (Orci et al., 1991). Ultrastructure examination of BFA treated of this in vitro Golgi stacks shows an anastomosing tubular network. The most reasonable explanation of this is that the distinct Golgi cisternae mix via the formation of this network. These results suggest that in the presence of BFA, where the membrane budding of non-clathrin coated vesicles is inhibited, exchange of membrane between two cisternae occurs. It is not clear if these effects also exist in the absence of BFA.

Besides membrane traffic between ER and Golgi complex, BFA also affects other parts of the secretory and the endocytic pathway. Low et al. (1991) demonstrated that in MDCK cells, where the Golgi complex but not the endosomal system is resistant to low concentrations of BFA, protein secretion at the apical cell surface is inhibited by BFA while basolateral secretion is enhanced. Hunziker et al. (1991) demonstrated that in the same cell line that IgA can be taken up from the basolateral surface in a normal fashion in the presence of BFA, but its release at the apical surface is inhibited. The effect of BFA on the distribution of two different components of the TGN, TGN38 and the mannose 6 phosphate receptor has also been examined. In the presence of BFA, both proteins enter membrane tubules before establishing a new steady-state distribution with early endosomal components (Lippincott-Schwartz et al, 1991; Wood et al., 1991).

1.6. Effect of nocodazole on the organization of the Golgi complex

It has become increasingly evident that the microtubule network plays a direct and crucial role in the organization of cytoplasmic organelles and in the vesicle-mediated traffic between these membrane bounded compartments. The Golgi complex is a key organelle involved in intracellular membrane traffic, and interaction of this organelle with microtubules has been shown by several studies (for reviews see Thyberg and

Moskalewski, 1985; Kreis, 1990) The Golgi complex co-localizes with minus ends of interphase microtubules, which are usually associated with the microtubule-organizing center. Evidence for an interaction of elements of the Golgi complex with microtubules during establishment and maintenance of perinuclear position is manifold. Both the Golgi complex and the microtubule-organizing center change their location in a coordinate way during cell locomotion (Singer and Kupfer, 1986), cell differentiation (Tassin et al., 1985), and cell fusion (Ho et al., 1990). The Golgi complex fragments into tubulo-vesicular clusters at the onset of mitosis, when the interphase microtubules depolymerize and the mitotic spindle forms (Lucocq et al., 1989). Agents that alter the distribution of interphase microtubules have profound effects on integrity and location of Golgi complex. For example, treatment of cells with nocodazole or similar drugs that induce of complete depolymerization of interphase microtubules leads to fragmentation of Golgi complex into distinct elements, which randomly disperse throughout the cytoplasm (Rogalski and Singer, 1984). These dispersed Golgi elements reaggregate upon removal of the drug and subsequent microtubule repolymerization. It is also suggested that the movement of Golgi elements along the microtubules is cytoplasmic dynein dependent (Kreis, 1990). Under condition where microtubules are depolymerized and Golgi stacks are dispersed throughout the cytoplasm, newly synthesized proteins are still transported through the Golgi cisternae (Rogalski et al., 1984). Prolonged incubation with nocodazole arrests cells in mitosis by inhibiting mitotic spindle formation (Jordan et al., 1992). Analysis of vitally stained cells reveals that Golgi elements move along the microtubules toward their minus ends during reclustering, and neither intermediate filaments nor microfilaments are involved in this process (Ho et al., 1989; Ho et al., 1990). Taken together, these studies indicate that intact interphase microtubules play an essential role in maintaining the structural integrity and location of the Golgi complex.

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Chapter 2. Compartmentation of the Golgi Complex: Comparative Behavior of Golgi Subcompartments in Response to Nocodazole and Brefeldin A Challenge

2.1. Abstract

We have characterized the distribution in Vero cells of three Golgi cisternal membrane proteins and one cis Golgi network (CGN) membrane protein during the process of Golgi disassembly and reassembly following the addition or removal of the drugs, nocodazole (microtubule depolymerizer), and brefeldin A (BFA, coat protein inhibitor). α- 2,6-sialyltransferase (SialylT) and N-acetylglucosaminetransferase-1 (GlcNAcT-1) C-terminally tagged with the myc epitope provided markers for the trans-Golgi/trans Golgi network (TGN) and medial-Golgi, respectively. Distributions of the recombinant proteins were assessed by double label immunofluorescence with respect to that of the endogenous protein, β-1,4-galactosyltransferase (GalT, trans-Golgi/TGN). In control cells, the various cisternal markers gave a compact, tubular, perinuclear staining pattern with essentially complete overlap in distribution while p53, the CGN marker, although showing the same perinuclear location, gave a qualitatively dissimilar staining pattern that was more that of tightly clustered dots. After 1 h nocodazole treatment, SialylT and GalT were found in scattered punctate structures that increased in number over time as less and less of these proteins were found perinuclearly. Initially these punctate structures were often negative for GlcNAcT-1. Over a 2 to 3-fold slower time course, GlcNAcT-1 colocalized with SialylT and GalT in the scattered punctate structures. About 7% of the cisternal positive punctate structures were positive for p53. p53 was found in a separate set of scattered punctate structures even at 4 h following nocodazole addition. Following nocodazole removal, all the cisternal markers accumulated perinuclearly into a reassembled Golgi at the same rate. After BFA treatment, all the cisternae markers dispersed to ER with

similar kinetics, albeit, in some cases by different tubular extensions of the Golgi. GlcNAcT-1 and GalT showed similar kinetics of Golgi reassembly following BFA removal while SialylT lagged somewhat behind. Our data suggest that CGN, medial-Golgi and trans-Golgi/TGN are distinct subcompartments that can be separated one from the other by drug treatment; any exchange of components between the Golgi subcompartments must be slow with respect to the observed kinetics of Golgi disassembly.

2.2. Introduction

Animal cells have a juxtanuclear Golgi complex which comprises a dynamic membranous network. The organelle plays an important role in processing, maturation, and sorting of newly synthesized secretory and membrane proteins from ER, and in recycling receptors involved in endocytosis (for reviews, see Palade, 1975; Pfeffer and Rothman, 1987). The Golgi complex may be considered to be composed of three distinct subcompartments, the CGN, cisternal Golgi which includes cis-, medial- and trans-Golgi and the TGN (Mellman and Simons, 1992). The CGN consists of transitional elements which are localized near the ER and is involved in receiving and sorting of newly synthesized proteins from the ER. The cisternal Golgi functions as a glycosylation compartment where addition and trimming of carbohydrate moieties takes place. The final compartment, TGN, plays an essential role in sorting plasma membrane, lysosomal and secretory proteins to their respective end destinations. The glycosylation enzymes which modify N-linked oligosaccharides are located within the cisternal Golgi in the order in which they act (for review, see Dunphy and Rothman, 1985; Kornfeld and Kornfeld, 1985). Briefly, the enzymes residing in CGN such as mannosidase I act to continue trimming of mannose residues, a process started in the ER, those in the medial cisternae such as GlcNAcT-1 catalyze the addition of sugars to a core N-linked oligosaccharide sidechain, and those located in the trans cisternae to TGN such as GalT and SialylT act to add to terminal sugars to N-linked sidechains. The most current morphological data indicate that contrary to expectation there is appreciable overlap in distribution of oligosaccharide processing enzymes located in the medial-Golgi and trans-Golgi/TGN. Nilsson et al. (1993) find that GlcNAcT-1 is located in both the medial- and trans-cisternae. About one third of GlcNAcT-1 and half of GalT were found in the trans-cisternae. The remainder of each was found, respectively, in the medial-cisternae and TGN.

Coat proteins and microtubules play distinct roles in the structural organization of the Golgi complex. COP I coated vesicles have been proposed to mediate transport from ER to Golgi and between different Golgi stacks (for review, see Rothman,1994). The drug BFA inhibits ER-Golgi protein transport and dramatically redistributes Golgi components to the ER resulting in Golgi disassembly by promoting COP I dissociation from membrane (for review, see Klausner, Donaldson and Schwartz, 1992). Uncoated tubules emanating from Golgi complex are observed shortly after addition of BFA suggesting that they are structural intermediates that carry the Golgi membrane to the ER (Lippincott-Schwartz et al., 1990; Hauri and Schweizer, 1992). Cytoplasmic microtubules are thought to play a central role in the pericentriolar localization of Golgi complex. Agents that alter the distribution of interphase microtubule have profound effect on the distribution and integrity of Golgi complex. A most dramatic example of this is the reversible fragmentation and scattering of the Golgi after nocodazole treatment. Nocodazole binds tubulin heterodimers and inhibits their polymerization, therefore progressively depolymerizing the microtubules (Robbins and Gonatas, 1964; Rogalski and Singer, 1984). Electron microscopy examination reveals that the Golgi fragments are composed of stacked cisternae which resemble the intact Golgi stacks (Thyberg and Moskalewski, 1985). Surprisingly, under this condition newly synthesized proteins are still transported through and modified by the Golgi complex (Rogalski et al., 1984). After removal of drug the Golgi fragments are translocated along the microtubules during reassembly of intact interphase Golgi complex (Ho et al., 1989). After prolonged incubation of cells with nocodazole the Golgi is found to be composed of small vesicular structures similar to the mitotic form of Golgi (Lucocq et al, 1989). Breakdown of interphase Golgi during mitosis is the direct consequence of activated cdc2 kinase (for review, see Moreno and Nurse, 1990) and is thought to be a COP I mediated vesiculation process (Misteli and Warren, 1994).

Here we have used the drugs nocodazole and brefeldin A as tools to examine how distinct the subcompartmentalization of Golgi proteins is. We describe the distribution of three well characterized cisternal type II transmembrane proteins of Golgi: SialylT, GalT, and GlcNAcT-1 and p53 (for review, see Hauri and Schweizer, 1992), a membrane protein of cis-Golgi network (CGN), following drug treatment of Vero cells. The major conclusion of our experiments is that *in vivo* SialylT and GalT must either be in the same Golgi subcompartment or in rapid equilibrium with each other and that GlcNAcT-1 is located predominantly in a separate subcompartment that exhibits little, if any, equilibration with more trans subcompartments. Based on these experiments, the p53 positive subcompartment is distinct from the cisternal structures.

3.3. Materials and methods

Materials

General purpose chemicals were purchased from Baxter Scientific Products (McGaw Park, IL) or Sigma Chemical Company (St. Louis, MO). Restriction enzymes were purchased from Promega (Madison, WI) or New England Biolab (Bainbury, MA). Electrophoresis chemicals were purchased from Bio-Rad (Hercules, CA). Photographic reagents and Kodak X-ray film were from Kodak (Rochester, NY). Cell culture reagent s were purchased from Gibco BRL (Grand Island, NY) and fetal bovine serum from Hy-Clone Laboratories, Inc. (Logan, UT). Protein Labeling Mix [^{35}S] Methionine was purchased from NEN/Du Pont (Boston, MA). 9E10 mouse monoclonal antibody against a myc peptide (Evan et al., 1985) was diluted from an ascites preparation. G1/93 mouse monoclonal against human p53 was a gift of Prof. Hans-Peter Hauri, Biozentrum, Basel, Switzerland. A rabbit polyclonal antibody against bovine GalT was a gift from Dr. Joel Shaper, Oncology, Johns Hopkins University School of Medicine, Baltimore, MD. HP24 mouse monoclonal antibody against human protein disulfide isomerase (Kaetzel, Rao and Lamm, 1987) was a gift of Dr. Charlotte Kaetzel, Pathology, Case-Western Reserve School of Medicine, Cleveland, OH. DM 1A mouse monoclonal antibody against chicken α-tubulin was purchased from Sigma Chemical Company.

Methods

Recombinant DNA The human GlcNAcT-1 cDNA (Kumar et al., 1990) with a myc epitope at its extreme carboxyl-terminus was prepared by Dr. T. Nilsson in pSRα expression vector (Nilsson et al., 1993) and was a gift of Dr. T. Nilsson (ICRF, London, UK). The full length human SialylT (generously provided by Dr. S. Munro, MRC, Cambridge, UK) was used as a PCR template to place the 9E10 myc epitope (Evan et al., 1985) at the carboxyl terminus. The primers were:

5' primer: 5'-GGATCCGGATCCCATATGATTCACACCAACCTGAAG-3',

3' primer: 5'-GGATCCGGATCCTTACAGGTCTTCTTCAGAGATCAGTTTCTG

TTCAGGCAGTGAATGGTCCGGAAGCC-3'.

The bases encoding the myc epitope are underlined. The PCR product was digested by BamH I and the resulting DNA was ligated into pSRα at the BamHI site. Recombinant plasmids containing SialylT-myc with correct orientation with respect to the promoter were selected by restriction enzyme digestion and subsequently confirmed by DNA sequencing.

Cell Culture and Transfection Monolayer Vero (African green monkey) cells were cultured in MEM medium supplemented with 10% heat inactivated fetal calf serum. All cells were routinely grown in 100-mm plastic tissue culture dishes. The cultures were maintained at 37°C in a humidified 5% CO_2 incubator.

For DNA transfection, cells were seeded at $1x10^6$ cells per 100-mm dish one day before transfection so the cells were about 70% confluence on the day of transfection. The plasmid DNA pSRα-GlcNAcT-1-myc or pSRα–SialylT-myc was purified using $CsCl_2$ gradient centrifugation according to standard methods and dissolved in distilled water at 1μg/μl final concentration. The calcium phosphate method was used (Chen and Okyama, 1987). Approximately 20 μg of DNA were used for each transfection. The precipitate was left in contact with the cells for 16 hr. Cells were then rinsed once with PBS and once with calcium- and magnesium-free medium before additional incubation in complete medium. In transient expression experiments, cells were analyzed by indirect immunofluorescence microscopy 24-36 hr after transfection. In stable expression experiments, cells were maintained in the above medium containing 500 μg/ml Geneticin (G418 sulphate) for two weeks before isolating individual clones. Clones were screened by immunofluorescence using 9E10 monoclonal antibody. About one out of three clones for both GlcNAcT-1-myc and SialylT-myc transfections were positive for perinuclear Golgi staining with 9E10

monoclonal antibody. SialylT-myc and GlcNAcT-1-myc Vero cells were maintained in the presence of 200 μg/ml Geneticin.

Metabolic Labeling With [^{35}S] Methionine and Immunoprecipitation Wild type or transfected Vero cells expressing SialylT-myc or GlcNAcT-1-myc grown in 60-mm dishes were incubated for 20 min at 37°C in MEM medium lacking methionine supplemented with [^{35}S] methionine (50 μCi/ml) and 10% dialyzed fetal bovine serum. The cells were rinsed with three times with PBS and then chased in complete MEM medium for 2h. For lysate preparation, cells were washed twice with ice cold PBS and then solublized in 500 μl of ice cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, 2 μg/ml leupeptin). Dishes were then scraped and cell debri pelleted by 5 min centrifugation in a chilled Eppendorf centrifuge. To each supernatant, 10 μg of 9E10 antibody was added. After rocking overnight at 4°C, 50 μl of protein A-Sepharose beads were added to each sample tube, and the tubes were rocked at 4°C for 1 h. Immunoprecipatate-Protein A-Sepharose complexes were pelleted by 2 sec centrifugation in an Eppendorf in the cold room and then washed 5 times with cold lysis buffer. Material was released from the beads by boiling each sample for 5 min in SDS sample buffer (62.5 mM Tris, pH 6.8; 2% (w/v) SDS; 10% (v/v) glycerol; and 5% β-mercaptoethanol) (Laemmli, 1970) and analyzed by 10% SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed to autoradiography film for 16 h..

Drug Treatments Nocodazole was obtained from Sigma Chemical Co. (St. Louis, MO) and stored as 10 mM stock solution at -20°C. BFA was purchased from Epicenter Technologies (Madison, WI). It was stored at -20°C as a stock solution of 5 mg/ml in methanol. Immediately before use, stock drug solutions were diluted to final concentration in complete culture medium. Coverslip cultured cells were incubated at 37°C with 10 μM nocodazole or 5 μg/ml BFA for various time periods. For drug removal, coverslip cultured

cells were transferred to fresh dishes containing complete culture medium three times and then incubated in drug-free complete culture medium at 37°C for various time periods.

Immunofluorescence Microscopy SialylT-myc Vero cells and GlcNAcT-1-myc Vero cells were grown attached to 10 mm round glass coverslips in complete medium for 48 hr before use. After appropriate drug treatment, cells were transferred directly to -20°C methanol for 3 min. Methanol fixed cells were rinsed three times with room temperature PBS and incubated in appropriate primary antibodies for 30 min at room temperature. Coverslip cultures were then rinsed three times with PBS and then incubated with FITC-conjugated donkey anti-rabbit IgG and Texas red goat anti-mouse IgG secondary antibodies (Jackson Immunoresearch, West Grove, PA) for double labeling. Excess secondary antibodies were removed by washing cells three times in PBS. Coverslips were then mounted in Mowiol. Antibodies were diluted in PBS. Cells were observed with a Zeiss IM-35 inverted microscope using a Zeiss planapochromat 63X (numerical aperture=1.40) oil immersion objective. Fluorescein and Texas red fluorescence were observed with selective Zeiss filter sets. Randomly selected fields of cells were photographed on Kodak Tmax 3200 film.

Scoring of Co-localization and Number of Punctate Structures Fluorescent micrographs were printed at an end magnification of 1200X. For scoring of co-localization, appropriate micrographs of similar contrast and intensity were paired, overlaid with a grid, and positive structures marked with a pen. The number of punctate structures was scored similarly excluding the immediate perinuclear Golgi complex. For each time point, approximately 30 cells were scored.

3.4. Results

In these studies we wished to examine the effect of nocodazole and BFA challenge on multiple portions of the Golgi complex. Vero GalT and p53 were localized using a crossreacting rabbit polyclonal antibody or a mouse monoclonal antibody, respectively. Because of the lack of available antibodies to other Vero Golgi proteins and the probable difficulty of their generation, we decided to stably express separately in Vero cells SialylT-myc or GlcNAcT-1-myc. The myc epitope reacts with the monoclonal antibody 9E10. In the respective SialylT and GlcNAcT-1 transfectants, the distributions of three of the four different markers could be characterized by appropriate pairwise double label immunofluorescent staining. Typically, GalT was paired with SialylT-myc or GlcNAcT-1-myc.

Construction and Characterization of Stable Vero Cell Lines Expressing SialylT-myc and GlcNAcT-1-myc

Vero clones stably expressing SialylT-myc or GlcNAcT-1-myc were prepared as described in Material and Methods. As shown in Fig. 1 A,D, the two clones used for these studies exhibited a strong perinuclear, Golgi immunofluorescence pattern when stained with anti-myc antibody (9E10). The distribution of both SialylT-myc (Fig. 1, A) and GlcNAcT-1-myc (Fig. 1, B) overlapped completely with GalT (Fig. 1, B,C). The Golgi immunofluorescence appeared compact and continuous. This result confirms at the immunofluorescence level the previous light and electron microscope observations of Nilsson et al. (1993) with HeLa cells that GlcNAcT-1-myc localized normally to the Golgi complex.

The molecular weight of SialylT-myc and GlcNAcT-1-myc synthesized in the stable Vero cells was determined by immunoprecipitation or immunoblotting. For immunoprecipitation, wild type, SialylT-myc, and GlcNAcT-1-myc Vero cells were

metabolically labeled by [^{35}S] methionine incorporation, immunoprecipitated with 9E10 antibody, and the precipitates then analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, lane A, wild type Vero yielded essentially no 9E10 immunoprecipitate. The GlcNAcT-1-myc cell precipitate consisted of a major band with a 54 kD molecular mass (Fig. 2, lane B, left arrowhead) in agreement with previous results (Nilsson et al., 1993). In both immunoprecipitation (Fig. 2, lane C) and immunoblotting experiments (data not shown), SialylT-myc cell lysates yielded two major electrophoretic bands. The slower migrating band was a doublet with molecular masses of 53 and 56 kD (Fig. 2, lane C, righthand arrowhead). The faster migrating band was again a doublet, here of 47 and 48 kD (Fig. 2, star). The molecular mass of SialylT-myc deduced from its amino acid sequence is 50 kD. SialylT is known to be glycosylated and has O-linked sugar sidechains and 1 to 2 N-linked sugar sidechains (Colley et al., 1992). Fully processed SialylT-myc then should have a molecular mass of about 56 kD. We propose that the slower migrating doublet consists of SialylT-myc which differs in the number of N-linked oligosaccharide sidechains. The faster migrating doublet may represent either SialylT-myc degradation products or be an immunoprecipitation contaminant. As small amount of this appear in lanes A (control) and B (GlcNAcT-1-myc immunoprecipitation), it is likely to be contamination along with the other fainter bands in the SialylT-myc immunoprecipitation.

We conclude that SialylT-myc and GlcNAcT-1-myc are normally synthesized and distributed in transfected Vero cells. For the sake of simplicity, we will refer to the immunofluorescent staining of these proteins in the cell as that of SialylT or GlcNAcT-1, respectively.

Nocodazole Fragmentation of Trans-Cisternae/TGN Precedes That of Medial-Cisternae

We monitored the distribution of three cisternae type II membrane proteins, SialylT (trans-Golgi/TGN), GalT (trans-Golgi/TGN) and GlcNAcT-1 (medial-Golgi), following nocodazole addition at 10 μM at 37°C. Before drug addition, the three cisternal markers

were co-localized in a compact perinuclear distribution (Fig. 1, A-D). 15 min after nocodazole addition, a small number of punctate structures positive for the marker proteins were observed, often close to the nucleus and Golgi complex (Fig. 1, E-F). Decidedly fewer punctate structures were positive for GlcNAcT-1 than SialylT or GalT. Microtubules had already depolymerized (data not shown). With time (30, 60, 120, 240 min; Fig. 1, I-X), the number of punctate structures positive for each cisternal protein increased while compact perinuclear Golgi staining disappeared. Qualitatively the redistribution of GlcNAcT-1 positive Golgi to a punctate staining pattern lagged behind that of SialylT and GalT (e.g., see, black arrowheads Fig. 1, S and T). Qualitatively, SialylT and GalT appeared to redistribute to punctate structures with similar kinetics. By 240 min, all three cisternal proteins were found in punctate structures that were scattered in no apparent pattern about the cytoplasm. As indicated by the white arrowheads in Fig. 1 (S and T), punctate structures that were positive for GalT were often negative for GlcNAcT-1 at time points through 120 min.

