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Uptake and Incorporation of an Epitope-tagged Sialic Acid Donor into Intact Rat Liver Golgi Compartments. Functional Localization of Sialyltransferase Overlaps with β-Galactosyltransferase but not with Sialic Acid *O*-Acetyltransferase

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The transfer of sialic acids (Sia) from CMP-sialic acid (CMP-Sia) to N-linked sugar chains is thought to occur as a final step in their biosynthesis in the *trans* portion of the Golgi apparatus. In some cell types such Sia residues can have O-acetyl groups added to them. We demonstrate here that rat hepatocytes express 9-O-acetylated Sias mainly at the plasma membranes of both apical (bile canalicular) and basolateral (sinusoidal) domains. Golgi fractions also contain 9-O-acetylated Sias on similar N-linked glycoproteins, indicating that O-acetylation may take place in the Golgi. We show here that CMP-Sia-FITC (with a fluorescein group attached to the Sia) is taken up by isolated intact Golgi compartments. In these preparations, Sia-FITC is transferred to endogenous glycoprotein acceptors and can be immunochemically detected in situ. Addition of unlabeled UDP-Gal enhances Sia-FITC incorporation, indicating a substantial overlap of β-galactosyltransferase and sialyltransferase machineries. Moreover, the same glycoproteins that incorporate Sia-FITC also accept [^{3}H]galactose from the donor UDP-[^{3}H]Gal. In contrast, we demonstrate with three different approaches (double-labeling, immunoelectron microscopy, and addition of a diffusible exogenous acceptor) that sialyltransferase and *O*-acetyltransferase machineries are much more separated from one another. Thus, 9-O-acetylation occurs after the last point of Sia addition in the *trans*-Golgi network. Indeed, we show that 9-O-acetylated sialoglycoproteins are preferentially segregated into a subset of vesicular carriers that concentrate membrane-bound, but not secretory, proteins.

INTRODUCTION

N-linked glycan biosynthesis is a complex process occurring in an ordered manner in defined compartments of the endoplasmic reticulum (ER) and Golgi apparatus (Kornfeld and Kornfeld, 1985; Griffiths and Simons, 1986; Roth, 1991). Compartmental organization of the Golgi apparatus has been described as a function of the glycosidases and glycosyltransferases contained in different cisternae (Goldberg and Kornfeld, 1983; Quinn *et al.*, 1983; Dunphy and Rothman, 1985; Griffiths and Simons, 1986). For example, the N-linked sugar chain-processing enzyme α-mannosidase I is located predominantly in the *cis*-portion of the Golgi in most cells (Balch *et al.*, 1986), α-mannosidase II and *N*-acetyl-glucosaminyltransferase I are

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more concentrated in the *medial* Golgi (Dunphy *et al.*, 1985; Velasco *et al.*, 1993; Burke *et al.*, 1994), and GlcNAc:β-galactosyltransferase and sialyltransferase(s) are usually considered to be in the *trans*-Golgi and *trans*-Golgi network, respectively (Roth *et al.*, 1985; Griffiths and Simons, 1986; Roth *et al.*, 1986; Chege and Pfeffer, 1990). In some cell types, there is evidence for significant overlap of these localizations (Roth *et al.*, 1986; Mellman and Simons, 1992; Hayes *et al.*, 1993; Hayes and Varki, 1993b; Nilsson *et al.*, 1993; Velasco *et al.*, 1993). Moreover, although in situ immunodetection of specific glycosyltransferases is useful to subdivide Golgi compartments, it is not sufficient for predicting their biosynthetic capabilities (Bergeron *et al.*, 1982; Hayes *et al.*, 1993). As shown in Figure 1, a fully functional Golgi compartment must contain not only the glycosyltransferase but also the suitable glycoconjugate acceptors, as well as specific endogenous transporters to take up and concentrate activated nucleotide donors (Bergeron *et al.*, 1982; Hirschberg and Snider, 1987; Milla and Hirschberg, 1989; Abeijon and Hirschberg, 1990; Hayes *et al.*, 1993). In addition, endogenous sugar nucleotide-degrading enzymes can regulate the internal pools of the donor (Martina *et al.*, 1995). Thus, specific transferases, nucleotide donors, and glycoconjugate acceptors coexisting in a Golgi compartment comprise the basic elements of the glycosylation machinery (see Figure 1). However, relatively little is known about the distribution of factors other than the transferases. We have recently shown that the coordinated action of these components of the glycosylation machinery can be recapitulated in in vitro "freeze-frame" reactions, which are initiated by labeling the endogenous glycoprotein acceptors in rat liver Golgi preparations with radioactive nucleotide donors (Hayes *et al.*, 1993; Hayes and Varki, 1993a,b). In these preparations, intercompartmental transport is absent, and the glycosylation reactions mediated by the exogenously added donors take place primarily in sealed compartments of the correct topological orientation. Thus, nonfunctional compartments or other subcellular contaminants do not interfere.