Quantitatively, the rate of redistribution of the trans/TGN proteins, SialylT and GalT, into punctate structures appeared to be 2-3 fold faster than that of the medial protein, GlcNAcT-1 (Fig. 3). As shown in Fig. 4A, the number of punctate structures positive for SialylT and GalT increased in parallel starting at about 3 per cell at 15 min following drug addition and ending at about 45 per cell at 240 min. The number of GlcNAcT-1 positive punctate structures per cell were decidedly fewer at time points through 120 min, e.g., at 120 min, about 40 GalT positive versus about 16 GlcNAcT-1 positive (Fig. 4B). At 240 min following nocodazole addition, the number of GalT and GlcNAcT-1 positive structures were equal at about 50 per cell. To determine whether individual punctate structures contained both GalT and SialylT or GalT and GlcNAcT-1, each individual structure was scored for co-localization of immunofluorescence. As shown in Fig. 5A, approximately 80% of the punctate structures positive for GalT were equally positive for SialylT. In

striking contrast only about 30% of the punctate structures positive for GalT were equally positive for GlcNAcT-1 at all time points between 15 and 120 min following drug addition; most were stained more intensely for GalT and about 25% were negative for GlcNAcT-1 staining (Fig. 5B). At 240 min following nocodazole addition, almost 90% of the GalT positive punctate structures were equally positive for both SialylT and GlcNAcT-1.

The data presented here clearly suggest that nocodazole induced fragmentation of the trans-Golgi/TGN precedes fragmentation of medial-Golgi. Moreover, the data suggest that the dispersed punctate fragments of the Golgi which are initially decidedly enriched in trans-Golgi/TGN cisternal markers serve as a delivery site for medial-Golgi components.

Reassembly of the Golgi Complex Following Nocodazole Removal

As shown in Fig. 6, A-D immediately after nocodazole removal, SialylT, GalT and GlcNAcT-1 were fully scattered into cytoplasmic punctate structures. By 15 min after drug removal, occasional perinuclear compact clusters of punctate structures were seen; although, most of the SialylT, GalT, and GlcNAcT-1 staining remained punctate in the cytoplasm (Fig. 6, E-H). After 15 min of drug removal, microtubules were already repolymerized and radiated outward from the cell center (data not shown). Further accumulation of punctate structures about the nucleus was apparent by 30 min post nocodazole removal and few peripheral punctate structures were observed (Fig. 6, I-L). At the 60 and 120 min time points, there was a progressive accumulation of cisternal Golgi proteins into a compact perinuclear distribution presumably accompanied by membrane fusion (Fig. 6, M-T). Qualitatively, there was little apparent difference in the distribution of SialylT, GalT and GlcNAcT-1 at all time points following nocodazole removal. Quantitatively, the kinetics of Golgi reassembly were the same for all three cisternal proteins (Fig. 7) and the number of punctate structures positive for each decreased in parallel (Fig. 8). During the whole process of Golgi reassembly, punctate structures were equally positive for the two protein pairings (scoring not shown).

In contrast to the process of Golgi fragmentation with nocodazole treatment, all three cisternal Golgi markers behaved identically during the process of Golgi reassembly suggesting this process occurs concomitantly with respect to the individual cisternae.

CGN and Cisternal Golgi Behave Separately with Respect to Nocodazole Treatment

To investigate the distribution relationship between Golgi cisternae and the CGN, we studied the effect of nocodazole on the distribution of GalT and p53, which resides in CGN (for review, see Hauri and Schweizer, 1992). Before nocodazole treatment, p53 was distributed in the same general area as that of GalT. Qualitatively, GalT had a more continuous and compact staining pattern than p53 which appeared more as a tight cluster of dots. (Fig. 9, A,B). After 240 min nocodazole treatment, both p53 and GalT appeared scattered in punctate structures. However, few, ~7%, of the p53 positive structures were positive for GalT (Fig. 9, C,D, white arrowheads: examples of non-co-localizing p53 and GalT punctate structures, black arrowheads: examples of co-localization). Furthermore, the p53 distribution fragmented faster than GalT (data not shown). Qualitatively, CGN as marked by p53 appeared to fragment upon nocodazole treatment more rapidly than cisternal Golgi (GalT) (not shown).

These data suggest that the CGN and cisternal Golgi are distinct compartments that respond individually to microtubule depolymerization.

BFA Induced Disassembly of Medial-Cisternae and Trans-Golgi/TGN Occurred at Similar Rates

. BFA treatment of living cells results in the rapid loss of the Golgi complex as a distinct organelle due to redistribution of Golgi content and membrane into the ER (for review, see Klausner et al., 1992). To test whether BFA treatment sequentially disassembles cisternal Golgi, we studied the kinetics of redistribution of three molecularly well characterized Golgi type II membrane proteins, SialylT, GalT and GlcNAcT-1.

Similar experiments have been done by Alcalde et al. (1992) using as markers GIMPc and GIMPt, two Golgi proteins defined by antibody reactivity.

Five min following addition of BFA at 5 μg/ml, tubular processes, similar to those described previously for medial and trans Golgi enzymes (Lippincott-Schwartz et al. 1989), extending from enlarged, perinuclear Golgi structures were observed for all three markers (Fig. 10, compare A-D with E-H). Some of the SialylT positive tubular processes were positive for GalT while other were not (black and white arrowheads, Fig. 10, E,F). For the Gal T and GlcNAcT-1 double labeling pairing, a similar pattern was true (Fig. 10, G,H). With longer incubation times, all markers gradually lost their perinuclear staining and by about 20 min reticular ER staining began to appear, particularly in the case of SialylT (Fig. 10, I-L). The more condensed staining patterns appeared more like a CGN pattern than a Golgi pattern. After 60 min, the majority of the proteins were dispersed into ER (Fig. 10, M-P) with GalT lagging a little bit. With 180 min total incubation, all of them showed a dispersed, reticular staining pattern throughout the cytoplasm characteristic of ER labeling (Fig. 10, Q-T). This co-localized with an ER marker, protein disulfide isomerase (data not shown). Quantitatively, the kinetics of dispersal to the ER of all three, initially compactly distributed, cisternal proteins did not differ greatly, albeit, GalT lagged somewhat behind (Fig. 11). The quantitation includes additional time points, 2 min, 40 min and 120 min, that are not included in the micrographs.

Overall, we conclude that all of the cisternal markers behaved similarly with respect to BFA treatment and that the initial formation of processes for each may occur independently.

Reassembly of Golgi Cisternae After BFA Removal

The perturbing effect of BFA upon the Golgi complex is entirely reversible (Lippincott-Schwartz et al., 1989). Ten min after BFA removal, the distribution of SialylT GalT, and GlcNAcT-1 remained largely disperse (compare Fig. 12, A-D with E-H),

although some of GalT tended to concentrate centrally in the area near the nuclei (Fig. 12F and 12 G). By 40 min, considerable difference existed between GalT/GlcNAcT-1 and SialylT. Both GalT and GlcNAcT-1 showed compact, perinuclear vesicular structures that may represent transport intermediates in ER to Golgi reassembly while SialylT did not (Fig. 12, I-L, for examples, see white arrowheads: I,J). The compact, perinuclear vesicular structures were generally positive for GalT and GlcNAcT-1 (for example, see black arrowheads: K,L) while in other cases they were not (for example, see white arrowheads: K,L). By 90 min, much of GalT and GlcNAcT-1 was in compact perinuclear structures characteristic of reformed Golgi while SialylT tended to remain more disperse and ER like in distribution (Fig 12, M-P). At 120 min, fully reassembled compact perinuclear Golgi staining was achieved for both GlcNAcT-1 and GalT while a clear ER contribution was detected in many of the SialylT staining patterns in addition to a compact perinuclear staining pattern (Fig. 12, Q-T). ER staining by SialylT was reduced to almost non-detectable level after a total incubation of 3 h without drug giving rise to fully reassembled Golgi (data not shown). Quantitatively the kinetics of Golgi reassembly for SialylT were slower than that of GalT and GlcNAcT-1 which were generally similar (Fig. 13). The quantitation includes additional time points, 5 min, 20 min, 60 min and 180 min, that are not included in the micrographs.

Overall, we conclude that the medial and trans-Golgi/TGN compartments as marked by GlcNAcT-1 and GalT behave similarly with respect to Golgi reassembly following BFA removal.

2.5. Discussion

In this study, we have characterized the distribution in Vero cells of three Golgi cisternal membrane proteins and one CGN membrane protein during the process of Golgi disassembly and reassembly following the addition or removal of the drugs, nocodazole and brefeldin A. SialylT and GlcNAcT-1 C-terminally tagged with the myc epitope provided markers for the trans-Golgi/trans Golgi network (TGN) and medial-Golgi, respectively. Distributions of the recombinant proteins were assessed by double label immunofluorescence with respect to that of endogenous GalT, trans-Golgi/TGN. With nocodazole treatment, SialylT and GalT were progressively scattered into punctate structures that initially contained little GlcNAcT-1 and were negative for p53, a CGN marker. GlcNAcT-1 slowly co-localized with SialylT and GalT in the scattered punctate structures while p53 was found in a separate set of scattered punctate structures. Following nocodazole removal, all the cisternal markers accumulated concomitantly into a reassembled Golgi. With BFA treatment, all the cisternae markers dispersed to ER with similar, but not identical kinetics; GalT lagged behind. In contrast SialylT lagged behind during Golgi reassembly following BFA removal. Our data indicate that CGN, medial-Golgi and trans-Golgi/TGN are distinct subcompartments that can be separated one from the other by drug treatment; any exchange of components between the Golgi subcompartments must be slow with respect to 1-2 h kinetics of Golgi disassembly.

A key aspect to these experiments is the use of multiple markers to the Golgi complex in the same cell. Obviously, without this, the differential response of Golgi subcompartments to microtubule depolymerization would never be detected. Our results strongly indicate that medial- and trans-Golgi/TGN are not in rapid equilibrium with respect to the overlapping distributions of GlcNAcT-1 and GalT between medial and trans-Golgi reported previously by Nilsson et al (1993). The distribution of individual components

between medial- and trans-Golgi must be relatively stable. Similarly the quite differential distribution of cisternal proteins and the CGN protein in response to nocodazole was reliant on multiple markers. More subtlely, though, the fact that Golgi processes may be extended independently in response to BFA that are positive for different trans-Golgi/TGN proteins could not be detected otherwise. It is only because the distribution of two different trans-Golgi/TGN proteins were characterized that this observation could be made. On the basis of microscopy, SialylT and GalT are thought to have similar distributions within trans-Golgi/TGN (Roth, 1985; Nilsson et al., 1993). Recent diffusible acceptor assays indicate that at least some galactosyltransferases and sialyltransferases are in the same Golgi subcompartment (Etchison, Srikrishna and Freeze, 1995). In the present study, the two behave identically with respect to their kinetics of redistribution in response to microtubule depolymerization, dissimilar to the kinetics of redistribution of proteins in other Golgi subcompartments. Yet based on the processes extended in response to BFA, at least a significant portion of the two must be present in different subregions of the trans-Golgi/TGN. There must be discontinuities to the distribution of the two. Clearly, an inherent limitation to all Golgi organization studies is the lack of well characterized, multiple protein markers across the entire Golgi complex. Here we have taken a step both to overcome this problem and to show the benefits of doing such. For our studies and those of others to have the greatest validity, the markers must be well characterized both molecularly and microscopicly with respect to their subGolgi distribution.

The pattern of Golgi fragmentation upon nocodazole addition is consistent with a three compartment model for Golgi organization. In these studies, the two trans-Golgi/TGN markers have identical, rapid kinetics for redistribution into scattered punctate structures. The medial marker scatters in its distribution 2-3 fold more slowly. The distribution of the CGN marker also scatters rapidly. Neither SialylT or GalT consistently lags behind GlcNAcT-1 in its distribution response to BFA addition and removal; with

BFA addition, GalT dispersal to the ER is somewhat slow while with BFA removal, SialylT redistribution from ER to Golgi is somewhat slow. If indeed SialylT and GalT are good markers for TGN, our results strongly indicate that the TGN and trans-Golgi behave in an overall sense as a unit with some subregionalization. The medial-Golgi would be a second unit and the cis-Golgi/CGN in analogy to the trans-Golgi/TGN would be the third unit. Mellman and Simons (1992) have previously proposed such a model for Golgi organization. The validity of our suggestions should be considered tentative until more abundant markers become available. Our studies suggest that the glue(s) that hold the Golgi together in a perinuclear, MTOC centered pattern are most sensitive to disaggregation at either edge of the organelle. In this regard, it should be noted that the inter-cisternal matrix complex described by Slusarewicz et al. (1994) is a medial not trans or cis complex. What the nature of the forces holding the trans-Golgi/TGN and cis-Golgi/CGN together with medial-Golgi can only be speculated at this moment.

Our data suggest that the fragmentation of the Golgi is not the result of lateral unfolding of intercisternae connecting regions without disrupting Golgi continuity as proposed by Rogalski and Singer (1984). Our results do suggest these tubular interconnections on which intact Golgi organization based may be severed and dispersed after microtubule depolymerization in agreement with Tassin et al (Tassin et al., 1985). Golgi fragmentation in response to nocodazole addition is not likely to be a direct consequence of budding processes. The punctate structures observed soon after drug addition are too large to be small vesicles. Moreover, these structures seem to be targeting sites for the gradual accumulation of medial-Golgi. This suggests that the Golgi stacklets observed in electron micrographs of nocodazole treated cells form slowly and progressively with the addition of medial components to trans-Golgi/TGN. Moreover these results imply the existence of a Golgi cisterna recognition mechanism(s) of unknown nature. Golgi reassembly following nocodazole removal is, in contrast, by a different mechanism in

which reassembly follows upon movement of punctate Golgi fragments positive for all three cisternal markers to the circumnuclear region. Previous work has suggested that proximal Golgi elements in cells (heterokaryons) may fuse by interconnecting bridge formation (Ho et al., 1990; Xiao and Storrie, 1991).

In contrast to the work of Alcalde et al. (1992) with BFA treated NRK cells, we find little evidence for the coordinate disassembly or reassembly of cisternal compartments. Using GIMPc and GIMPt, Alcalde et al. (1992) found that disassembly was cis first and that reassembly following drug removal was also cis first. Here we found little consistent evidence for sequential disassembly or reassembly. Our two trans-Golgi/TGN markers did not show consistent kinetics one way or the other when compared with the medial marker. Presumably the difference in results between our work and that Alcalde et al. (1992) relates either to the exact markers used or to the differences in cell type studied. Our results do suggest that the onset of Golgi dispersal to ER can occur at the same time from multiple tubular process which come from different portions of the Golgi and from different subregions within a portion. This conclusion is based primarily on the staining characteristics of processes in double immunofluorescence labeling; early processes were positive for one or both markers in that Golgi subcompartment and could be from the same or different subcompartments of the Golgi.

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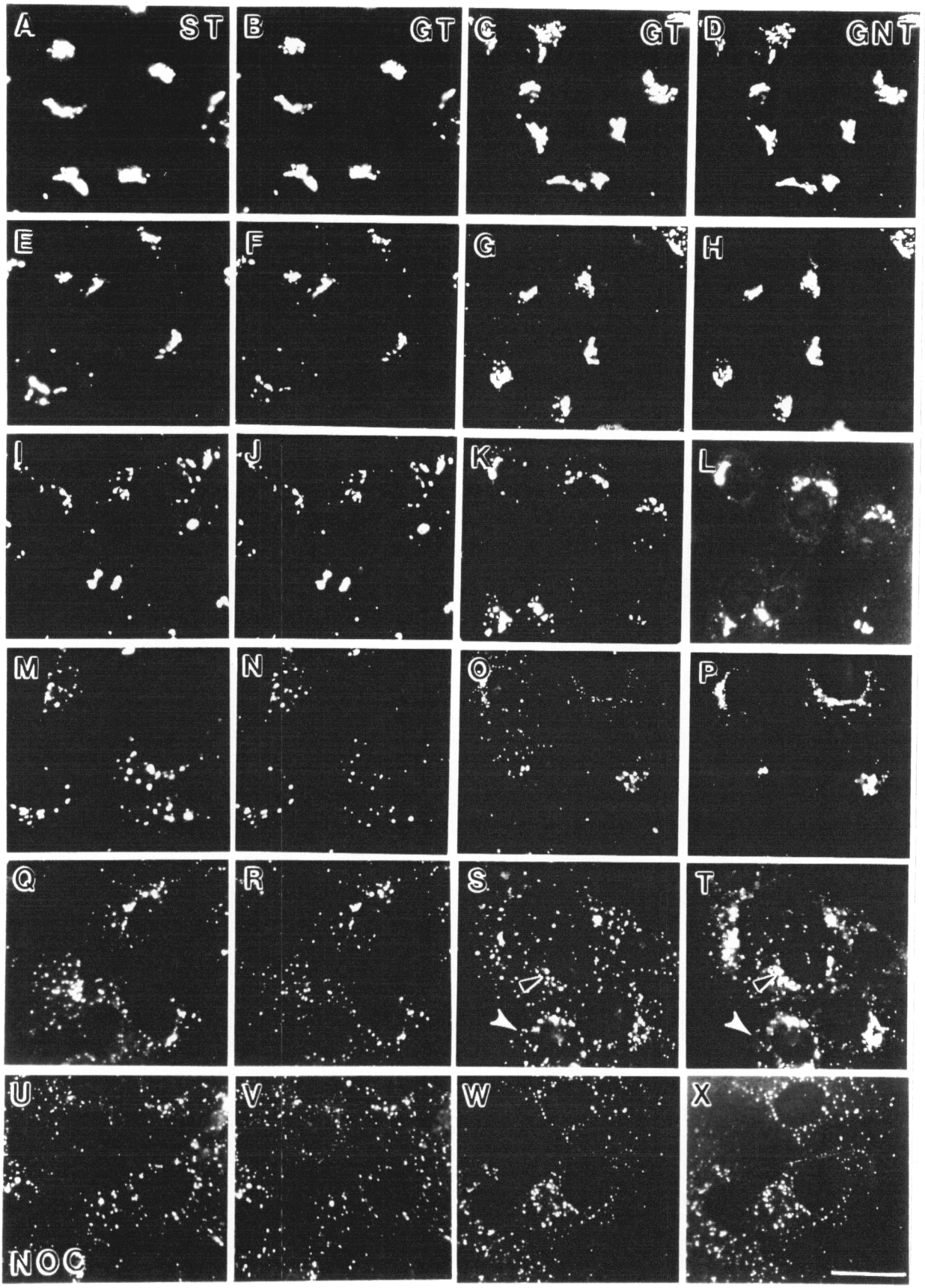
Fig. 2-1. Effect of nocodazole treatment on the distribution of SialylT, GalT, and GlcNAc1. SialylT-myc or GlcNAcT-1-myc Vero cells were treated with 10 μM nocodazole for 0 min, control (A-D), 15 min (E-H), 30 min (I-L), 60 min (M-P), 120 min (Q-T), and 240 min (U-X). Cells were fixed with methanol. SialylT-myc Vero cells were stained for double immunofluorescence microscopy using monoclonal anti-SialylT-myc antibody 9E10 (Texas red channel; A, E, I, M, Q, U) and a rabbit polyclonal anti-GalT antibody (FITC channel; B, F, J, N, R, V). For GlcNAcT-1-myc Vero cells, the distribution of GalT and GlcNAcT-1 was monitored by anti-GalT antibody (FITC channel; C, G, K, O, S, W) and 9E10 antibody (Texas red channel; D, H, L, P, T, X). The distribution of GalT in the two cell lines was comparable for each time point. Note that the redistribution rate for SialylT and GalT was nearly identical, while the redistribution of GlcNAcT-1 was significantly slower than hat of GalT. Black arrowheads in S and T point to differences in the perinuclear, compact Golgi-like staining of GalT and GlcNAcT-1. White arrowheads in S and T point to punctate structures in the cell periphery that are strongly stained for GalT but not for GlcNAcT-1. Bar=20 μm.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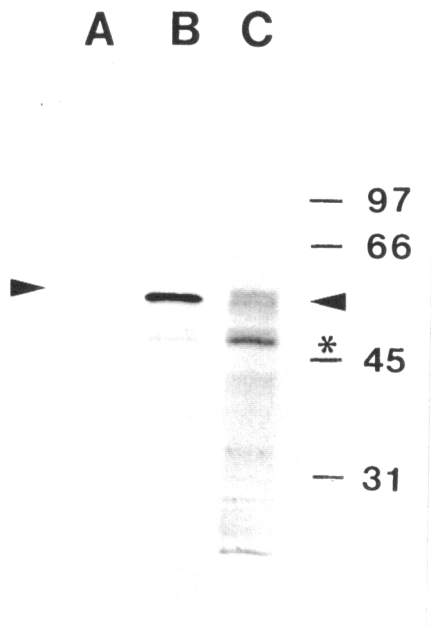


Fig. 2-2. Immunoprecipitation characterization of GlcNAcT-1-myc and SialylT-myc from stable Vero cells. Wild type, GlcNAcT-1-myc and SialylT-myc Vero cells cultured in 60 mm dishes were labeled by 20 min incubation with [^{35}S]-methionine and chased in presence of cold methionine for 2 h. Cells were lysed and supernatants immunoprecipitated with mouse 9E10 monoclonal antibody (anti-myc epitope). The immunoprecipitated material was analyzed by 10% SDS gel. One-third of each sample was applied to the gel. The gel was exposed to film for 16 h. Lanes A, B, and C show immunoprecipitated proteins from wild type, GlcNAcT-1-myc , and SialylT-myc Vero cells, respectively. Arrowheads indicate the electrophoretic species corresponding to expected GlcNAcT-1-myc or SialylT-myc. Star indicates a likely major immunoprecpitate contaminant. Bars denote positions of four different molecular weight marker (kD).

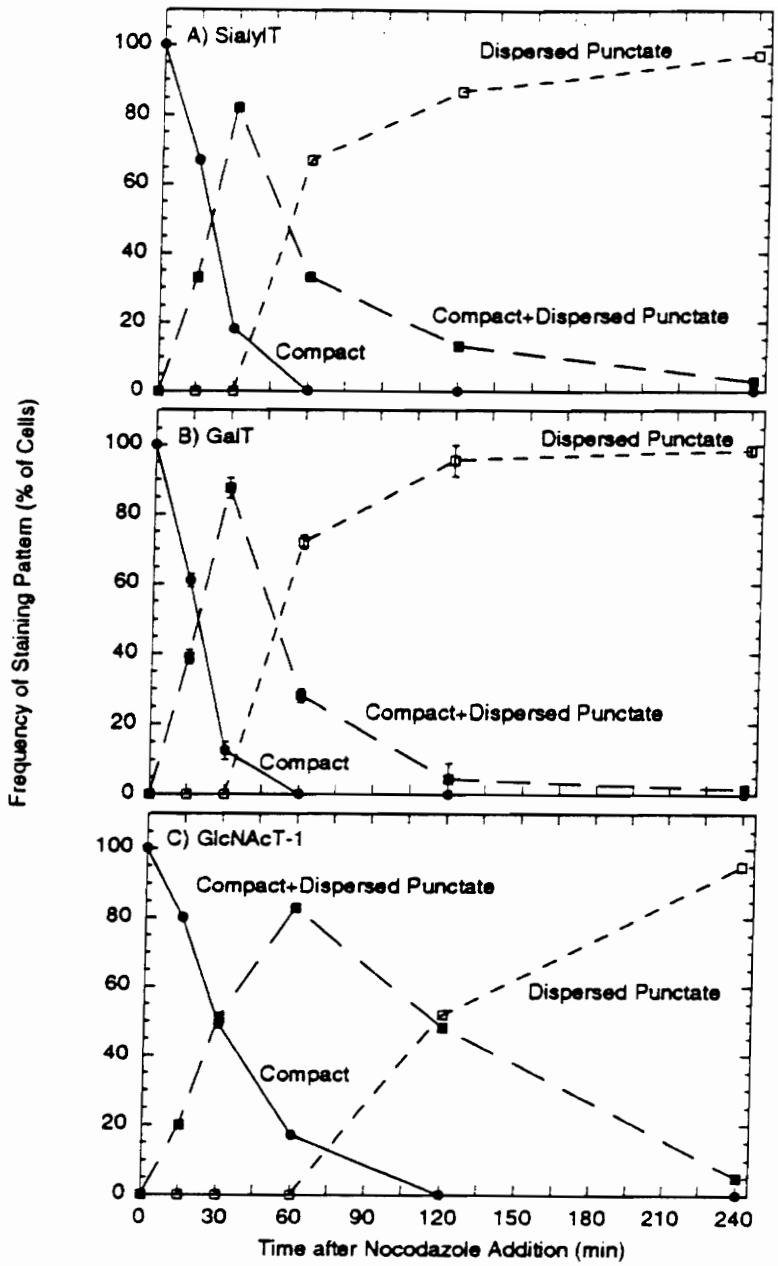


Fig. 2-3. Comparative kinetics of redistribution of SialylT (A), GalT (B), and GlcNAcT-1 (C) after nocodazole addition. Micrographs from the experiments described in Fig. 2-1 were scored for overall marker protein distribution pattern at each time point. The overall distribution patterns are divided into three categories: 1) most of fluorescence is centrally located (Compact), 2) most of fluorescence is in punctate structues (Dispersed Punctate), 3) the fluorescence pattern is a combination of the previous two (Compact+Dispersed Punctate). For every time point, the SialylT, GalT, and GlcNAcT-1 distributions were scored and the percent localization pattern calculated. For GalT the data were averaged from two separate experiments. The error bars indicate the data range. About 30 cells were scored for each transferase.

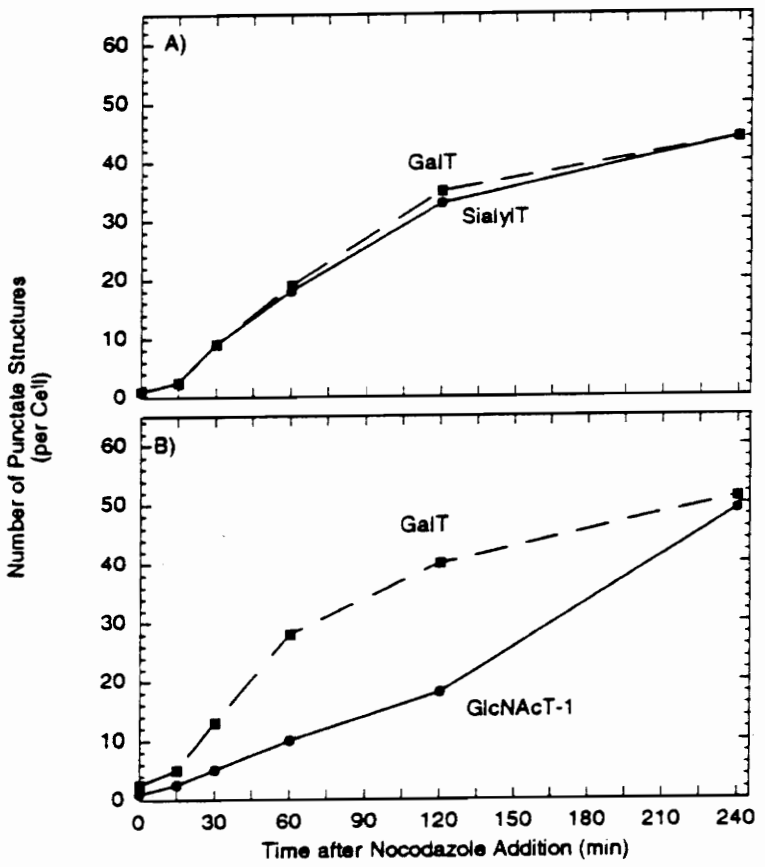


Fig. 2-4. The number of punctated structures per cell after nocodazole treatment. The punctate structures positive for SialylT, GalT, and GlcNAcT-1 were scored and the average number per cell calculated. (A), Punctate structure number for SialylT and GalT. (B), Punctate structure number for GalT and GlcNAcT-1

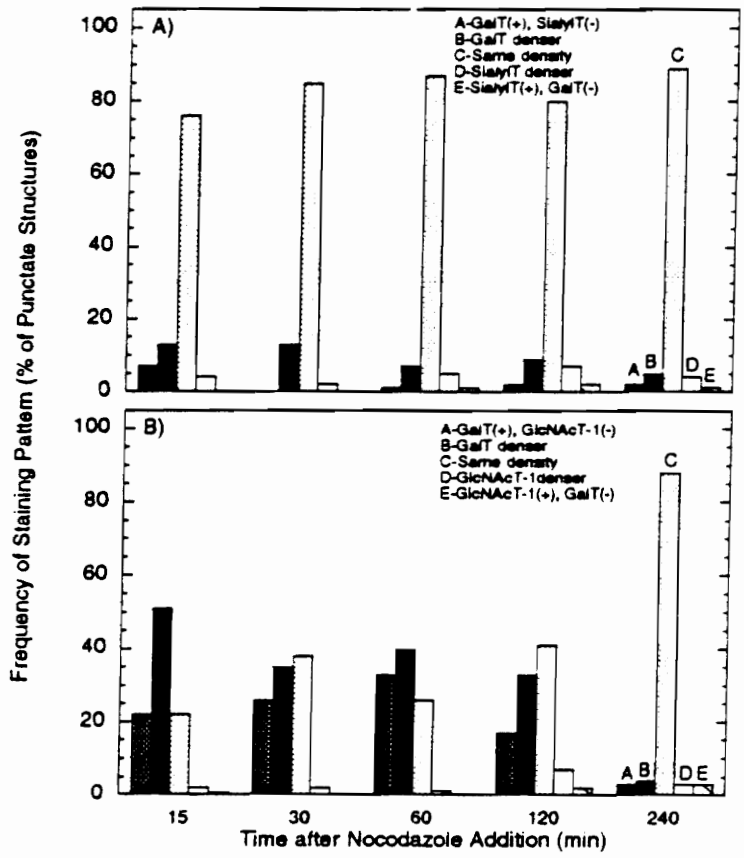
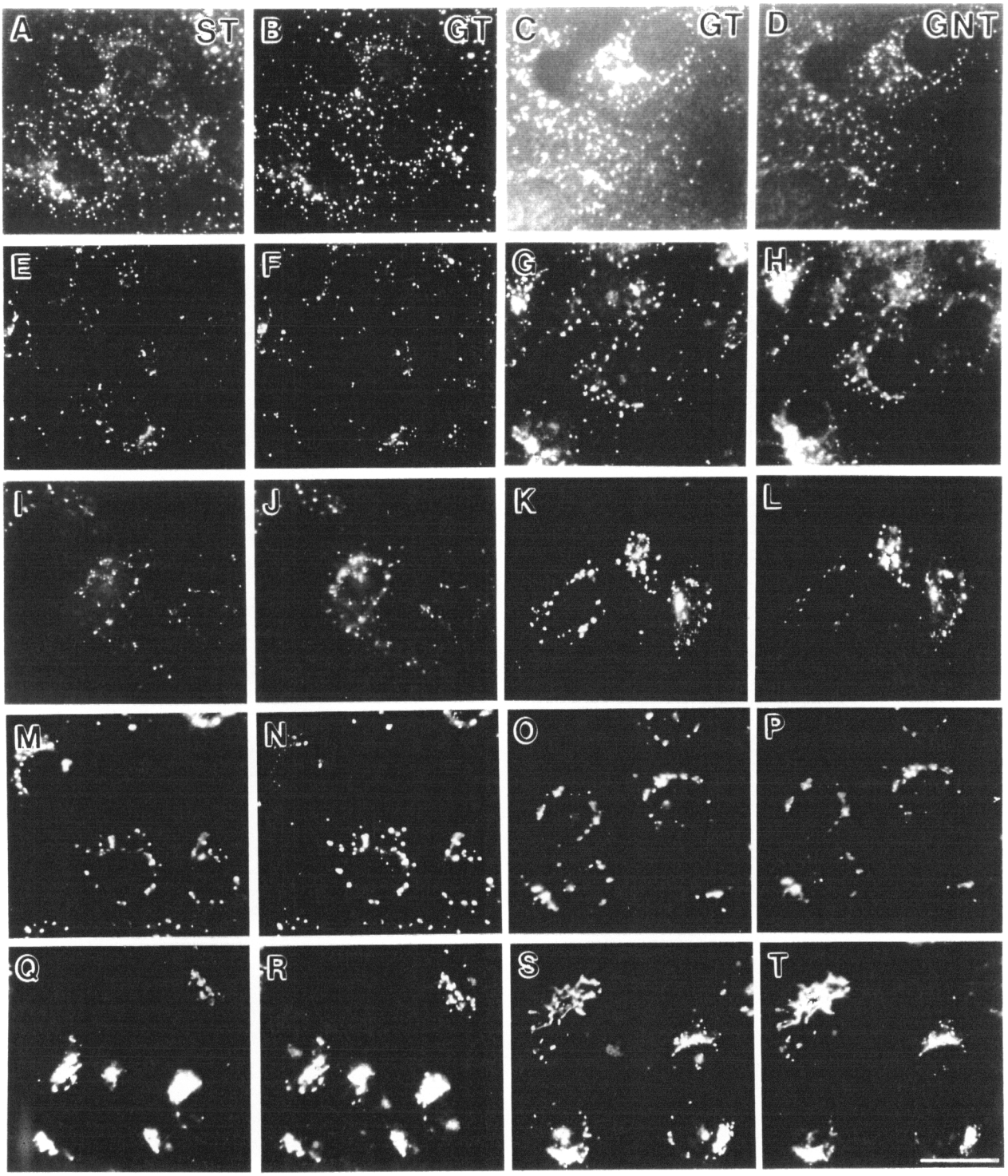


Fig. 2-5. Frequency of colocalization of GalT and SialylT and GalT and GlcNAcT-1 in nocodazole induced punctate structures. (A), for nocodazole treated SialylT-myc Vero cells, the punctate structures were scored for co-localization. The scoring was divided into five categories: A, GalT positive and SialylT negative; B, GalT staining more intense than SialylT; C, GalT and SialylT staining same intensity; D, SialylT staining more intense than GalT; E, SialylT positive and GalT negative. (B), for nocodazole treated GlcNAcT-1-myc cells, the punctate structures were scored for co-localization as described in (A).

Fig. 2-6. Reassembly of Golgi complex after removal of nocodazole. SialylT-myc or GlcNAc-1-myc Vero cells were treated with 10 μM nocodazole for 240 min to completely fragment the Golgi complex. After brief washes, the cells were incubated in drug-free medium for 0 min, control (A-D), 15 min (E-H), 30 min (I-L), 60 min (M-P), and 120 min (Q-T). Cells were processed for double immunofluorescence as described in Fig 2-1. Note that the reassembly process for all three protein markers were similar. Bar=20 μm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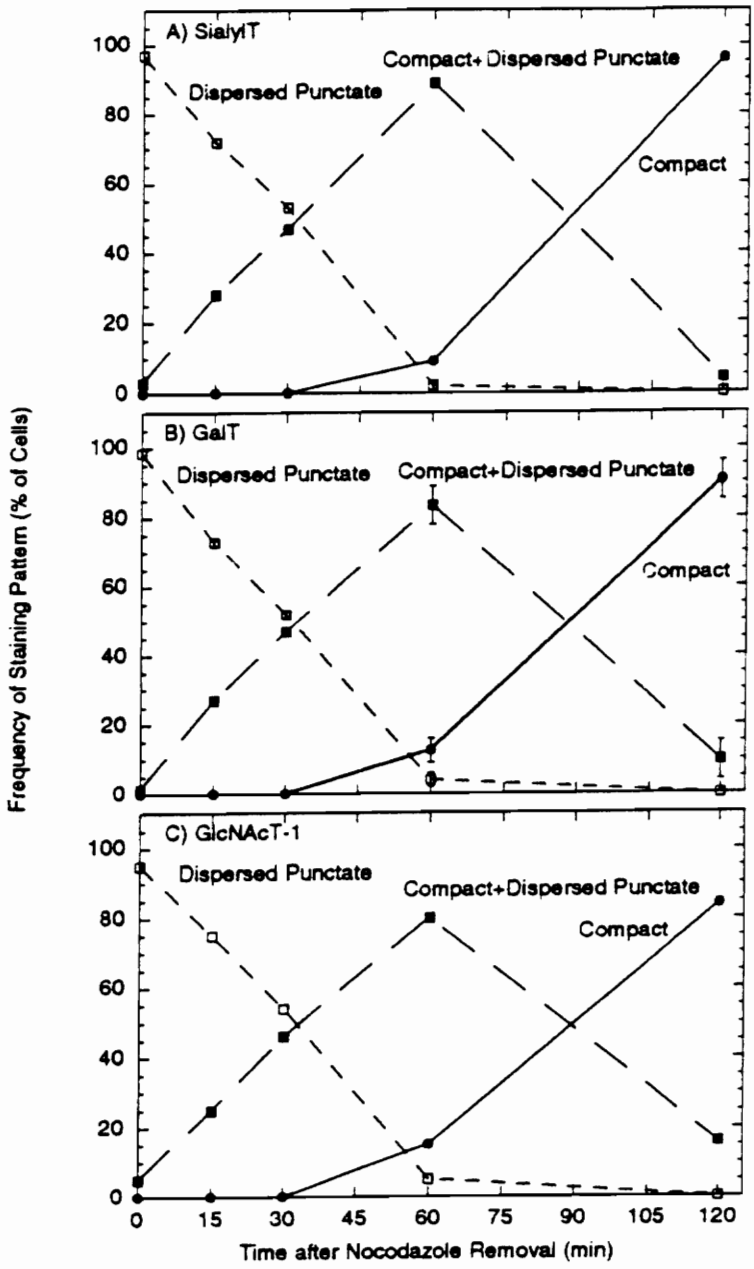


Fig. 2-7. Comparative kinetics of redistribution of SialylT (A), GalT (B), and GlcNAcT-1 (C) after nocodazole removal. Micrographs in the experiments described in Fig. 2-6 were scored for overall marker protein distribution patterns at each time point. The scoring method is described in the legend of Fig. 2-3.

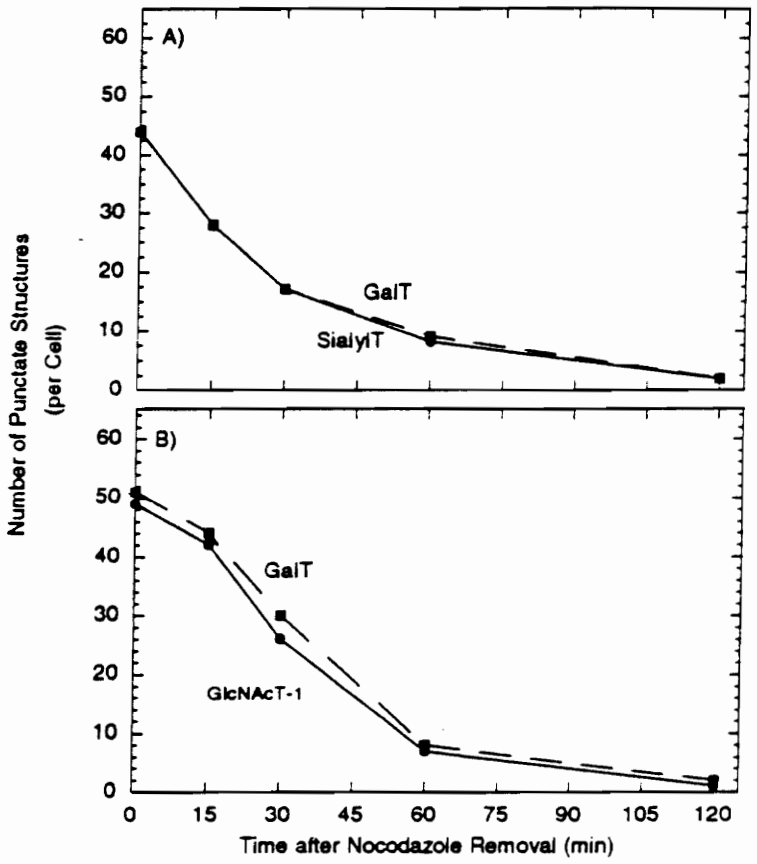


Fig. 2-8. The number of punctate structures per cell after nocodazole removal. (A), Punctate structure number for SialylT and GalT. (B), Punctate structure number for GalT and GlcNAcT-1

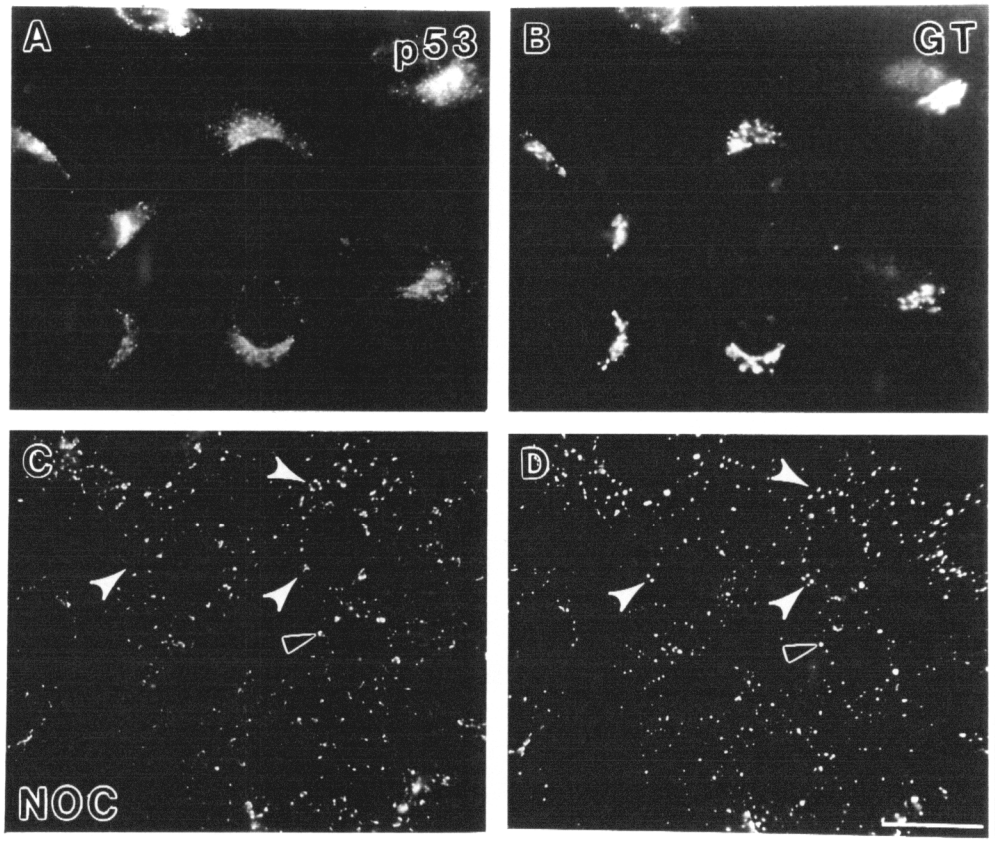
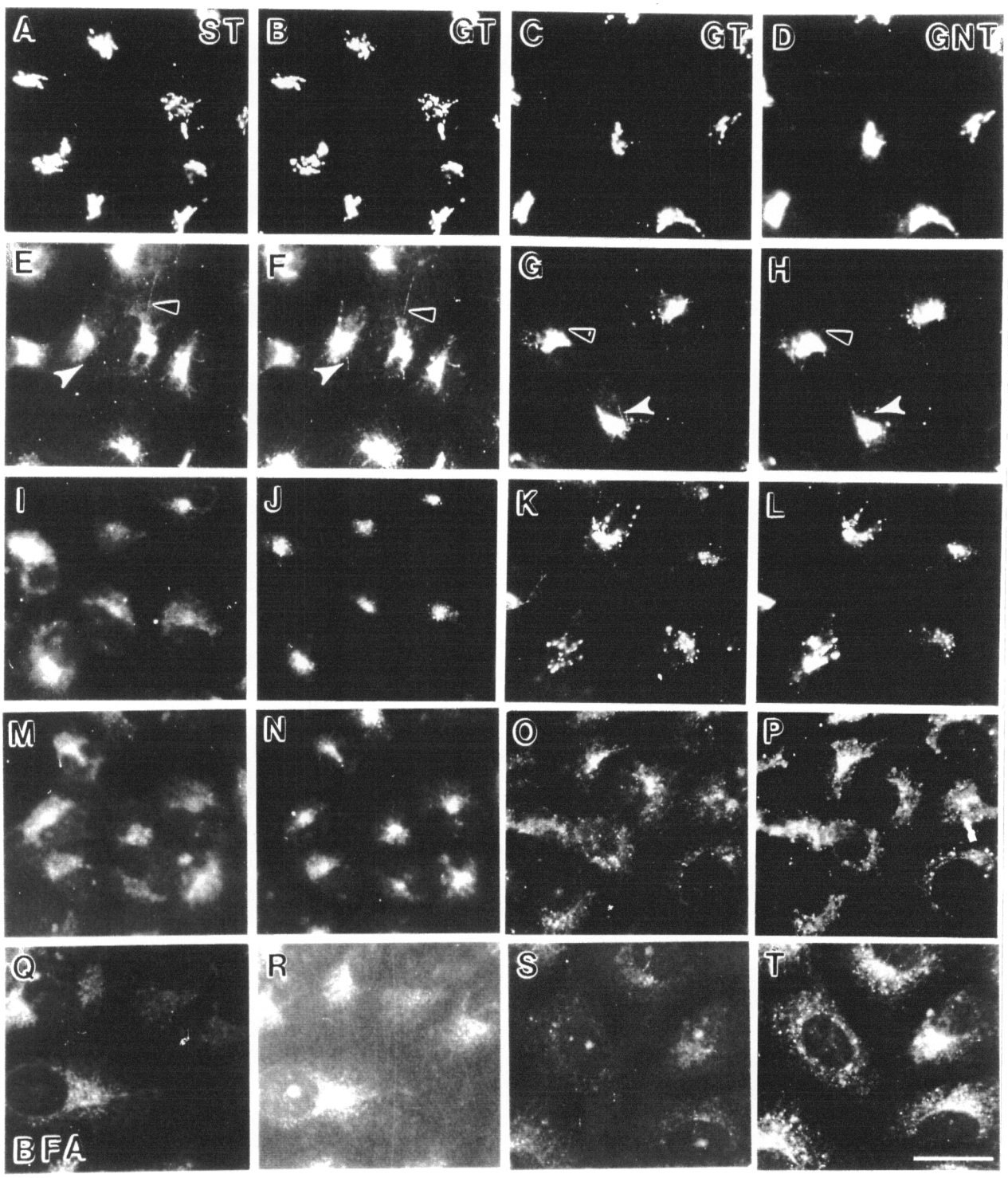


Fig. 2-9. Effect of nocodazole on the localization on p53 and GalT. Cells with treated for zero time, control (A and B), or 4h (C and D) were processed for indirect immunofluorescence using monoclonal anti p53 antibody (G1/93) (A and C) and polyclonal anti-GalT antibody (B and D). White arrowheads indicate examples of punctate structures which were not colocalized. Black arrowheads indicate examples of punctate structures which were colocalized. Bar=20 μm.