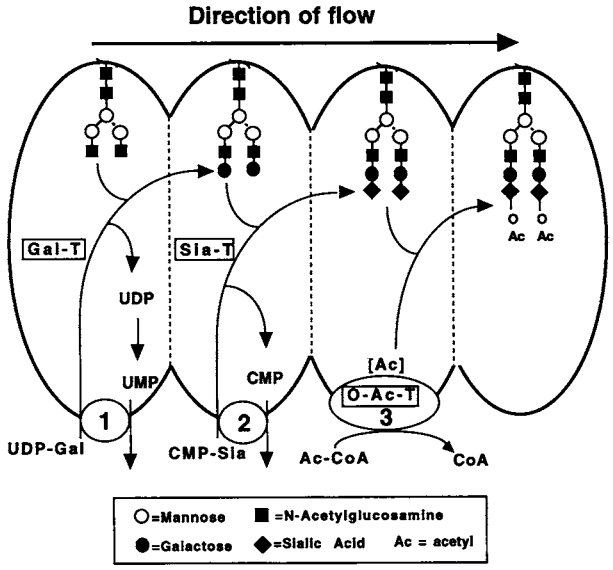

Figure 1. Stepwise addition of galactose, sialic acid, and O-acetyl groups to N-linked sugar chains on newly synthesized membrane glycoproteins in compartments of the Golgi apparatus. UDP-Gal and CMP-Sia are imported from the cytoplasm into Golgi compartments by antiport mechanisms (1 and 2), where they are concentrated. Within the Golgi lumen, transfer of Gal or Sia can take place only if the appropriate glycosyltransferases—galactosyltransferase (Gal-T) and sialyltransferase (Sia-T)—and the correct endogenous acceptors are available. O-acetylation of sialic acids occurs by transmembrane transfer of acetyl groups donated from acetyl-CoA by *O*-acetyltransferase(s) (3, *O*-Ac-T), which transfer the acetyl group to sialic acids at both 7- and 9- positions. Each of these reactions can be recapitulated in vitro by adding labeled nucleotides to isolated intact Golgi preparations. The dotted lines emphasize the question addressed in this study: Do the compartments mediating these reactions overlap functionally?

Sialic acids (Sia)[1] are a family of negatively charged sugars, usually found at the terminal position in animal glycans (Varki, 1992). Sialylation is usually considered the final step in the glycosylation of N- and O-linked oligosaccharides and is believed to occur either in the *trans*-cisternae or in the *trans*-Golgi network (TGN; Roth *et al.*, 1985, 1986). However, Sia residues can be further modified by the addition of O-acetyl groups, most commonly at the 9-position of the side chain (Varki, 1992). These modifications of sialic acids can affect a variety of biological phenomena, including complement activation (Varki and Kornfeld, 1980), viral pathogenesis (Schultze and Herrler, 1994; Zimmer *et al.*, 1995), embryonic development (Blum and Barnstable, 1987; Varki *et al.*, 1991), and the formation of tumor antigens (Cheresh *et al.*, 1984). With the development of improved analytical techniques (Schauer, 1987; Manzi *et al.*, 1990a) and better probes for their detection (Klein *et al.*, 1994; Zimmer *et al.*, 1994), modifications of Sia were more widespread than previously thought (Klein *et al.*, 1994). For example, we have shown that α2-6-linked Sia residues on N-linked chains of rat liver glycoproteins carry extensive O-acetylation at the 7- and 9-positions (Butor *et al.*, 1993).