Fig. 2-10. Effect of BFA treatment on the distribution of SialylT, GalT, and GlcNAc-1. SialylT-myc or GlcNAcT-1-myc Vero cells were treated with 5 μg/ml BFA for 0 min, control (A-D), 5 min (E-H), 20 min (I-L), 60 min (M-P), and 180 min (Q-T). Cells were fixed and stained as described in Fig. 2-1. Black arrowheads in E-H point to extended tubular processes which were positive for both GalT and SialylT or GalT and GlcNAcT-1. White arrowheads in E-H point to extended tubular processes which were negative for SialylT but positive for GalT or negative for GlcNAcT-1 but positive for GalT. Bar=20 μm.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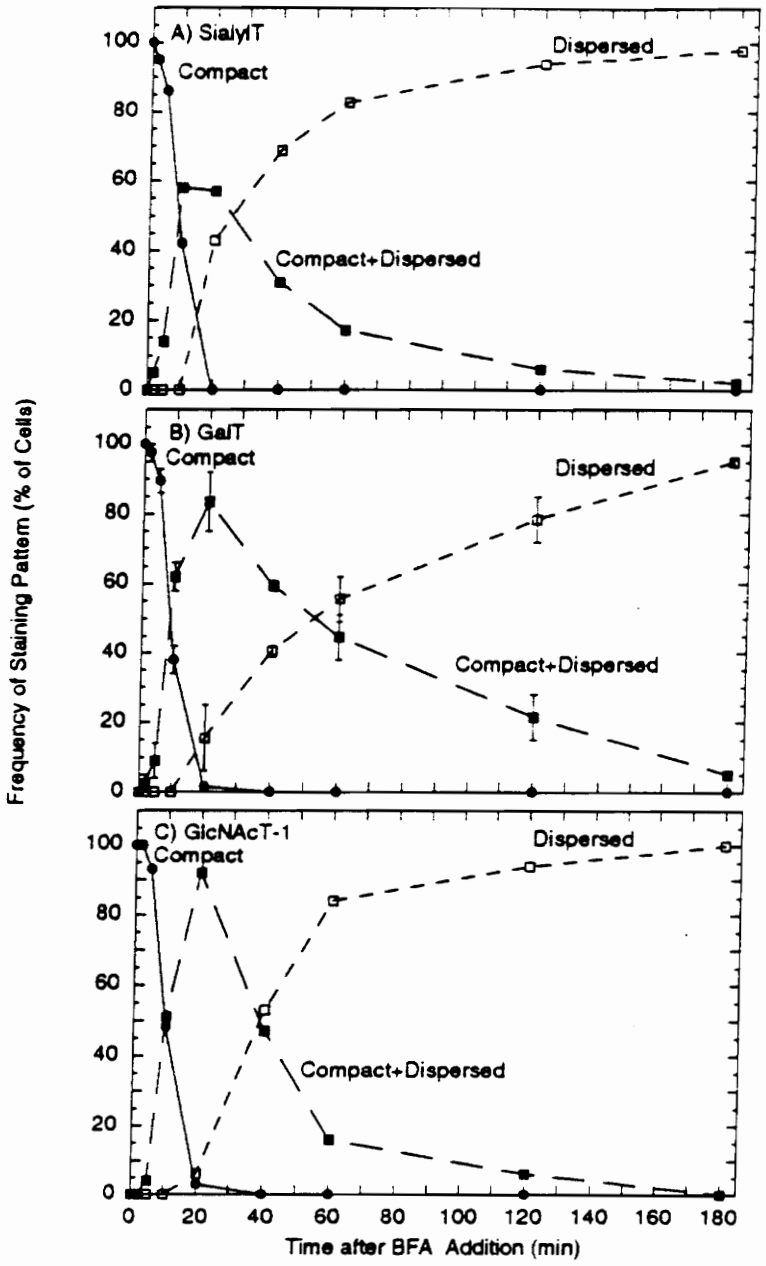
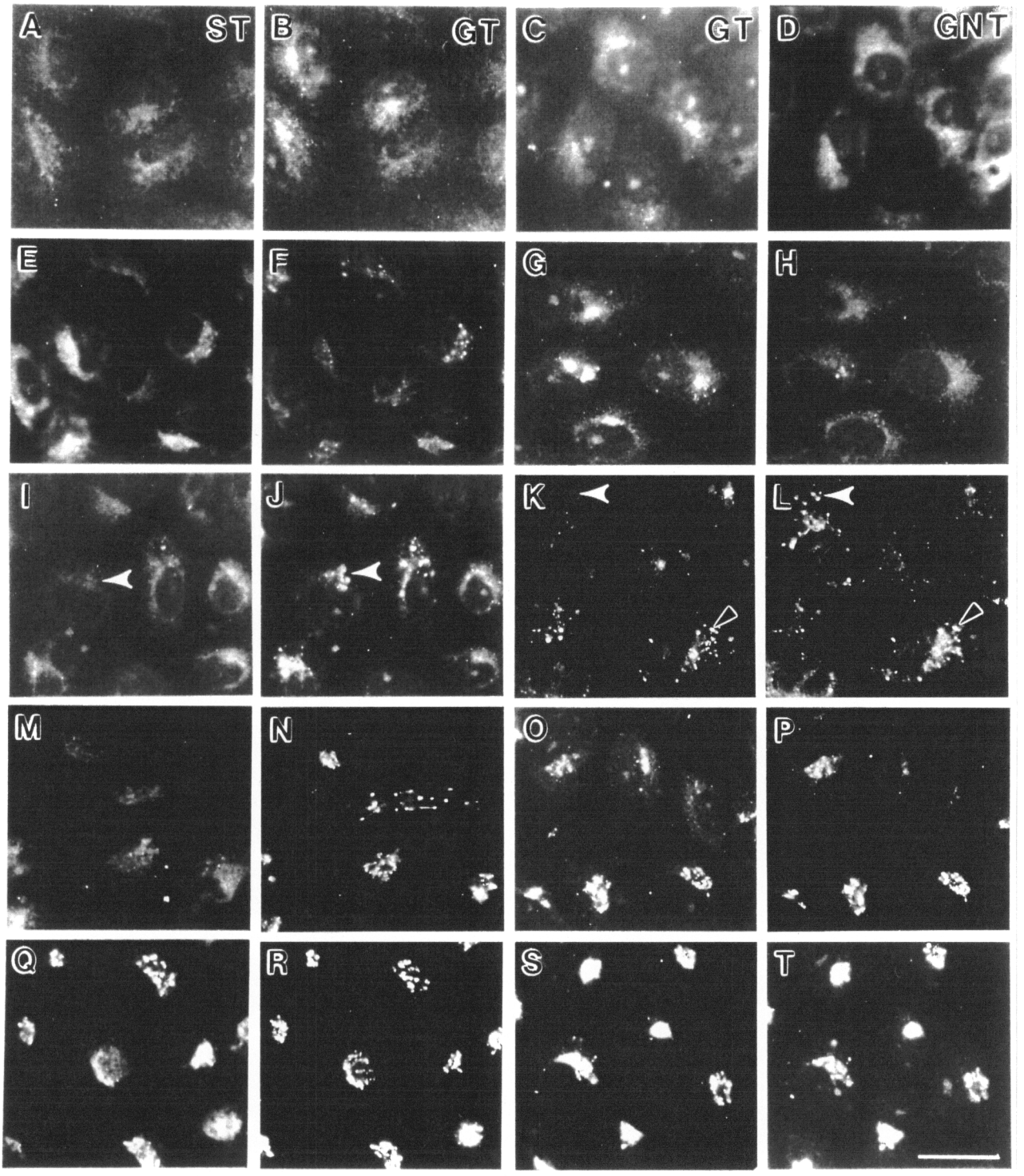


Fig. 2-11. Comparative kinetics of redistribution of SialylT (A), GalT (B), and GlcNAcT-1 (C) after BFA addition.. Micrographs in the experiments described in Fig. 2-10 were scored for overall marker protein distribution patterns at each time point. The overall distribution patterns are divided into: 1) most of fluorescence is centrally located Golgi-like staining (Compact), 2) most of fluorescence is dispersed ER-like staining (Dispersed), or 3) the fluorescence pattern is a combination of the previous two (Compact+Dispersed). For GalT, the data were averaged from two separate experiment and range bars were included in the figure.. About 30 cells were scored for each transferase distribution.

Fig. 2-12. Reassembly of Golgi complex after removal of BFA. SialylT-myc or GlcNAc-1-myc Vero cells were treated with 5 μg/ml BFA for 180 min . After brief washes, the cells were incubated in drug-free medium for 0 min, control (A-D), 10 min (E-H), 40 min (I-L), 60 min (M-P), and 120 min (Q-T). Cells were fixed and processed for double immunofluorescence as described in Fig 2-1. Black arrowheads in K and L point to compact, perinuclear vesicular structures which were positive for both GalT and GlcNAcT-1. White arrowheads in I and J point to compact, perinuclear vesicular structures which were negative for SialylT but positive for GalT. White arrowheads in K and L point to compact, perinuclear vesicular structures which were negative for GalT but positive for for GlcNAcT-1. Bar=20 μm.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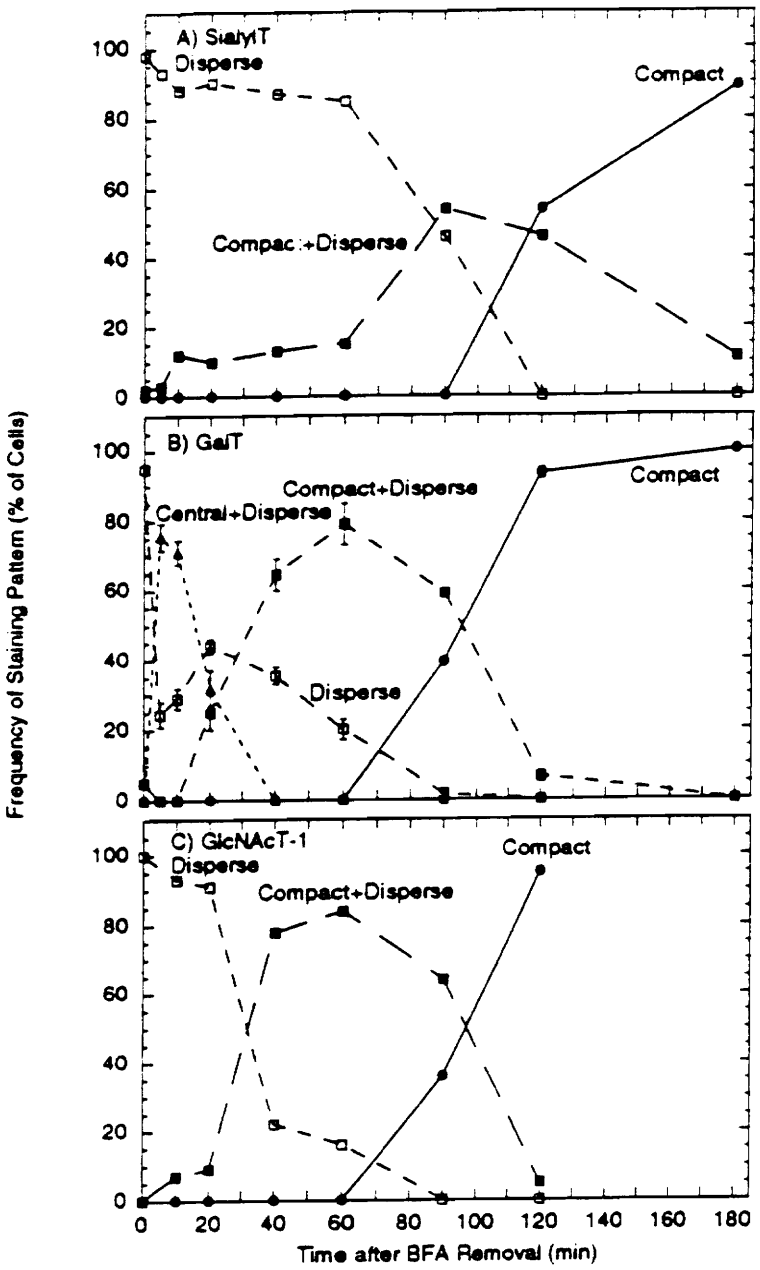

Fig. 2-13. Comparative kinetics of redistribution of SialylT (A), GalT (B), and GlcNAcT-1 (C) after BFA removal. Micrographs from the experiments described in Fig. 2-12 were scored for overall marker protein distribution patterns at each time point. The overall distribution patterns are divided into: 1) most of fluorescence is centrally located Golgi-like staining (Compact), 2) most of fluorescence is dispersed ER-like staining (Dispersed), or 3) the fluorescence pattern is a combination of previous two (Compact+Dispersed). In early time points, GalT tended to be central localized (Central+Disperse), which was distinguished from compact Golgi staining. For GalT the data were averaged from two

Chapter 3. Alterations in the Cytoplasmic Domain of Golgi Type II Membrane Proteins, by an Epitope Tagging Approach, Often Result in Their Accumulation in ER

3.1. Abstract

N-acetylglucosaminetransferase-1 (GlcNAcT-1, medial-Golgi), β-1,4-galactosyltransferase (GalT, trans-Golgi/TGN) and α-2,6-sialyltransferase (SialylT, trans-Golgi/TGN) are resident type II membrane proteins of Golgi complex. The wild-type forms of these proteins all have a short cytoplasmic domain of 6 to 24 total amino acids with that of GlcNAcT-1 being the shortest. Presumably these short tails, particularly that of GlcNAcT-1, are evolved to fit within the intercisternal distances of the Golgi. To delineate the importance of the length of these cytoplasmic tail domains in Golgi targeting and retention, we constructed a series of chimeric cDNA so as to generate hybrid proteins in which different epitope tags and spacer sequences served to lengthen the cytoplasmic tail. These sequences also served as reporter groups for assessing the accessibility of the tail sequences when the chimeric proteins were expressed in cells. No known targeting features are present in the added sequences. By immunofluorescence, we found that the cytoplasmic tail could be lengthened considerably (3-4 fold) and SialylT and GalT still accumulated in a perinuclear, Golgi-like distribution with little ER background. One construct, VSV-SialylT, localized essentially exclusively to the Golgi complex. For SialylT, the longest constructs (40-42 amino acids) located relatively well to the Golgi complex while for GalT, the longest constructs (32-34 amino acids) located exclusively to the ER. Surprisingly, the epitope tags of several different GalT constructs was inaccessible to antibody in fixed cells. Any lengthening of the cytoplasmic domain of GlcNAcT-1 resulted in considerable to exclusive accumulation of the chimeric proteins in ER. No cell surface accumulation of any of the chimeric proteins was detected. The specific sequence of the epitope tag was

important; the positively charged VSV epitope tag was preferred over negatively charged myc or FLAG tags. Depending on the exact tail alteration, we found that all three transferases accumulated in the ER with no detectable Golgi or cell surface accumulation. In some cases accumulation in the intermediate compartment or CGN was observed. Most surprisingly, in the one homologous case studied, expression of chimeric human GlcNAcT-1 in HeLa cells, ER accumulation of GlcNAcT-1 led to disruption of pre-existing Golgi. Based on these results, we propose, as the simplest explanation of the data, that alteration of the cytoplasmic tail of Golgi resident proteins can decrease their rate of exit from ER, presumably due to premature oligomerization, and may lead to capture of Golgi proteins in the ER.

3.2. Introduction

In eukaryotic cell, proteins destined for the secretory compartments or for secretion are translocated to the endoplasmic reticulum co-translationally and then processed through a series of membrane bounded compartments on the way to their final destination. It is believed that the transport of many proteins from ER to cell surface is by a default pathway (Pfeffer and Rothman, 1987). However, a subset of proteins contains specific targeting signals which are used by the cell to direct these proteins to specific subcompartments. In general, the motifs responsible for the targeting or retention of the proteins in unique subcompartment along the secretory pathway can be divided into three different categories based on their function in targeting: selective retrieval, selective retention and selective forward transport. Selective retrieval is best described for some ER resident proteins. Pelham and his colleagues (for review, see Pelham, 1991) has identified a KDEL/HDEL peptide motif at the carboxyl-terminus of some soluble ER proteins, which is required for their ER localization via receptor mediated retrieval of mislocalized proteins from Golgi back to ER (Lewis et al, 1990a; Lewis and Pelham, 1990b). Other investigator find a retrieval mechanism for some ER membrane proteins. This is by specifically placed lysine or arginine residues in the cytoplasmic tail of these membrane proteins (Schutze et al., 1994). Good examples of selective forward targeting are soluble lysosomal enzymes. These are targeted to lysosomes by receptors recognizing the mannose 6-phosphate found on the N-linked oligosaccharides of these proteins (Kornfeld and Mellman, 1989; Storrie, 1987). A striking example of selective retention occurs in the Golgi complex, where resident type II Golgi membrane proteins appear to be retained by a non-saturable mechanism (for review, see Gleeson et al., 1994; Nilsson et al., 1993a). The Golgi complex is a highly organized organelle composed of a series of subcompartments. Mellman and Simons have proposed that these are three: the cis Golgi network (CGN)/cis-

Golgi, medial-Golgi and the trans-Golgi/trans Golgi network (TGN). The Golgi is a major glycosylation compartment and contains most of enzymes involved in this process. Several glycosyltransferases have been localized to distinct cisternae by combinations of immunolocalization and biochemical methods (for review of older literature see, Kornfeld and Kornfeld, 1985; Kornfeld and Mellman, 1989). N-acetylglucosaminetransferase I (GlcNAcT-1), β-1,4-galactosyltransferase (GalT), and α-2,6-sialytransferase (SialylT) have been localized to medial-Golgi and trans-cisternae /TGN, respectively (Dunphy et al., 1985; Roth et al., 1982; Roth et al., 1985; Nilsson et al., 1993). In double immunolabeling of thawed cyrosections, GlcNAcT-1 and GalT overlapped in distribution with about one third of GlcNAcT-1 labeling being in trans-Golgi cisternae and two thirds in medial-Golgi cisternae while for GalT half was in trans-Golgi cisternae and half was in TGN (Nilsson et al., 1993).

Over the last several years, genes coding for Golgi glycosyltransferases and glycosidase have been cloned and sequenced as well those for some viral proteins that reside in the Golgi during the infection process. All of the endogenous Golgi glycosyltransferases and glycosidases have the same type II membrane topology with a short N-terminal cytoplasmic domain, typically 6 to 12 amino acids, a transmembrane domain of about 17 amino acids, a relatively short stem region and a large lumenal catalytic domain. No obvious homology in their primary sequences has been found which might imply a common Golgi targeting or retention signal (for reviews, see Paulson and Colley, 1989; Gleeson et al., 1994). By domain swapping and site directed mutagensis, several groups have narrowed the Golgi localization signal of type II membrane proteins to within and surrounding the transmembrane domain. For GlcNAcT-1, the transmembrane domain is a major determinant of Golgi residency (Tang et al., 1992). Flanking sequences, often positively charged, also contribute to Golgi residency and are important in preventing the leakage of GlcNAcT-1 to the cell surface (Burke et al., 1992; Burke et al., 1994).

Similarly for GalT which exists as two forms in many cells that differ only in the length of their cytoplasmic tail, a short form of 11 amino acids and a long form of 24 amino acids (Russo et al., 1989), the transmembrane domain to Golgi retention (Russo et al., 1992). Nilsson et al. (1992) find that as few as 11 residues from the lumenal side of the transmembrane domain of short GalT are sufficient to retain a cell surface protein in Golgi complex in transfected cells. For SialylT, Wong et al. (1992) showed that the 17-residue transmembrane domain could confer a Golgi localization when transferred to the corresponding region of the cell surface membrane protein dipeptidyl peptidase IV (Wong et al., 1992). However, the actual sequence of the transmembrane domain appears not to be important. As shown first by Munro (1991) a sequence of a "nonsense" hydrophobic sequence (17 leucines) will substitute for the wild type transmembrane domain and still give normal Golgi localization. Work principally by Colley and colleagues (Colley, Lee and Paulson, 1992; Dahdal and Colley, 1993) indicates that it is the correct spacing by the transmembrane domain of short, proximal flanking sequences that is what is important in Golgi retention of SialylT. The cytoplasmic tails of all three of these most studied glycosyltransferases are short, 6-11 amino acids, have positively charged residues near the membrane and a net positive charge at pH 7. Viral membrane proteins and chimeric constructs containing elements from these viral proteins can also be Golgi resident proteins. Some viruses bud into the Golgi complex during their infectious cycle. Three examples that might be given include the M protein of infectious bronchitis virus, E2 protein of rubella, and bunyavirus G1 protein. The M protein crosses the membrane three times and its first transmembrane domain appears to be the important sequence in determining residence (Machamer and Rose, 1987). The E2 protein is a type I membrane protein in which the transmembrane segment is important in residence (Hobman, Woodward and Farquhar, 1995). Bunyavirus G1 protein is also a type I membrane protein; here, the transmembrane domain together with a portion of the cytoplasmic domain is crucial for Golgi residence

(Matsuoka, Chen and Compans, 1994) . All these localization signals appear to act as retention rather than retrieval signals for the Golgi proteins (for reviews, see Machamer, 1993; Gleeson et al., 1994). Overexpression of Golgi proteins results in ER accumulation rather than cell surface expression (for review, see Nilsson and Warren, 1994). This can not be readily explained by a saturable receptor-mediated mechanism. Receptor saturation would be expected to lead to leakage of the protein to the cell surface rather than to accumulation in the ER, the compartment of biosynthesis of the protein.

Three different models have been proposed to account for the importance of the transmembrane domain and other sequences in Golgi retention of proteins and the apparent non-saturability of Golgi protein retention within the cell. 1) Formation of large insoluble aggregates or lattices: according to this model, resident Golgi proteins form large insoluble complexes via interactions of their transmembrane domains that are unable to enter transport vesicles (Machamer, 1991; Machamer 1993). Consistent with this proposal, the Golgi retained chimeric protein Gm1 (VSV-G protein in which transmembrane domain is replaced with the first transmembrane domain from infectious bronchitis virus M protein) forms large, sodium dodecyl sulfate (SDS) resistant oligomers when expressed in COS cells (Swift and Machamer, 1991; Weisz et al, 1993). However, this behavior has not been observed for the IBV M protein. 2) Kin recognition: this model proposes that Golgi enzymes are homodimers (stabilized by lumenal domain interactions) which are retained by interactions between the transmembrane domains of adjacent "kin" homodimers in the same cisternae, thus forming long heterooligomers (Nilsson et al., 1993, 1994). Retention is believed to be enhanced by interaction of cytoplasmic domains of Golgi proteins with an intercisternal matrix (Slusarewicz et al., 1994). Consistent with this model, enzyme inactivation target size experiments suggest that Golgi membrane proteins are indeed dimers (Fleischer et al, 1993) 3) Bilayer-mediated sorting: this model is based on the observation that the transmembrane domains of endogenous Golgi enzymes are shorter (17 residues on

average vesus 21 for plasma membrane proteins). It holds that Golgi membrane proteins are inherently less stable in thicker, cholesterol and sphingolipid-rich membranes (e.g., plasma membrane) and as a result, partition to the thinner cholesterol/sphingolipid-poor bilayers of the Golgi (Bretscher and Munro, 1993). All these models predict overexpressed Golgi proteins will accumulated in early compartments of the secretory pathway rather than the cell surface. For aggregation and kin recognition models the overexpressed Golgi proteins are at an abnormally high protein concentration during their synthesis in the ER and hence pre-Golgi associations and oligomerization are promoted. The aggregated protein hence does not exist the ER and is degraded slowly in situ. For the bilayer-mediated model, the overexpressed protein can not partition to the Golgi membrane; the amount of Golgi lipid is fixed in amount and hence the capacity of the Golgi is limited.

In this study, we have taken an epitope tag approach to explore the roles of cytoplasmic tails of three well characterized Golgi type II membrane proteins in determining their subcellular localization. By adding different tags to the NH_2 terminus of Sialyltransferase (TGN/trans Golgi), galactosyltransferase (TGN/trans Golgi), and N-acetylglucosaminetransferase 1 (medial Golgi), we could not only ask what the effect of altering tail length and sequence was on the subcellular distribution but also ask whether the cytoplasmic tail was accessible to antibody binding or masked. None of the tags contain a known targeting sequence. For the two trans-Golgi/TGN glycosyltransferases, we found by immunofluorescence that the cytoplasmic tail could be lengthened considerably (3-4 fold) and a significant portion of the protein still accumulated in a perinuclear, Golgi-like distribution. With respect to GlcNAcT-1, it tolerated increased tail length poorly with accumulation in CGN and ER. For Golgi localized GalTs, we found the epitope tagged cytoplasmic tail was often masked in fixed cells. Depending on the exact tail alteration, we found that all three transferases accumulated in the ER with little, if any, apparent perinuclear or cell surface accumulation. Most surprisingly, in the one homologous

expression case studied, ER accumulation of a GlcNAcT-1 chimeric protein lead to disappearance of pre-existing Golgi. Based on these results, we propose, as the simplest explanation of the data, that alteration of the cytoplasmic tail of Golgi resident proteins can decrease their rate of exit from ER, perhaps due to premature oligomerization, and may lead to the capture of other Golgi proteins in the ER.

3.3 Material and Method

Materials

General purpose chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Restriction enzymes were purchased from Promega (Madison, WI) or New England Biolabs (Bainbury, MA). Electrophoresis chemicals were purchased from Bio-Rad (Hercules, CA). Photographic reagents and Kodak X-ray film were from Kodak (Rochester, NY). Cell culture reagents were purchased from Gibco BRL (Grand Island, NY) and fetal bovine serum from Hy-Clone Laboratories, Inc. (Logan, UT). Protein Labeling Mix [^{35}S] Methionine was purchased from NEN/Du Pont (Boston, MA). 9E10 mouse monoclonal antibody against a myc peptide (Evan et al., 1985) was diluted from an ascites preparation. M2 monoclonal mouse against the FLAG epitope (Hopp et al., 1988) was purchased from Kodak IBI (Kodak IBI, New Haven, CT). 9E10 mouse monoclonal antibody against myc has been described (Evan et al, 1985). P5D4 mouse monoclonal antibody against the C-terminal 15 amino acids of VSV-G protein has been described previous (Kreis, 1986). A mouse monoclonal antibody against amino acids 98-106 of HA protein has been described (Wilson et al, 1984). Polyclonal rabbit antibody against bovine GalT was kindly provided by Dr. Shaper. Polyclonal rabbit antibody against the EAGE peptide of β-COP I has been described (Duden et al, 1992; Pepperkok et al., 1994)

Methods

Cell Culture and Transfection Monolayer Vero cells (African green monkey kidney cells, ATCC CCL81), HeLa cells(ATCC CCL 185) and BHK cells were grown in MEM medium supplemented with 10% heat inactivated, fetal bovine serum. Cell were transfected using a standard calcium phosphate method (Chen and Okyama, 1987). For immunofluorescence experiments, cells were plated on coverslips (10mm) in 60 mm dishes (~50% confluent) and transfected with 5-10 μg supercoiled DNA which was purified by $CsCl_2$

centrifugation. 24 -36 h after transfection cells were analyzed by indirect immunofluorescence. The number of cells expressing the transfected protein ranged from 5% to 40% depending on the cell type with Vero being low and BHK and HeLa higher.

For stable expression of chimeric proteins in the Vero cells, 48 hours after transfection cells were transferred to and then maintained in the above medium containing 500 μg/ml Geneticin (G-418 sulphate, Gibco Laboratories, Grand Island, NY) for three weeks before analysis of expression patterns by immunofluorescence.

Recombinant DNAs All constructs were made using standard cloning procedures (Sambrook et al., 1989).

Sialyltransferase constructs: The human a-2,6-sialytransferase kindly provided by S. Munro (MCR, Cambridge, UK) has a VSV-G tag at its carboxyl terminus and the plasmid contains a BamHI site just before the initiation ATG. The amino acid sequences of the cytoplasmic domain of all SialylT constructs is summarized in Fig. 1.

myc-pΔtail-SialylT: the first 6 N terminal amino acids of SialylT, the BamHI site and the C-terminal VSV-tag were replaced using PCR to give a N-terminal myc epitope. The 5' primer was:

5'-GGATCCGGATCCACCATGGAACAAAAACTGATCTCAGAAGAGGACCTGAAGAAAAA GTTCAGCTGCTGCG-3'.

The underlined nucleotides encode a myc sequence. The 3' primer was:

5'-GGATCCGGATCCTTAGCAGTGAATGGTCCGGAAGCC-3'.

The amplified DNA was digested by BamHI and subcloned to pSRα (DNAX, Palo Alto, CA).

VSV-SialylT: a VSV-G tag was attached the N-terminus of SialylT by PCR. The sequence of 5' primer was:

5'-GGATCCGGATCCACCATGATTTACACCGATATAGAGATGAACAGGCTGGGA AAGATTCACACCAACCTGAAGAAAAAG-3'.

The sequence of the 3' primer was:

5'-GGATCCGGATCCTTAGCAGTGAATGGTCCGGAAGCC-3'.

The underlined sequence encodes a VSV-G epitope. The amplified DNA was digested by BamHI and subcloned to pSRα

In order to facilitate additional constructs, three tagging vectors pKS-FLAG, pKS-myc, pKS-HA and based on the pKS Bluescript vector (Stratagene, La Jolla, CA) were made by insertion of complimentary oligonucleotides into the Sal I site of pKS. The inserted sequence included in order: a eukaryotic translational initiation site, a tag sequence, a GlyGly helix breaker, and a Nde I site (CATATG) for cloning original cDNA at the first ATG initiation site downstream of the tag. For pKS-FLAG, the translated insertion sequence is MDYKDDDDKGGHM with the FLAG epitope sequence underlined. For pKS-myc, the translated insertion sequence is MEQKLISEEDLGGHM with the myc tag sequence underlined. For pKS-HA, the translated insertion sequence is MDYPYDVPDYAGGHM with the HA tag sequence. The use of this plasmid is described later in the Methods.

FLAG-SialylT and *myc-SialylT*: In order to subclone SialylT directly downstream of the FLAG and myc epitopes, the bases ACC before the initiation ATG were mutagenisised to CAT by PCR to create a NdeI site (CATATG) which can be inserted at the Nde I site in the pKS-epitope plasmid series. Primers used in PCR were:

The sequence of 5' primer was:

5'-TCCCCCGGGCATATGATTCACACCAACCTGAAG-3'

The sequence of 3' primer was:

5'-GGATCCGGATCCTTAGCAGTGAATGGTCCGGAAGCC-3'.

The amplified DNA was digested by NdeI and BamHI, subcloned into pKS FLAG and pKS myc which were cut by the same restriction enzymes. These two epitope tagged

constructs were digested by SalI and BamHI and subcloned to expression vector pSRα cut with Xho I and BamHI.

FLAG-spacer-SialylT and *myc-spacer-SialylT*: the cDNA for SialylT was cut by BamHI and subcloned into pKS-FLAG and pKS-myc at the BamHI site, the translation of SialylT is inframe with the tags. Between the tags and SialylT sequence was a spacer sequence: VDGIDKLDIDFLQPGGST (deduced amino acids) which derived from the polycloning site of pKS plasmid. The resulting constructs were subcloned into expression vector pSRα.

Galactosyltransferase constructs: The cDNA of bovine short GalT (Russo et al, 1989) provided as a kind gift by J. Shaper (Oncology, Johns Hopkins University School of Medicine, Baltimore, MD) was inserted into pKS Bluescript at the Sal I and Xba I cloning sites. The resulting plasmid pKS-GalT was used for several subsequent manipulations. The unique Pst I site at nucleotide 16 in the coding sequence of GalT was used to insert complimentary oligonucleotides coding for different tags. The amino acid sequences of the cytoplasmic domain of all GalT constructs is summarized in Fig. 2.

FLAG-pΔtail-GalT: To put the FLAG epitope tag nearly adjacent to the transmembrane domain, a PCR mutagenesis method was used. The 5' primer used was:

5'-GGATCCGGATCCGCCATGGACTACAAGGACGACCATGACAAGGGCGCCTC

CTCGTGGCTGTCTGC-3'.

The underlined nucleotides encode the FLAG epitope sequence. The T3 primer of the plasmid was used as 3' primer for PCR. The amplified DNA was digested by BamHI and inserted at the Bam HI site of expression vector pSVL (Pharmacia LKB, Piscataway, NJ).

myc-pΔtail-GalT and *double-myc-GalT:* To put a myc tag close to the transmembrane domain of GalT, a portion of the wild type tail sequence was deleted. GalT cDNA was recloned into pKS at the BamHI cloning site. Clones with the polylinker Pst I

site located upstream of GalT cDNA were selected by restriction enzyme analysis. DNA encoding the N-terminal most 5 amino acids of GalT was then replaced by myc epitope by insertion of a double stranded olignulceotides at the Pst I site of the digested plasmid. The double stranded oligonucleotide used was

5'-CACTATGGAGCAGAAGCTCATCTCCGAGGAAGACCTAGGCGGCCTGCA-3'

5'-GGCCGCCTAGGTCTTCCTCGGAGATGAGCTTCTGCTCCATAGTGTGCA-3'

which translates to MEQKLISEEDLGG. The underlined amino acids are the myc epitope. The additional two glycines served as a helix breaker to expose the epitope. The orientation of the insert was established by DNA sequencing. One construct accidentally had two tandem oligonucleotides insertion both in correct orientation and is referred to as *double-myc-GalT*. Both constructs were subcloned into pSVL expression vector.

myc$_i$-GalT (i stand for internal): pKS-GalT was digested with PstI. The double nucleotide shown above was into the PstI site. The correct orientation of the insert was established by DNA sequencing. This construct places a myc epitope coding sequence internally with splitting of the wild type tail domain sequence. The resulting construct was inserted into pSVL expression vector.

VSV$_i$-GalT: A sequence coding for the VSV epitope was inserted internally into the cytoplasmic tail sequence of pKS-GalT using the following double stranded oligonucleotide:

5'-GCGCCAAATCTACACAGATATCGAGATGAACCGCCTTGGAAAAGATCCTCTGCA-3'

5'-GAGGATCTTTTCCAAGGCGGTTCATCTCGATATCTGTGTAGATTTGGCGCTGAC-3'

in which the underlined nucleotides encode the VSV epitope QIYTDIEMNRLGK. The correct orientation was confirmed by restriction enzyme digestion and DNA sequencing. The resulting DNA was cloned into pSVL eukaryotic expression vector .

HA -spacer-GalT and *FLAG-spacer-GalT:* cDNA coding for GalT was cut by Pst I and BamH I and then ligated into pKS- HA or pKS-FLAG cut by the same enzymes. The orientation of the insert was established by restriction enzyme digestion and DNA sequencing. The spacer sequence between the tag and actual GalT comes from the polycloning region of pKS encodes VDGIDKLDIDFL. The translation of the tags and GalT were inframe.

GlcNAcT-1 constructs:

cDNA coding GlcNAcT-I (Kumar et al., 1990) was kindly provided by Dr. P. Stanley (Albert Einstein College of Medicine, Bronx, NY). To facilitate plasmid construction, the nucleotides before the initiation ATG were mutagenisised to CAT to provide a Nde I site by PCR with the following primers:

5' primer: 5'-CCCATCGATATCCATATGATCAAGAAGCAGTCTGCTGGCC-3'

3' primer: 5'-GGATCCGGATCCCTAATTCCAGCTAGGATCATAGCCC-3'

myc-GlcNAcT-1 and FLAG-GlcNAcT-1: The amplified DNA was digested by was digested by NdeI and BamHI and cloned to pKS-myc or pKS-FLAG, also digested with NdeI and BamHI. The myc-GlcNAcT-1 and FLAG-GlcNAcT-1 coding sequences were subcloned into expression vector pRSα at the XhoI and BamHI sites.

myc-spacer-GlcNAcT-1 and FLAG-GlcNAcT-1: The amplified DNA was digested by EcoRV and BamHI, ligated to pKS-myc or pKS-FLAG digested by Sma I and BamHI. This has the effect of including in the coding sequence a spacer sequence of 16 amino acids contributed by the polylinker region. The myc-spacer-GlcNAcT-1 and FLAG-spacer-GlcNAcT-1 coding sequences were subcloned into expression vector pRSα at the XhoI and BamHI sites.

The sequence of all the constructs were confirmed by DNA sequencing.

Immunofluorescence Microscopy Transfected cells were analyzed by immunofluorescence 24-48 h after transfection. All manipulations were at room temperature. Cells were washed three times with PBS after fixation and antibody incubation. Cells on coverslips were fixed and permeablized in -20°C methanol for 3 min. Coverslips were inverted over 30 µl of the appropriate primary antibody and incubated for 20 min. Secondary antibodies conjugated to either rhodamine or fluorescein were used to visualize primary antibody. Fluorescence microscopy was performed using a Zeiss Axiophot microscope. Images were either captured with a CCD camera or photographed to Kodak film.

In Vitro Transcription and Translation pKS-myc$_1$-GalT was linearized by Sal I. The linearized plasmid was transcribed in vitro using T7 polymerase (Pharmacia LKB, Piscataway, NJ) in the presence of an RNase inhibitor (RNasin, Promega, Madison, WI). The transcript was capped by including 0.5 mM of the cap analogue m^7GpppG (Promega, Madison, WI) in the transcription reaction. The mRNA was used to program in vitro translation reaction using the reticulocyte lysate system (Promega, Madison, WI) containing [^{35}S] methionine as described by the manufacturer.

For immunoprecipitation, in vitro translation reactions were diluted five-fold with dilution buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, 2 µg/ml leupeptin). Half was incubated with mouse IgG as a control and the rest incubated with 9E10 mouse monoclonal antibody. The immune complexes were precipitated with protein A-Sepharose, washed three times with dilution buffer, and then analyzed by SDS-PAGE followed by antoradiography. The total volume of each sample was applied to the gel wells and the autoradiography exposure was 12-16 h.

3.4. Results:

The epitope tags used in this study include: myc (9E10 antibody; Evan et al., 1985), HA (Wilson et al., 1984), VSV (P5D4 antibody; Kreis, 1986), and FLAG (M2 antibody; Hopp et al., 1988). These are well characterized, react with high affinity mouse monoclonal antibodies, and have been used on many occasions with no discernible effect on the localization or function of the tagged proteins (for examples, see Munro and Pelham, 1986; Smith and Blobel, 1993; Soldati and Periard, 1991). They contain no known targeting feature. The tags and spacer sequence with the exception of the VSV epitope carry a net negative charge at cytosolic pH, ~7.0. The naturally occurring cytoplasmic domain sequences of SialylT, GalT, and GlcNAcT-1 all have a net positive charge with the positive charge being concentrated in the membrane proximal few amino acids. These are thought to be important in maintaining the topology of the protein in the membrane and may have a function in the Golgi retention signal. In constructing chimeric proteins tagged in the cytoplasmic domains of the glycosyltransferases, we were careful to leave unchanged the transmembrane domain proximal few amino acids. The N-terminal most amino acids are not important in the targeting of these proteins to the Golgi (Dahdal and Colley, 1994; Nilsson et al., 1991; Tang et al., 1991). Based on the Chou-Fasman algorithm, the epitope tags and spacer sequence in the chimeric proteins have variable tendencies to form α-helices. The wild type cytoplasmic domains are also predicted to have variable extents of α-helicity.

<u>Replacement of Partial Cytoplasmic Tail of SialylT by myc Epitope Resulted in CGN and ER Accumulation</u>

Based on the short length of wild type cytoplasmic tails, we reasoned that epitope attachment in such a manner to give minimal increase in cytoplasmic tail length should give a normally distributed SialylT. Hence, a construct, myc-pΔtail-SialylT in which the myc

epitope (MEQKISEEDL) replaced the first six amino acids (MIHTQL) of SialylT, was prepared (Fig. 1, construct 1). The three membrane proximal lysines remained. Dahdal and Colley (1993) have shown that deletion of amino acids 2-6 of the SialylT cytoplasmic tail has no detectable effect on Golgi accumulation. myc-pΔtail-SialylT is five amino acids longer than wild type. The subcellular localization of myc-pΔtail-SialylT was characterized by immunofluorescence in Vero cells 36 h after transfection. To our surprise, the immunofluorescence pattern was not Golgi-like (Fig. 4, A). Instead it showed granular perinuclear concentration of staining that might well be CGN and a reticular staining of the rest of the cytoplasm, characteristic of ER. Expression of myc-pΔtail-SialylT in Vero cells had no effect on the normal perinuclear distribution of the Golgi coat protein β-COP I, a cis-Golgi marker in Vero cells (Duden et al., 1991; Pepperkok et al., 1993). These results imply that either the intact cytoplasmic sequence was required for efficient transport or that addition of the myc tag severely interfered with myc-pΔtail-SialylT transport to the Golgi. It is more likely that the mislocalized myc-pΔtail-SialylT resulted from the placement of the myc tag near the transmembrane domain rather than from the partial deletion of wild type cytoplasmic tail sequence.

Placement of myc and FLAG Epitope Tags at the N-terminus of Intact SialylT Had a Reduced Effect on Golgi Accumulation

To test if moving the negatively charged epitope sequence out from the membrane might lead to better Golgi localization of cytoplasmically tagged SialylT chimeric proteins, we prepared FLAG-SialylT (Fig. 1, construct 2) and myc-SialylT (Fig. 1, construct 4).In these, the epitope tag is separated from the normal cytoplasmic tail sequence by a GlyGlyHis, helix breaker sequence. When transiently expressed in Vero cells, both FLAG-SialylT (Fig. 4, B) and myc-SialylT (Fig. 4, E) appeared to give a combination of perinuclear staining which colocalized with β–COP (data not shown) and a faint but readily detectable reticular cytoplasmic and nuclear envelop staining characteristic of ER. Some of

the chimera must be retained in ER. The above results demonstrated that placement of the myc and FLAG tags further out from the membrane resulted in more efficient transport of chimeric SialylT from the ER.