O-acetyl residues are first transferred to Sias that are already incorporated into N-linked chains (Butor *et al.*, 1993). The *O*-acetyltransferase(s) can be detected in vitro by labeling endogenous acceptors of rat liver

[1] Abbreviations used: CHE-Fc, chimeric protein made of InfCHE (Influenza C hemagglutinin-esterase with the fusion peptide eliminated by mutation) and the Fc portion of human immunoglobulin (Ig)G1; CHE-FcD, diisopropyl fluorophosphate (DFP)-treated CHE-Fc (esterase activity irreversibly inactivated); FITC, fluorescein isothiocyanate; Gal, galactose; pIgA-R, polymeric Ig-A receptor; Sia, sialic acid.

Golgi preparations with [^{3}H]acetyl-CoA. [^{3}H]Acetyl groups seem to be donated to a transmembrane intermediate on the cytosolic face of the Golgi, which then transfers the groups to a nascent sialoglycoprotein on the luminal side (Higa *et al.*, 1989). The polypeptide(s) responsible for this transfer reaction have so far resisted all attempts at purification or molecular cloning. Thus, the subcellular location of O-acetylation remains unknown. Sialylation can also be recapitulated in vitro in the same intact cytosol-free rat liver Golgi preparations by adding CMP-[^{3}H]Sia (Hayes and Varki, 1993b). However, autoradiographic detection of the incorporated tritium does not have the resolution necessary for precise morphological localization of the reactions. Here, we use a fluoresceinated derivative of CMP-sialic acid (Gross and Brossmer, 1988) to follow the sialylation reaction and a recently described recombinant probe derived from the Influenza C hemagglutinin-esterase (Klein *et al.*, 1994) to detect 9-O-acetylated sialoglycoproteins. By observing the outcome of sialylation and O-acetylation on both endogenous and exogenous acceptors, we demonstrate that the two reactions show a substantial functional segregation. Furthermore, we show that 9-O-acetylation of Sias is preferentially found on membrane-bound glycoproteins. This population of proteins is known to be transported from the *trans*-Golgi network to the plasma membrane by distinct vesicular carriers (Saucan and Palade, 1994).

MATERIALS AND METHODS

Materials

CMP-9-fluoresceinyl-Neu5Ac (CMP-Sia-FITC) was obtained from Boehringer Mannheim (Indianapolis,IN). Unless otherwise stated, all other nonradioactive chemical reagents were purchased from Sigma (St. Louis, MO). CMP-[^{3}H]Sia (10 Ci/mmol), CMP-[^{14}C]Sia (2.5 Ci/mmol), and [*acetyl*-^{3}H]acetyl-CoA (2.28 Ci/mmol) were synthesized as previously described (Higa and Paulson, 1985; Manzi *et al.*, 1990b). UDP-[^{3}H]galactose (20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The chimeric protein made of InfCHE (Influenza C hemagglutinin-esterase with the fusion peptide eliminated by mutation; CHE-Fc) recombinant chimeric molecule was produced by stably transfected 293 cells and purified as described elsewhere (Klein *et al.*, 1994). After purification, an aliquot of CHE-Fc was routinely inactivated with 1 mM of diisopropyl fluorophosphate (DFP) to give DFP-treated CHE-Fc (esterase activity irreversibly inactivated; CHE-FcD), a probe specific for 9-O-acetylated sialic acids. Monospecific antibodies against α-mannosidase II (Man II; Velasco *et al.*, 1993) and the polymeric-IgA receptor (Saucan and Palade, 1994) have been previously described and characterized. Anti-rat α2-6sialyltransferase was a kind gift from Dr. K. Colley (University of Chicago, IL).

Isolation of Golgi Apparatus-enriched Subcellular Fraction

Golgi compartments from rat liver were purified by following Leelavathi's procedure (Leelavathi *et al.*, 1970), as modified by Tabas and Kornfeld (Tabas and Kornfeld, 1979). The Golgi-enriched fraction (typically 50–100-fold enriched for β-galactosyltransferase activity) was collected from the interface between 1.0 and 0.5 M sucrose solutions of the second gradient step. This preparation was used without further washing after serial dilution with maleate buffer to the range of 0.6 M of sucrose, which was optimal for both biochemical and ultrastructural studies, including preservation of cisternal morphology. The final preparation was also routinely treated with 1 mM DFP (Muchmore and Varki, 1987) to inactivate endogenous O-acetylesterases.

Incorporation of Labeled Sialic Acid into Endogenous Acceptors

Golgi-enriched fractions were incubated at room temperature (RT) with 1 μM of either radiolabeled CMP-[^{3}H]-Sia or CMP-[^{14}C]-Sia or with CMP-Sia-FITC in the presence or absence of indicated concentrations of either unlabeled (10–30 μM) or radioactive (0.01–2.0 μM) acetyl-CoA or UDP-Gal. After 15 min, the incubations were chased by the addition of 20 μM of unlabeled CMP-Sia. To examine whether transport of donors and incorporation of Sia was occurring in sealed Golgi compartments, similar studies were done in the presence of 0.1% Triton X-100 (Hayes *et al.*, 1993). After incubation, aliquots of the Golgi-containing mixtures were centrifuged at 100,000 × *g* for 30 min, the supernatant was discarded, and the pellets containing Golgi membranes were either prepared for immunoelectron microscopy of FITC-labeled sialoglycoconjugates (see below) or solubilized in lysis buffer (Triton X-100 1% in PBS pH 7.4 containing 1 mM EDTA, 50 μg/ml leupeptin, and 4 μg/ml pepstatin) or sonicated in water and precipitated with 4% perchloric acid for determining incorporation of radiolabel into endogenous sialoglycoproteins.

Immunochemical Assays for FITC-labeled Sialoglycoconjugates

Conventional immunochemical assays such as ELISA, immunoprecipitations, immunodepletion, and Western blots for the FITC-labeled sialoglycoconjugates were done with a monospecific rabbit polyclonal antibody directed against FITC (Molecular Probes, Eugene, OR). For ELISA assays, equal amounts of proteins were used to coat 96-well plates (Costar, Cambridge, MA) overnight at 4°C, and blocking was done with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) for 30 min at RT. After incubation with anti-FITC (1:500 in PBS containing 1% BSA) for 1 h at RT, the wells were washed and incubated with EIA-grade horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Ig; Bio-Rad, Hercules, CA) at 1:1000 in PBS containing 1% BSA for 1 h at RT. After washing further, the reaction was developed with *o*-phenylenediamine (0.5 mg/ml) in 0.1 M citrate/phosphate buffer, pH 5.5, and 0.03% of hydrogen peroxide. The reaction was quenched with 8 M H_2SO_4, methanol was added to obtain a homogeneous liquid phase, and the color was read in an ELISA plate reader with a 492 nm filter. For immunoprecipitation/immunodepletion assays, Golgi preparations incubated with CMP-Sia-FITC and either [acetyl-^{3}H]Ac-CoA or UDP-[^{3}H]galactose were extracted by lysis buffer as described above, and 0.5 ml aliquots were incubated overnight at 4°C with a 1:500 dilution of the anti-FITC antibody (or a control rabbit serum). Immune complexes were then purified batch-wise with 50 μl of protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). This immunoprecipitation was repeated three more times sequentially. Aliquots of 30 μl were removed from the supernatants of each round of the immunodepletion procedure and monitored for radioactivity by liquid scintillation counting. Western blots were developed with suitable alkaline phosphatase-conjugated secondary antibody (Bio-Rad) and nitro blue tetrazolium/bromo-chloroindolyl phosphate (Life Technologies, Grand Island, NY) as a chromogenic substrate.

Immunofluorescence Studies

Rat liver was perfused with 4% formaldehyde (freshly prepared from paraformaldehyde) contained in 100 mM phosphate buffer, pH

7.4. The liver was then removed, minced into 1 mm^3 pieces, and fixed by immersion in 4% formaldehyde for 15 min, followed by fixation in 8% formaldehyde for an additional 45 min. The tissue was then washed briefly in 100 mM phosphate buffer pH 7.4, trimmed and immersed in 2.3 M sucrose containing 20% polyvinylpyrrolidone (10,000 molecular weight), mounted on cryopins, and frozen in liquid N_2. Semithin cryosections (0.5–1.0 μm) were then cut on a Reichert Ultracut E ultramicrotome, equipped with an FC-4 cryostage, and incubated in CHE-FcD probe, followed by a goat anti-human FITC conjugate (Jackson Immunoresearch, West Grove, PA).