Placement of a VSV Epitope Tag at the N-terminus of Intact SialylT Had Little, if Any, Effect on Golgi Accumulation

To test if the partial mislocalization of myc- and FLAG-SialylT might be due to the negative charge of the epitopes, we prepared the similar construct (VSV-SialylT, Fig. 1, construct 3) tagged with a VSV-11 sequence which is essentially neutrally charged at pH 7.0 (Table 1). The immunofluorescence staining pattern of VSV-SialylT showed a near-to-normal Golgi localization. Almost all fluorescence was concentrated in a juxtanuclear region and qualitatively this juxtanuclear fluorescence appeared compact and continuous, traits typical of Golgi staining in Vero cells (Chapter 2); little to no staining was detected outside of the juxtanuclear region (Fig. 4, C). VSV-SialylT co-localized with β-COP I (Fig. 4, C and D, black arrowheads). The β-COP I fluorescence in the non-transfected cell is brighter than normal (Fig. 4, D). The predicted cytoplasmic domains of FLAG-SialylT, VSV-SialylT, and myc-SialylT vary in length from 21 to 23 amino acids. The fact that VSV-ST was almost exclusively Golgi localized while the others were not implies that the charge carried by N-terminal sequences in the cytoplasmic domain can be important in determining the subcellular distribution of a glycosyltransferase.

SialylT Chimeras with Long Cytoplasmic Domains Showed Golgi Accumulation

To further investigate the effect of cytoplasmic tail length and charge on SialylT targeting, a spacer sequence composed of 18 amino acids was inserted between the FLAG (FLAG-spacer-SialylT; Fig. 1, construct 5) and myc (myc-spacer-SialylT; Fig. 1, construct 6) tags and the intact SialylT cytoplasmic tail. These constructs had a cytoplasmic domain of 40 or 42 amino acids and the first negatively charged residue in the VDGIDKLDIDPLQPGGST spacer sequence was 18 amino acids from the transmembrane

domain (Fig.1, constructs 5 and 6). The resulting constructs were transiently expressed in Vero cells. The localization patterns for FLAG-spacer-SialylT (Fig. 4, F) and myc-spacer-SialylT (data not shown) were similar to each other and showed very significant Golgi-like juxtanuclear accumulation that co-localized with β-COP I. There was a weak ER background. These data suggested that SialylT could tolerate a long cytoplasmic domain without dramatically affecting subcellular localization. This may result from spacing any negative residues out from the membrane. Stable Vero cell lines expressing FLAG-spacer-SialylT were constructed to test chimeric protein localization in a long-term expression situation. Transfected Vero cells were screened by immunofluorescence after pooling Geneticin resistant clones three weeks post transfection. By double staining with monoclonal anti-FLAG antibody and polyclonal anti-β-COP I, about 2% of the cells in the population is FLAG positive. The immunofluorescence was perinuclear concentrated and co-localized with that of β-COP I (Fig. 5, A and B). SialylT could tolerate long, 40 or 42 amino acids, cytoplasmic domains quite well in both short-term or long-term expression.

Epitopes Carried by a Number of GalT Chimeric Proteins Were Masked When Expressed in Cells

Bovine and murine GalT has two related forms that differ only in the length of their respective NH_2-terminal cytoplasmic domains and result from alternative transcription initiation (Russo et al., 1989). Short GalT is agreed by all to be a trans Golgi resident enzyme while long GalT may also be expressed at the cell surface (Lopez et al., 1991). In this study, the short form of bovine GalT was used.

Several constructs with different tags placed near the transmembrane domain and differing in length of the cytoplasmic domain, 11-29 amino acids, were made. The constructs were: FLAG-Δtail-GalT, FLAG epitope replaced 9 of 11 amino acids of the wild type tail leaving an Arg membrane proximal (Fig. 2, construct 1); myc-pΔtail-GalT, a myc epitope replaced the first five amino acids of the GalT tail (Fig. 2, construct 2); myc_i-

GalT, a myc epitope was inserted between amino acids six and seven of the GalT cytoplasmic domain (Fig. 2, construct 3); VSV_i-GalT, a VSV-G tag was inserted between amino acids six and seven of the GalT cytoplasmic domain (Fig. 2, construct 4). When expressed in Vero cells, no epitope-specific staining was seen (data not shown). Conceivably the cytoplasmic domain was masked. To test this, we decided to express our GalT constructs in BHK cells and double stain with anti-epitope antibodies and anti-GalT antibody that reacted with the lumenal domain. This antibody was directed against bovine GalT and crossreacted with endogenous Vero cell GalT. BHK cell populations transiently expressing any of these four constructs upon double label immunofluorescence staining showed about 5-10% cells to be GalT with a perinuclear Golgi-like staining pattern, i.e., transfected, (Fig.6, B and D). None of the transfected cells gave a positive immunofluorescence signal for the respective epitope (FLAG epitope, Fig. 6, A; myc, Fig. 6, C). This confirmed the hypothesis that the epitopes were masked. In *in vitro* transcription-translation experiments, the myc epitope carried by the construct myc_i-GalT was readily immunoprecipitated; gel electrophoretic analysis of the immunoprecipitate revealed a 48 kD band which was expected size of myc_i-GalT from its amino acid sequence (Fig. 7, lane C). This band was specifically precipitated by 9E10 anti-myc antibody. No radioactive material was detected if 9E10 was omitted from the immunoprecipitation (Fig. 7, lane A). As a positive control, it was shown that 9E10 immunoprecipitated a myc tagged 170 kD protein (Fig. 7, lane B). This eliminated the possibility that the chimeric protein was incorrectly tagged and again implied that the epitope tags carried each of the four GalT constructs were masked in the fixed cells. It should be noted that no significant difference in epitope behavior related to charge was detected; all behaved the same.

Replacement of GalT Cytoplasmic Tail Sequences with Long Epitope Tagged Domains Resulted in ER Accumulation of Antibody Accessible Chimeric Proteins

When making myc_i-GalT constructs, one clone had a double myc insertion correctly orientated for translation. This construct designated as double-myc-GalT (Fig. 2, construct 7) was expressed in BHK cells. By double labeling, both anti-myc 9E10 (Fig. 6, E) and anti-GalT (Fig. 6, F) gave a strong fluorescence signal. The immunofluorescence pattern showed nuclear envelope and reticular cytoplasmic staining, characteristics of ER localization. Presumably the accessibility of it is N-most myc epitope which is accessible to antibody and the more internal myc epitope functions as a spacer sequence. To confirm this idea, two other constructs: myc-spacer-GalT (Fig. 2, construct 5) and HA-spacer-GalT (Fig. 2, construct 6) with a 12 amino acids spacer sequence between the epitope and the sixth amino acid of GalT, were made. When expressed in BHK cells, both were recognized quite well by 9E10 and anti-HA antibody (data not shown). The staining pattern was ER similar to that of double-myc-GalT. From this, we conclude that there is a threshold length of ~30 amino acids to the cytoplasmic domain of GalT beyond which transport to the Golgi is strongly inhibited.

Placement of myc and FLAG Epitope Tags at the N-terminus of Intact GlcNAcT-1 Resulted in ER Accumulation

All know medial glycosyltransferases or glycosidases have a short predicted cytoplasmic domain of five or six amino acids. FLAG and myc tags were directly placed at the NH_2-terminus of GlcNAcT 1. This added 12 or 14 amino acids to the cytoplasmic domain. The resulting chimeric cDNAs: FLAG-GlcNAcT 1 (Fig. 3, construct 1) and myc-GlcNAcT-1 (Fig. 3, construct 2) were transiently expressed in Vero cells. After a 36 h expression period, the cells were double stained with anti tag antibodies (9E10 for myc and M2 for FLAG) and polyclonal anti-β-COP I antibody. The immunofluorescence localization of the two chimeric proteins was similar. For FLAG-GlcNAcT-1, perinuclear granular CGN-like staining was found in the same area of the cell as a more compact β-

COP staining pattern, at the same time cytoplasmic staining extending to the cell periphery was detected (compare, Fig. 8, A and B). When a spacer sequence composed of 18 amino acids was inserted between the epitope tag and the wild type cytoplasmic tail and resulting FLAG-spacer-GlcNAcT-1 (Fig. 3, construct 3) and myc-spacer-GlcNAcT-1 (Fig. 3, construct 4) constructs expressed in the Vero cells, they showed similar reticular cytoplasmic distributions and staining of the nuclear envelope, both traits characteristic of ER localization (FLAG-spacer-GlcNAcT-1, Fig. 8, C). The distribution of β-COP I was normal (Fig. 8, D). Long-term transfectants expressing myc-GlcNAcT-1 were selected in the presence of Geneticin for three weeks. Drug resistant clones were pooled and screened for epitope staining patterns with 9E10. About 5% of the cells were positive. Two different staining patterns were seen. One was marked by a granular, perinuclear staining characteristic of CGN together with ER-like cytoplasmic staining (Fig. 9, A). The other was exclusively ER (Fig. 9, B). The first expression pattern was similar to the transient situation.

Together these data suggest that unlike SialylT and GalT, the medial cisternal enzyme GlcNAcT-1 does not tolerate well increased tail length. The immunofluorescent distributions of all three sets of chimeric proteins are summarized in Fig. 10. In no case was cell surface staining noted.

Mislocalization of GlcNAcT-I Can Affect the Localization of Other Golgi Proteins

The previous experiments have all examined the expression of a chimeric protein in a heterologous cell system. To test the effect of expression in an homologous cell system, we expressed myc-spacer-GlcNAcT-1, a human chimeric protein, in HeLa cells, a human cell line. Not surprisingly, the myc-spacer-GlcNAcT-1 localized in an ER-like pattern that rimmed the nucleus in these less well spread cells (Fig. 11, A and C). Unexpectedly, β-COP I in the transfected cells did not localize in a perinuclear manner, rather staining was diffuse in the cytoplasm (Fig. 11, B, arrowheads). Likewise, the endogenous trans-

Golgi/TGN enzyme GalT was no longer perinuclear, but rather gave a weak, diffuse staining about the nucleus, presumably an ER localization while neighboring non-transfected cells showed a normal Golgi localization (Fig. 11, D, arrowheads point to transfected cells). These results suggest that ER mislocalization of GlcNAcT 1 can have a profound effect on both protein transport and the structure of pre-existing Golgi.

3.5. Discussion:

We added different epitope tags and spacer sequences to the cytoplasmic NH_2 terminal domain of SialylT (TGN/trans Golgi), GalT (TGN/trans Golgi), and GlcNAcT-1 (medial Golgi) to ask not only the effect of altering tail length and sequence on subcellular distribution but also whether the cytoplasmic tail was accessible to antibody binding or masked. The added sequences contained no known targeting sequence. By immunofluorescence, we found that the cytoplasmic tail of the two trans-Golgi/TGN glycosyltransferases could be lengthened 3-4 fold and a significant portion of the protein still accumulated in a perinuclear, Golgi-like distribution. GlcNAcT-1 did not tolerate increased tail length and accumulated in CGN and ER with addition of epitope sequences. For Golgi localized GalTs, we found the epitope tagged cytoplasmic tail was often masked. Most surprisingly, in the one homologous expression case studied, ER accumulation of a GlcNAcT-1 chimeric protein lead to disappearance of Golgi. The chimeric proteins were expressed both transiently and in some cases stably. Long-term stable transfectants were made that gave Golgi localization, CGN and ER localization, and ER localization. The chimeric protein depending on clone might be localized either to the CGN and ER or to ER exclusively. Presumably this relates to the level of expression. Based on these results, we propose, as the simplest explanation of the data, that alteration of the cytoplasmic tail of Golgi resident proteins can decrease their rate of exit from ER, presumably due to premature oligomerization, leading to the capture of other Golgi proteins in the ER.

SialylT can be epitope tagged in its cytoplasmic domain and accumulate normally in Golgi. VSV-SialylT demonstrated a normal localization at the immunofluorescence level. This was the only chimeric protein out of the 17 tested that did not show ER- or CGN-like localization of a significant portion of the total expressed protein. This chimera should be a useful protein for studying the in vivo dynamics of the Golgi using a resident marker; it

should be accessible to microinjected fluorescent Fab antibody fragments as a reporter group. Our results point to the probable significance of the charge properties of the cytoplasmic domain of Golgi type II membrane proteins. Wild type cytoplasmic domains are short and positively charged with the positive charge being concentrated near to the membrane. The VSV epitope added was essentially neutral. Placement of either of two negatively charged epitope sequences, FLAG and myc, at the same position in a chimeric SialylT protein resulted in significant ER accumulation of the chimera. Similarly, replacement of much of the cytoplasmic tail of SialylT, albeit leaving the three membrane proximal lysines, with the negatively charged myc epitope sequence resulted in ER localization. Beyond this statement, little in the way of general rules regarding the nature of the added sequence and its effects on Golgi versus CGN/ER localization can be given. Each of the three Golgi proteins behaved differently in response to cytoplasmic tail tagging (see Fig. 3-10, for summary). For example, adding additional negatively charged sequence to SialylT to give a cytoplasmic domain of 40 to 42 amino acids actually resulted in better Golgi accumulation and a decided decrease in ER accumulation. GalT seemed insensitive with respect to FLAG versus VSV versus myc epitope. It localized to Golgi with ER background irrespective of epitope and with addition of a negatively charged spacer sequence accumulated exclusively in the ER. For GlcNAcT-1 any addition of FLAG or myc tried had a negative impact on transport from the ER. In summary, these experiments clearly indicate that the nature of the cytoplasmic domain of Golgi type II membrane proteins plays a major role in determining their transport from ER. This is a result contrary to previous expectations based on deleting portions of the tail or domain swapping with tail segments of similar length from cell surface or lysosomal type II membrane proteins (Burke et al., 1994; Dahdal and Colley, 1993; Nilsson et al., 1991; Wong et al., 1992)

It is highly improbable that any of the added epitope or spacer sequences contains ER localization signals. The major evidence against this is two-fold: 1) no match is found

when comparing them with the known ER retention signals. 2) if we suppose the existence of a putative ER retention signal then the sequence would be expected to consistently cause ER retention of the three glycosyltransferases. This does not happen. The effect of mutations in the cytoplasmic domain on protein transport out of the ER has been reported previously for several viral glycoproteins such as influenza virus hemagglutinin (Doyle et al, 1986), VSV-G protein (Doms et al., 1988), and bunyavirus G1 protein (Matsuoka et al., 1994). The last one is particular interesting, because it is a Golgi localized type I viral glycoprotein. Mastsuoka et al. demonstrated that deletion of part of the G1 cytoplasmic tail reduced efficiency of mutant protein transport from ER to Golgi. The authors suggested that mutation in the G1 cytoplasmic domain might interfere with its interaction with cytoplasmic factors involved in protein transport. This may not be applicable to our case.

Since gross misfolding of protein will block its transport out of ER, this must be considered as a possible explanation for our results. Mutations in the cytoplasmic tail of VSV-G which affected its transport out of ER did not have any detectable effects on protein folding and biological function (Doms et al., 1988). In the one previous published report in which the length of the cytoplasmic tail of a medial Golgi enzyme was substantially increased (Nilsson et al., 1994), the authors report that the ER form of the enzyme is enzymatically active. Hence it can not be considered to be grossly misfolded. In this report the cytoplasmic tail of Iip33 (47 amino acid) was swapped for that of GlcNAcT-1 in hopes of causing ER retention due to the tri-Arg ER retrieval signal carried by the Iip33 tail. This chimera indeed was ER localized. Based on our results, we suggest that the localization may not necessarily be due to the tri-Arg sequence. The Iip33-GlcNAcT-1 when expressed in cells did not receive any Golgi modifications and did not appear to cycle between the ER and Golgi (Nilsson et al., 1994). Both of these cases suggest that alterations in the cytoplasmic domain does not affect folding of the much larger lumenal domain. Based on the behavior of Iip33-GlcNAcT-1, none of our ER localizing chimeric proteins are likely to

have been transported to the Golgi and then retrieved back to the ER. The importance of cytoplasmic domain length in our experiments may relate then not to the behavior of the proteins in Golgi but rather to their behavior in ER.

The selective masking of three different epitope tags (FLAG, VSV, myc) when placed in a total of four different ways in the cytoplasmic domain of GalT may be explained on the basis of the formation of a mixed GalT oligomer in the Golgi. In vivo epitopes placed within 2 to 8 amino acids of the membrane domain of GalT were not recognized by the appropriate anti-epitope antibody while an in vitro transcription-translation product was. In vivo when the epitopes were placed 21 to 23 amino acids out from the membrane they were recognized. The epitopes themselves are 8 to 14 amino acids in length. This situation was not encountered for SialylT or GlcNAcT-1 chimeras. GalT is the only one of the three transferases that exist in vivo as endogenous short (11 amino acid cytoplasmic domain) and long (24 amino acid cytoplasmic domain) forms (Russo et al., 1989; Harduin-Lepers, Shaper and Shaper, 1993). Assuming that Golgi proteins can form oligomers through their transmembrane domains and parts of the flanking regions, the short GalT and long GalT should produce a mixed protein lattice where the cytoplasmic epitopes carried by short GalT chimeras may be masked by the cytoplasmic domain of long GalT. Mixed oligomerization could explain the masking of the epitopes carried by FLAG-Δtail-GalT, myc-pΔtail-GalT, myc_i-GalT and VSV_i-GalT.

Pre-mature oligomerization provides a possible explanation for the ER accumulation of many of the chimeric proteins. Although the transmembrane domain of Golgi type II membrane proteins often plays a major role in retention of the protein, other regions of the protein, most importantly the membrane flanking regions, may also contribute. In the case of an oligomerization model, they may supply further attractive forces including hydrogen binding and ionic interactions. It is possible that many of added sequences could supply some of the bonding contributions needed to cause the chimeric protein to pre-maturely

oligomerize in early compartments in the secretory pathway including ER and CGN. The epitope tag and spacer sequences were frequently negatively charged and might participate in salt bridges with divalent cations such as Ca^{++} and Mg^{++} or in additional H-bonding. Based on the previous work of Nilsson et al. (1994) with Iip33-GlcNAcT-1, the proteins are unlikely to have ever left the ER (see above for more detailed discussion). Co-oligomerization might also explain the ER retention of endogenous GalT when human myc-spacer-GlcNAcT 1 was transiently expressed in human Hela cells. Here both GalT and β-COP I were abnormally localized. The β-COP I distribution appeared to be almost entirely diffuse. This result was species specific and was not observed for this or other ER localizing chimeras in Vero cells which are of African green monkey origin. The mislocalization of β-COP I suggests that this GlcNAcT-1 mutant dramatically affected protein transport between ER and Golgi and may have caused the disappearenc of pre-existing Golgi. This extends the previous report of Nilsson et al. (1994) that ER mislocalization of Iip33-GlcNAcT-1 caused mislocalization of mannosidase II to the ER but not GalT. Nilsson et al. (1994) suggested that this was due to kin recognition by enzymes destined for the same Golgi cisternae leading to pre-mature oligomerization in the ER.

3.6. References

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Table 1: Physical Properties of Wild Type Cytoplasmic Tails and Added Sequences

Sequence	Length	Charge	pI	Chou-Fasman Helicity
Wild type sequences				
SialyIT	9	3.07	10.30	Yes
GalT				
short-GalT	11	1.88	10.20	C-terminal
GalT extension (long-GalT)	13	0.91	9.01	N-terminal
GlcNAcT-1	6	1.91	10.01	Yes
Epitopes				
FLAG	8	-3.09	3.70	N-terminal
HA	9	-2.09	3.23	No
myc	10	-3.08	3.83	Yes
VSV (P5D4)				
VSV-11	11	-0.09	6.26	Yes
VSV-14	14	0.91	8.86	No
Spacer	14	-2.92	3.86	No

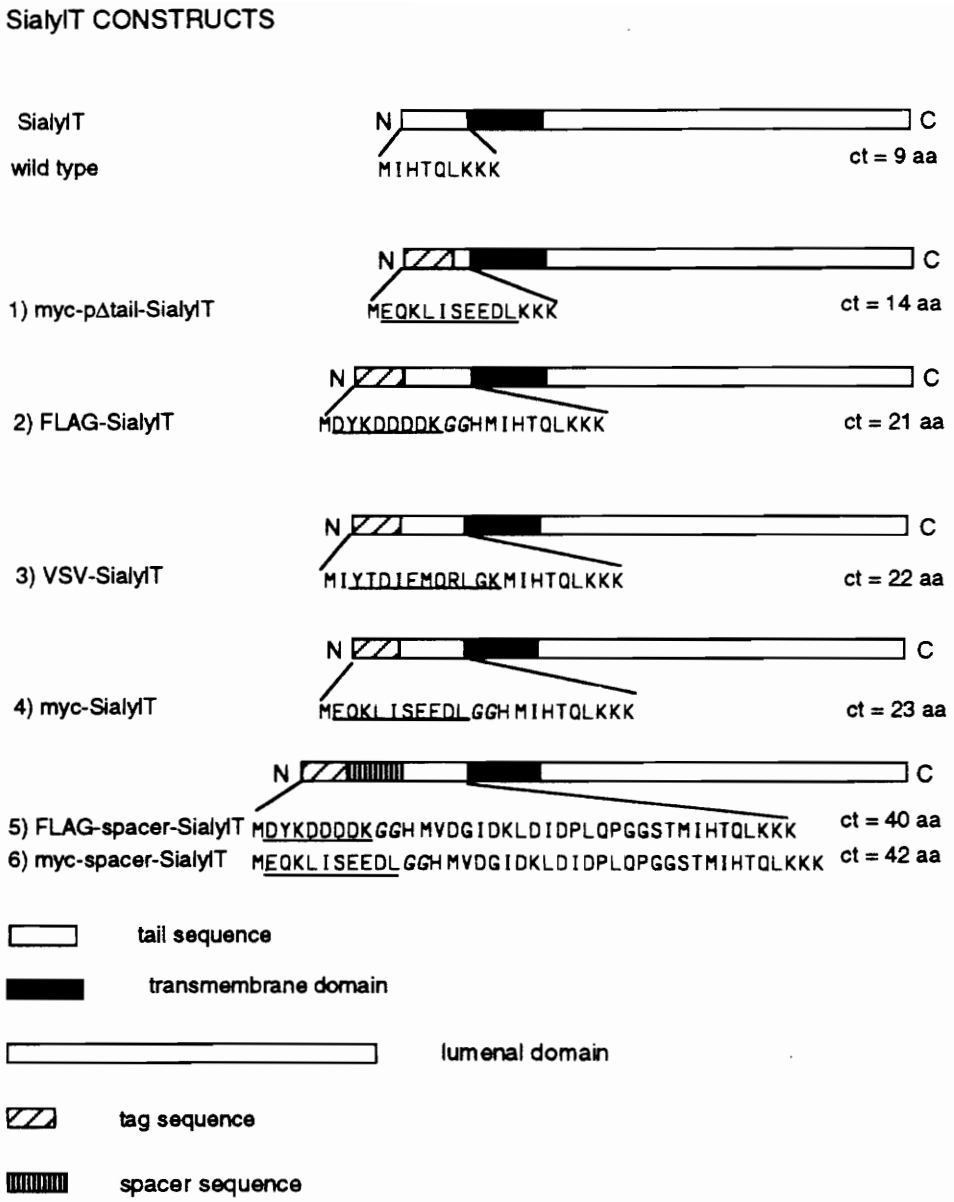


Fig. 3-1. Schematic diagram of SialylT constructs. The key to portions representing the cytoplasmic tail, transmembrane, and lumenal domain of SialylT as well as epitope tag and spacer sequences is given at the bottom of the figure. The actual amino acid composition and length of cytoplasmic tail domains are shown for each construct. The G (glycine) shown in italics is used as a helix breaker.

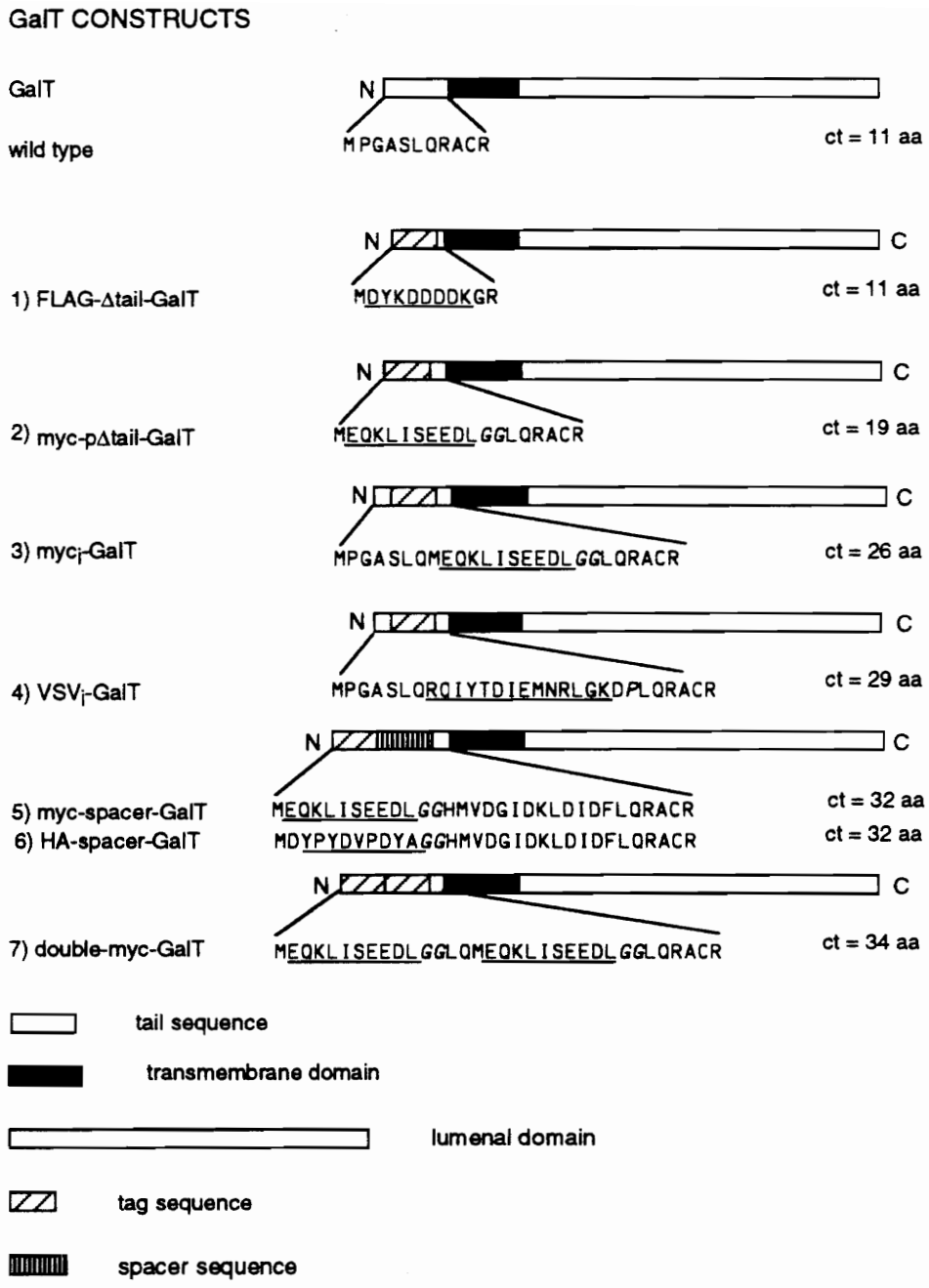


Fig. 3-2. Schematic diagram of GalT constructs. The key to portions representing the cytoplasmic tail, transmembrane, and lumenal domain of GalT as well as epitope tag and spacer sequences is given at the bottom of the figure. The actual amino acid composition and length of cytoplasmic tail domains are shown for each construct. The G (glycine) shown in italics is used as a helix breaker. The P (proline) shoen in italics is used as a helix breaker.

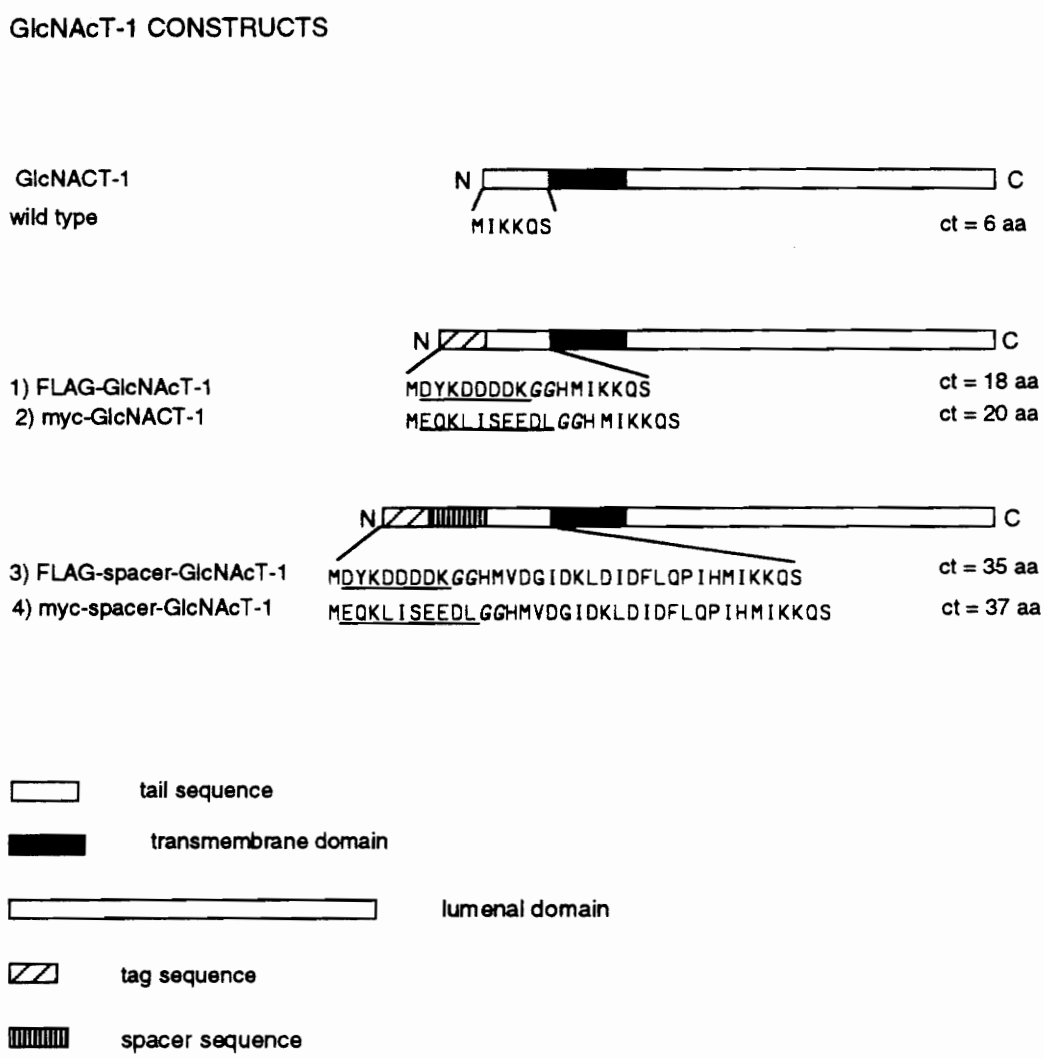
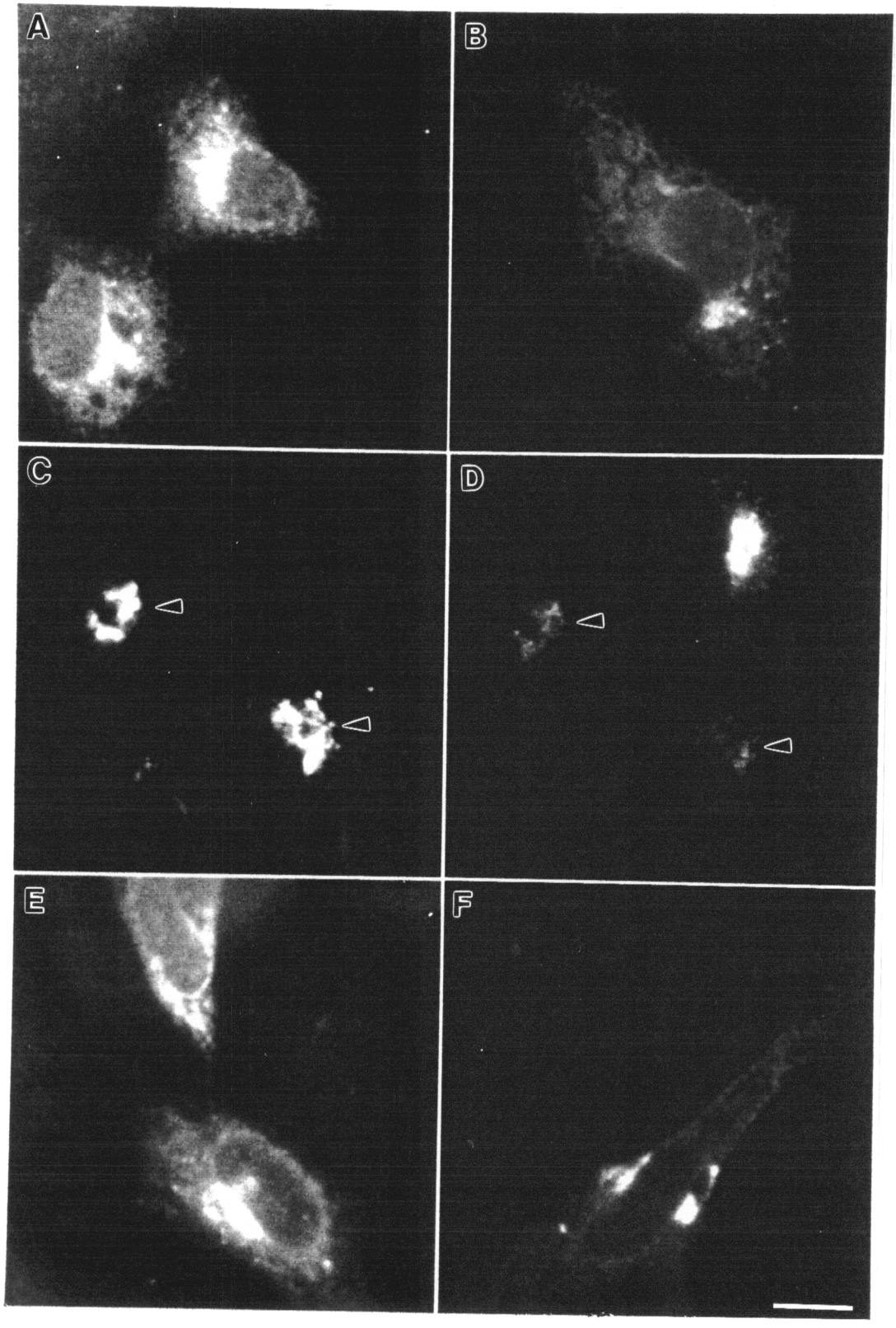


Fig. 3-3. Schematic diagram of GlcNAcT 1 constructs. The key to portions representing the cytoplasmic tail, transmembrane, and lumenal domain of GlcNAcT-1 as well as epitope tag and spacer sequences is given at the bottom of the figure. The actual amino acid composition and length of cytoplasmic tail domains are shown for each construct. The G (glycine) shown in italics is used as a helix breaker.

Fig. 3-4. Immunofluorescence localization of epitope tagged SialylT chimeric proteins in Vero cells. Vero cells transfected with cDNA encoding myc-pΔtail-SialylT (A), FLAG-SialylT (B), VSV-SialylT (C), myc-SialylT (E), and FLAG-spacer-SialylT (F).were fixed 36 h after transfection and processed for immunofluorescent staining with monoclonal antibodies recognizing the epitopes. VSV-SialylT transfected cells were double labeled with a polyclonal antibody against β-COP I (D). The first antibodies were visualized either by secondary antibody coupled to rhodamine (A, B, C, E, and F) or FITC (D). Arrowheads in C and D point to transfected cells. Bar=10μm.


A
B
C
D
E
F

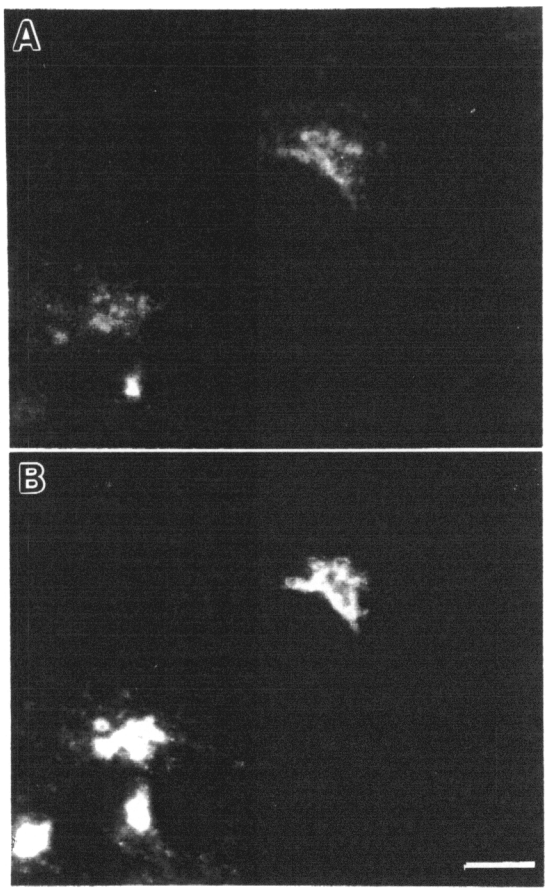


Fig. 3-5. Immunofluorescence localization of FLAG-spacer-SialylT stable transfectants expressed in Vero cells. Vero cells stably transfected with FLAG-spacer-SialylT were fixed, permeabilized and labeled with mouse monoclonal M2 antibody against the FLAG epitope (A) and rabbit polyclonal against β-COP I (B) followed by secondary antibodies coupled to rhodamine (A) and FITC (B). Bar=10μm.

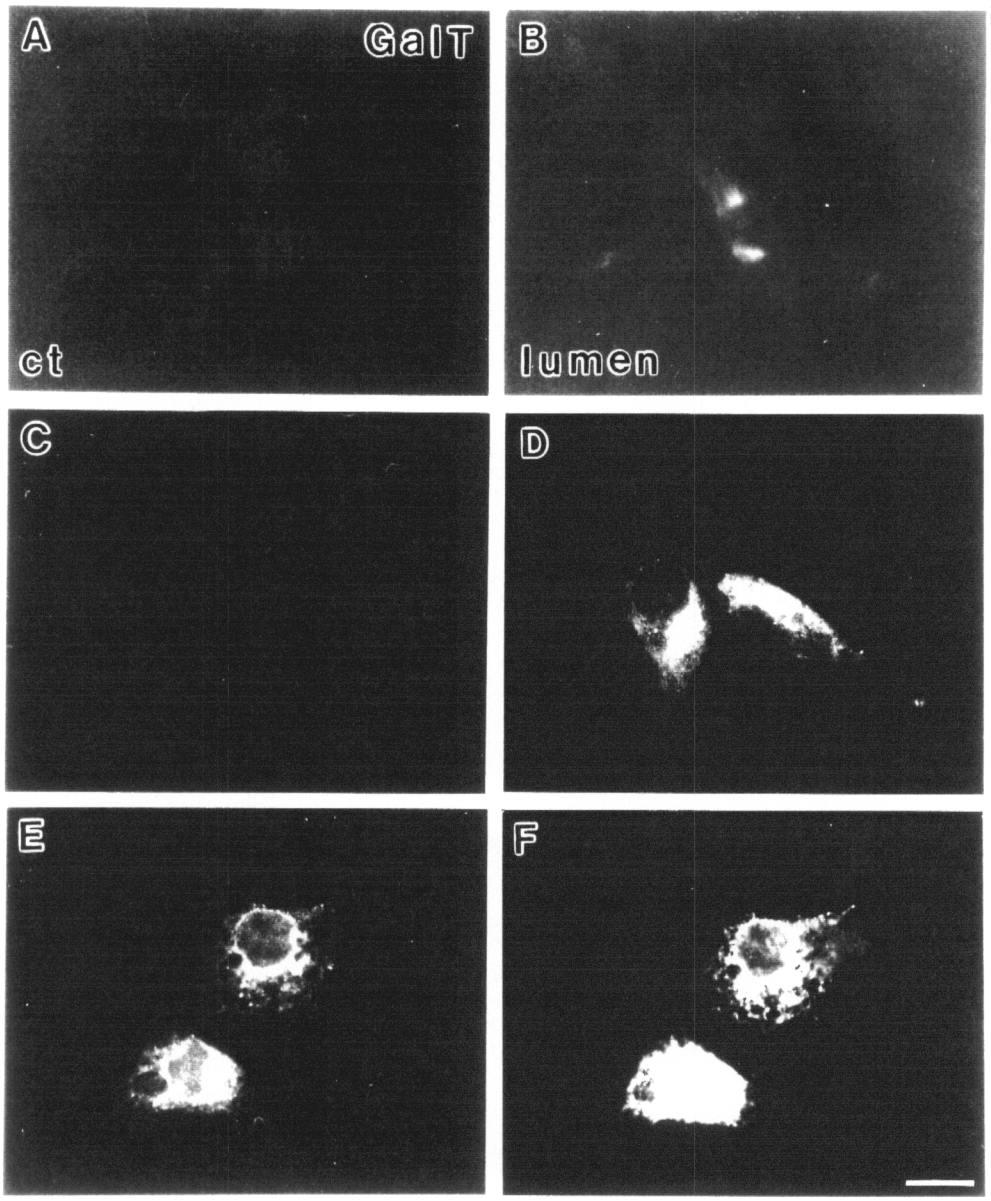


Fig. 3-6. Immunofluorescence localization of GalT chimeric proteins expressed in BHK cells. BHK cells transfected with cDNA encoding FLAG-Δtail-GalT (A and B), myc_i-GalT (C and D), double-myc-GalT (E and F) were fixed 36 h after transfection and double labeled with monoclonal antibodies (M2 for FLAG [A] and 9E10 for myc [C, E]) and rabbit polyclonal anti-GalT (B, D, F). The first antibodies were visualized either with secondary antibodies coupled to rhodamine (A, C, and E) or FITC (B, D, and F). The Golgi-like staining seen B is found in two regions on either side of the nucleus. Bar=10μm.

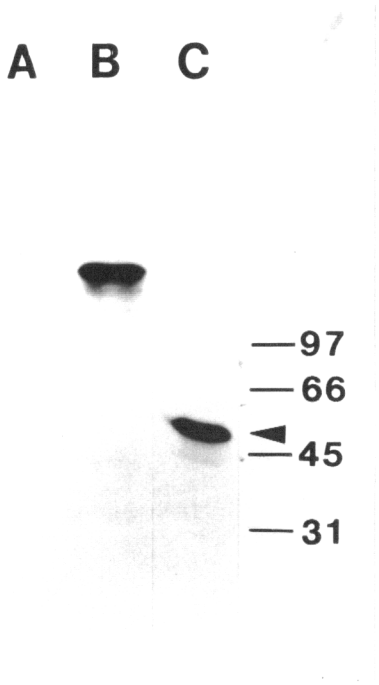


Fig. 3-7. Immunoprecipitation charaterization of *in vitro* transcribed and translated myc_1-GalT. myc_1-GalT was in vitro transcripted and translated as described in Material and Method. The translated proteins were precipitated either with control mouse antibody (lane A) or 9E10 (lane C) and electrophoresed in a 10% SDS polyacrylamide gel. The 9E10 immunoprecipitate reveal a band of 48 kD (lane C, arrowhead); no band was observed with control antibody. Lane B is a positive control in which 9E10 immunoprecipitated a myc tagged 170 kD protein (CLIP-170). MW standards indicated on right side of gel.

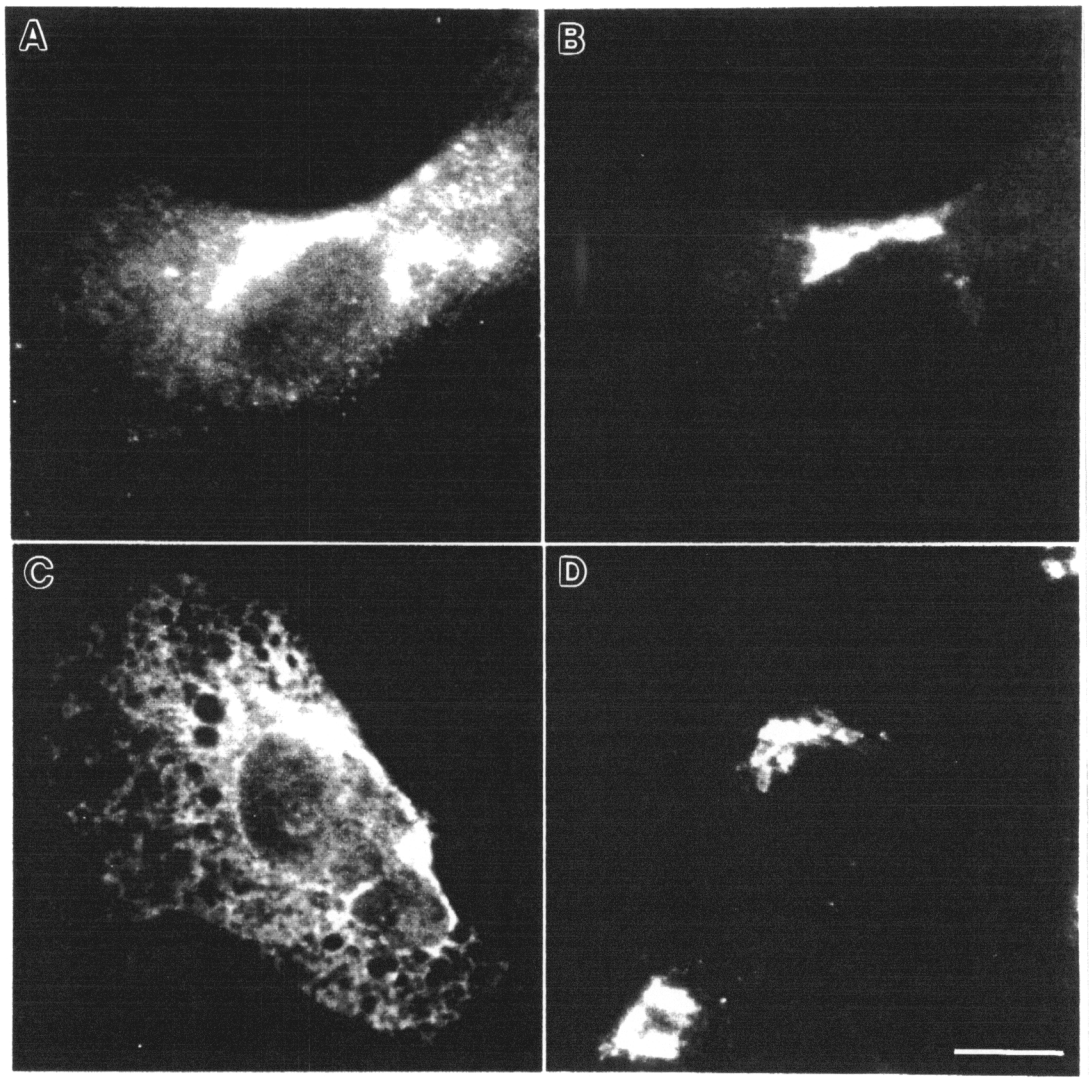


Fig. 3-8. Immunofluorescence localization of GlcNAcT-1 chimeric proteins in Vero cells. Vero cells transfected with cDNA encoding FLAG-GlcNAcT 1 (A and B), FLAG-spacer-GlcNAcT 1 (C and D) were fixed 36 h after transfection and double labeled with monoclonal antibody M2 recognizing the epitopes (A and C) and polyclonal antibody against β-COP I (B and D). The first antibodies were visualized either with secondary antibodies coupled to rhodamine (A and C) or FITC (Band D). Bar=10μm.

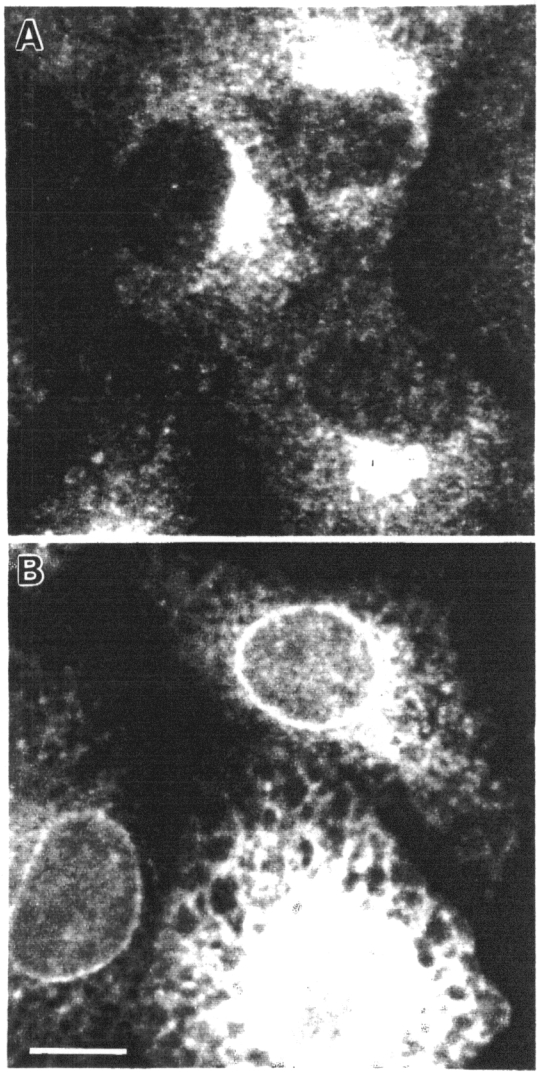


Fig. 3-9. Immunofluorescence localization of myc-GlcNAcT-1 stable transfectants expressed in Vero cells. Vero cells stably transfected with myc-GlcNAcT-1 were fixed, permeabilized, and labeled with mouse monoclonal 9E10 antibody (A and B) and secondary antibodies coupled to rhodamine. A and B showed two different expression patterns from the same transfection experiment. Bar=10μm.

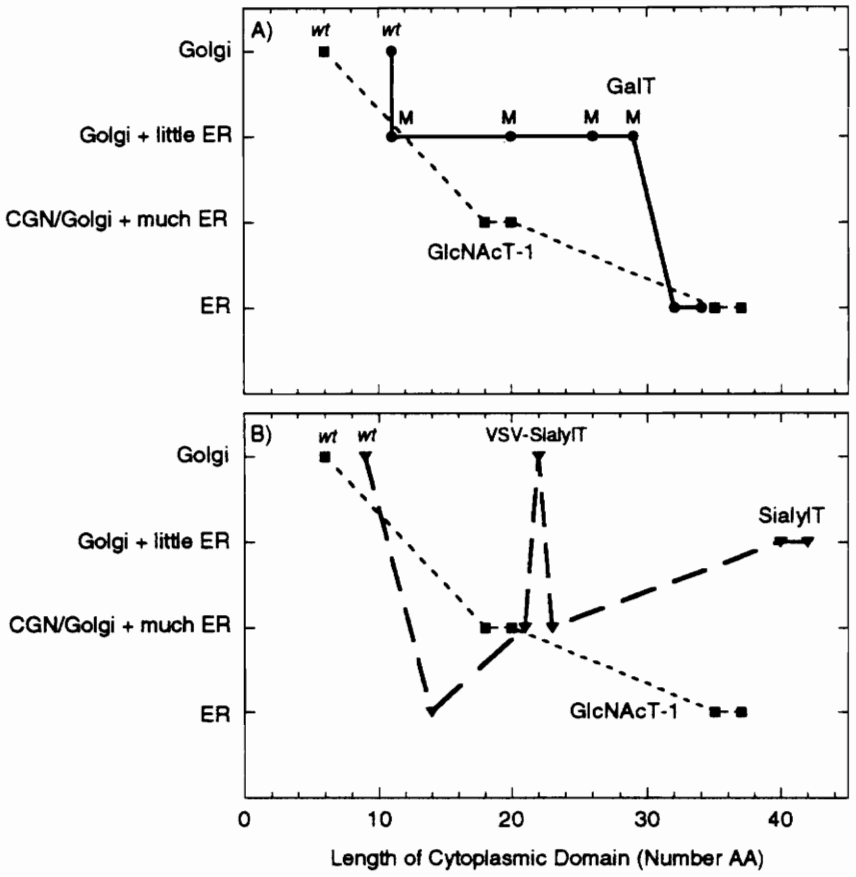


Fig. 3-10. Correlation of the length of cytoplasmic tail for various chimeric proteins with their subcellular localization. (A) Shows the cytoplasmic tail length of GlcNAcT-1 and GalT. (B) Shows the cytoplasmic tail length of GlcNAcT-1 and SialylT. The GlcNAcT-1 data were replotted from A.

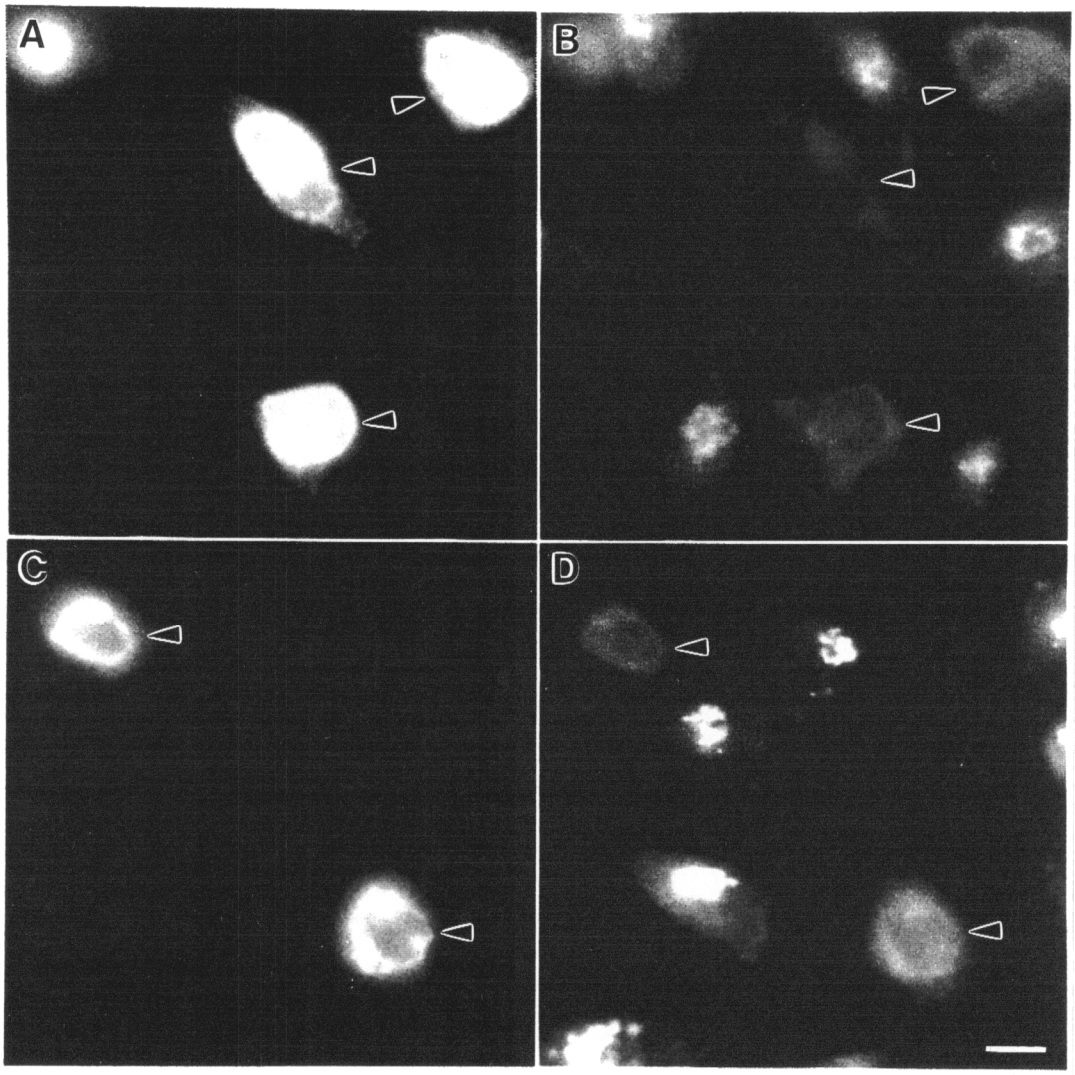


Fig. 3-11. Mislocalization of myc-spacer-GlcNAcT 1 disrupted Golgi accumulation of β-COP I and GalT in Hela cells. Hela cells transfected with cDNA encoding FLAG-spacer-GlcNAcT-1 were fixed 36 h after transfection, either double labeled with 9E10 (A, arrowheads) and polyclonal antibody against β-COP I (B, arrowheads), or double labeled with 9E10 (C, arrowheads) and polyclonal antibody against GalT (D , arrowheads). The first antibodies were visualized either with secondary antibodies coupled to rhodamine (A and C) or FITC (Band D). Bar=10μm.

Chapter 4. General discussion

The goals of this study were: 1) use drug challenge as a tool to describe Golgi subcompartments, and 2) to understand the roles of cytoplasmic tail of three Golgi resident proteins in their targeting. Both aspects contribute to our understanding of structural as well as functional organization of Golgi complex.

4.1. Comparative behavior of Golgi cisternae to drug challenge

The first part of my thesis is to characterize the distribution of Golgi membrane proteins and a CGN membrane protein during Golgi disassembly and reassembly following addition and removal of nocodazole and BFA. Various drugs have been proven to be useful to elucidate the dynamic equilibrium on which Golgi membrane organization is based. Brefeldin A has been shown to inhibit protein secretion and dramatically distributes the Golgi membrane into ER by a mechanism that requires energy and microtubules (Klausner et al., 1992). Nocodazole can progressively depolymerize microtubules and subsequently fragment the Golgi complex (Robbins and Gonatas, 1964). Our approach was to analysis in parallel changes in localization of various cisternal and a cis-Golgi network membrane proteins in response to BFA and nocodazole. We assume that these changes are the result of changes in the organization and integrity of the Golgi compartments in which the proteins reside.

Construction of Vero Cell Lines With Multiple Golgi Markers

The technical success of these double-label experiments relied on two stable Vero cell lines expressing human GlcNAcT 1-myc and SialylT-myc. This overcome the problem of lack of antibodies which recognize multiple cisternae markers in the same specie. The clones selected for this work expressed GlcNAcT-1-myc or SialylT-myc in every cell at approximately equivalent levels, allowing us to quantitatively score the localization

patterns. The monoclonal antibody 9E10 is a high affinity antibody (Evan et al, 1985). It gives a strong signal that is comparable to that of polyclonal antibody against GalT. It has been shown that expression and localization of lumenally tagged Golgi transferase is identical to its endogenous counterpart (Nilsson, 1993a), The ultrastructural localization of the Golgi transferases in Vero cells will be await double label immunogold studies at the electron microscope level.

Disassembly and Reassembly of Golgi After Nocodazole and BFA Treatment Suggest Distinct Compartmentalization of Golgi Complex

With nocodazole treatment, the Golgi disassembly process demonstrated the following characteristics: 1), The trans cisternae/TGN which host GalT and SialylT fragmented into punctate structures much faster than that of medial cisternae marked by GlcNAcT-1. 2), after sufficiently long incubation, GlcNAcT-1 compartment progressively fragmented and targeted to the pre-existing fragments marked by trans cisternae/TGN proteins. 3), CGN marked by p53 was found in a separate set of scattered punctate structures after incubation with nocodazole. With BFA treatment, all the cisternal markers dispersed to ER with similar, but not identical kinetics; GalT lagged behind. In contrast SialylT lagged behind during Golgi reassembly following BFA removal. Our data indicate that CGN, medial-Golgi and trans-Golgi/TGN are distinct subcompartments that can be separated one from the other by drug treatment; any exchange of components between the Golgi subcompartments must be slow with respect to 1-2 h kinetics of Golgi disassembly.

Nocodazole Induced Golgi Fragmentation is Consistent with a Three Compartment Model for the Golgi

Two trans-Golgi/TGN markers, SialylT and GalT, have identical and rapid kinetics for redistribution into scattered punctate structures. The medial-Golgi marker GlcNAcT-1 slowly scatters into these punctate fragments. The CGN marker p53 also scatters rapidly, but it redistributes into a different set of punctate structures. The data suggest a three

compartment organization of Golgi complex cis/CGN, medial, trans/TGN consistent with the model proposed by Mellman and Simons (1992).

Disassembly and Reassembly of Golgi Complex Followed Different Pathway

After nocodazole addition, SialylT and GalT were progressively scattered into punctate structures that initially contained little GlcNAcT-1. GlcNAcT-1 slowly co-localized with SialylT and GalT in the scattered punctate structures after sufficient long time treatment. Following nocodazole removal, all the cisternal markers accumulated concomitantly into a reassembled Golgi. Our data suggest that the fragmentation of the Golgi is not the result of lateral unfolding of intercisternae connecting regions without disrupting Golgi continuity as proposed by Rogalski and Singer (1984). Our results also suggest after nocodazole treatment that the tubular interconnections on which intact Golgi organization is based may be severed and dispersed after microtubule depolymerization in agreement with Tassin et al (Tassin et al., 1985). Golgi fragmentation in response to nocodazole addition is not likely to be a direct consequence of budding processes. The punctate structures observed soon after drug addition are too large to be small vesicles. Moreover, these structures seem to be targeting sites for the gradual accumulation of medial-Golgi. This suggests that the Golgi stacklets observed in electron micrographs of nocodazole treated cells form slowly and progressively with the addition of medial components to trans-Golgi/TGN. Moreover these results imply the existence of a Golgi cisternal recognition mechanism(s) of unknown nature. Golgi reassembly following nocodazole removal is, in contrast, by a different mechanism in which reassembly follows upon movement of punctate Golgi fragments positive for all three cisternal markers to the circumnuclear region.

4.2. Modification of cytoplasmic tail of resident Golgi proteins and their effects to the protein targeting

Central to the study of protein sorting in the eukaryotic secretory pathway is a precise understanding of the signals and mechanisms which allow specific localization of soluble and transmembrane proteins. Previous studies by several groups have narrowed the localization signal for the Golgi retention of GlcNAcT-1, GalT, and SialylT down to their transmembrane domain and part of flanking regions (for review, see Machamer, 1993; Gleeson et al., 1994)). In the second part of my research, the roles of cytoplasmic tail of these three enzymes in Golgi targeting and retention were explored using an epitope tagging approach. Our results showed that addition of more amino acids to the NH_2-terminus cytoplasmic domain of these enzyme would frequently cause the chimeric proteins to accumulate in ER/CGN. The added sequences contain no known targeting sequence. The significance of the data and a possible model of Golgi protein retention will be discussed.

Differential Response of Three Golgi Proteins to the Altered Cytoplasmic Domain Sequences

To determine whether the length of cytoplasmic tails of Golgi protein is important for their Golgi targeting and retention, two types of sequences carrying epitope tags were placed to the NH_2-terminus of three Golgi proteins. One was relative short sequences which were composed of about 10 amino acids, the other was longer and composed of approximately 30 amino acids which included the tag. The three transferases behaved differently in response to cytoplasmic tagging. GlcNAcT-1 was most sensitive to the lenghtened cytoplasmic tail. Placing the short sequences on the NH_2- terminal cytoplasmic domain moderately affected its Golgi localization and portion of molecules mislocated to CGN/ER, the long sequence placement accumulated almost all of chimeric GlcNAcT-1 in ER. On the contrary, SialylT could tolerate long sequences quite well. Its cytoplasmic tail

could be lengthened four fold and a significant portion of the protein still accumulated in a perinuclear, Golgi-like distribution. Finally, the behavior of GalT seems somewhat in between SialylT and GlcNAcT-1.

The Mislocalization and the Amino Acid Composition of Tags.

Most of the tags used in this study more or less mislocated Golgi proteins in ER/CGN. By comparing their amino acid composition and the severeness of mislocalization effect, some clues on mislocalization effects were revealed. Comparing the subcellular localization of a series chimeric proteins with similar tail length: myc-SialylT, FLAG-SialylT, and VSV-SialylT, VSV-SialylT gave a normal perinuclear Golgi-like localization while neither FLAG-SialylT nor myc-SialylT did. The obvious difference of amino acid composition is that VSV tag is essentially neutral, while both myc and FLAG epitopes are highly negative charged. Considering that cytoplasmic domains of wild type transferases are positively charged with the positive charge concentrated near to the membrane, we speculate that neutral or positively charged epitopes should have less effect on a Golgi protein's normal localization.

Possible Explanation for Mislocation By Cytoplasmic Tagging

Regarding possible mechanisms responsible for retention of Golgi protein, current opinion favors that when Golgi proteins arrive at correct cisternae they will form huge oligomers by the interaction of transmembrane and flanking region. The resulting oligomer can not enter transport vesicles resulting in their Golgi protein retention (Nilsson et al, 1993b; 1994). Oligmerization is mediated by regions within and around the transmembrane domain; the cytoplasmic domain may supply further attractive forces including hydrogen binding and ionic interactions. It is possible that many of added sequences could supply some of the bonding contributions needed to cause the chimeric protein to pre-maturely oligomerize in early compartments in the secretory pathway including ER and CGN. This

suggests that the newly synthesized chimera has never leave the ER or CGN, along with the observation that Iip33-GlcNAcT-1 never leaves the ER (Nilsson et al., 1994)

Proposal for Future Experiments to Evaluate the Suggested Retention Model

There is no direct evidence showing that Golgi proteins form oligomers within cisternae under *in vivo* conditions. We will use a promising approach to study this problem: *in situ* mobility measurement of the Golgi protein. As predicted by kin-oligomerization or insoluble aggregation models, the mobility of the protein will be significantly reduced after protean lattice formation. One of 17 our constructs, VSV-SialylT, showed a normal Golgi localization. This chimera should be a useful protein for studying the in vivo dynamics of the Golgi as a resident marker; it should be accessible to microinjected fluorescent Fab antibody fragments as a reporter group. It can be used as a molecular tool through which the mobility of Golgi proteins can be measured. This construct will be stably expressed in Vero cell by a tetracycline inducible expression system. After microinjection of rhodamine labeled P5D4 Fab fragment, mobility of protein can be measured as fluorescence recovery after LASER bleach or by diffusion photoactivation. The mobility of this resident Golgi protein will be compared with that of transient Golgi protein, VSV-G (Storrie et al, 1994). The result will definitely contribute to our understanding on the Golgi retention mechanisms.

4.3. References

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Publication

1, Storrie, B., W. Yang, E.H.K. Stelzer, and T.E. Kreis. 1993. The mobility of a model intracellular transmembrane protein (ts-O45-G) can be assessed by fluorescence recovery after photobleaching. Mol.Biol.Cell4, 203a (abstract).

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3,W Yang, R. Pepperkok, P. K. Bender, T. E. Kreis and B. Storrie. Alterations in the Cytoplasmic Domain of Golgi Type II Membrane Proteins, by an Epitope Tagging Approach, Often Result in Their Accumulation in ER(in preparation)