Immunoelectron Microscopy

Golgi-enriched preparations, incubated as previously described, were fixed at 4°C by adding 8% formaldehyde contained in 100 mM phosphate buffer pH 7.4 (vol/vol) for 15 min, after which fractions were pelleted at 13,000 × *g* and resuspended for an additional 45 min in 8% formaldehyde. The material was then repelleted, resuspended in 1% agarose, trimmed, cryoprotected, mounted on cryopins, and frozen in liquid N_2 as described above. Ultrathin cryosections (<80 nm) were cut, mounted on Formvar/carbon-coated 400-mesh nickel grids, and stained as follows. After blocking in 10% fetal calf serum (FCS), the grids were floated in 10-μl drops of the primary antibodies (polyclonal anti-FITC, anti-Man II, or anti-sialyltransferase), followed by colloidal gold (5 nm)-conjugated goat anti-rabbit IgG. Some sections were also incubated with the CHE-FcD probe and followed by goat anti-human IgG-gold (10 nm) conjugates. After washing, the grids were post-embedded in a mixture containing 0.2% uranyl acetate, 0.2% methylcellulose, and 3.2% polyvinylalcohol (10,000 molecular weight) and observed in either a JOEL 1200EX-2 or Philips CM-10 transmission electron microscope.

Incorporation of Sialic Acids into Exogenous Acceptors

4-methylumbelliferylβ-lactoside (4-MU-lactose) was used as an acceptor for sialyltransferases in Golgi compartments. 4-MU-lactose was dissolved in dimethyl sulfoxide (DMSO; stock solution 50 mM) and incubated at increasing concentrations in the range of 0.5–10 mM with suspensions containing Golgi compartments and 1 μM CMP-[^{3}H]-Sia for 15 min at RT (incubation volumes varied from 0.5 ml to 1.0 ml). After a chase with unlabeled CMP-Sia as described above, maleate buffer was added to a final volume of 5 ml, and the suspension was centrifuged at 100,000 × *g* for 30 min. The supernatant was discarded, and the pellet containing Golgi membranes was disrupted into 70% ethanol with a sonicator. The residue was pelleted by centrifugation at 13,000 rpm for 10 min, and the soluble material containing labeled products was dried with a shaker evaporator. This was resuspended in deionized water and applied to a C_{18} SPICE cartridge (Analtech, Newark, DE) prewashed with methanol, distilled water, and 0.1 M NH_4COOH, pH 6, to purify the labeled 4-MU-derivatives, as described (Etchison *et al.*, 1995). Material bound and eluted from the cartridge was then analyzed in a C_{18} reverse-phase high performance liquid chromatography (HPLC) on a 4.6 × 25 cm Microsorb MV-C_{18} column (Rainin, Emeryville, CA). The column was eluted isocratically with 0.1 M NH_4COOH pH 6 for 5 min, followed by a 0–50% methanol gradient in the same buffer over 45 min at a flow rate of 1.0 ml/min. Fractions (1 ml) were collected and radioactivity was monitored by liquid scintillation counting. In some experiments, the radiolabeled 4-MU derivatives were purified for further analysis of the labeled Sias, which were released by either sialidase treatment or mild acid hydrolysis with 2 M acetic acid at 80°C for 2 h (Manzi *et al.*, 1990a). Sialidase treatment was done with either *Arthrobacter ureafaciens* sialidase (Calbiochem, La Jolla, CA) or Newcastle disease virus sialidase as described (Powell and Varki, 1996). Released Sias were purified by reapplication to a C_{18} SPICE cartridge, which no longer retained the label. Free sialic acids in the run-through fractions were dried, resuspended in 20 μl of distilled water, treated or not with 0.1 M NaOH at 37°C for 15 min for de-O-acetylation, and separated in a descending paper chromatography system with Whatman 3 MM paper eluted with pyridine:ethyl acetate:acetic acid:water (5:5:1:3) for 16 h. In this system, Neu5Ac separates from $Neu5,9Ac_2$ and other modified sialic acids. After the run, strips of 1 cm were cut and soaked in 400 μl of water, and radioactivity was monitored by liquid scintillation counting.

Immunoisolation of Vesicular Carriers

Rat liver Golgi light fractions were processed through immunoisolation with magnetic beads coated with an antibody against the carboxy terminus of the polymeric IgA receptor (pIgA-R), essentially as described (Saucan and Palade, 1994). Starting material, nonbound, and bound fractions were solubilized in Laemmli sample buffer, separated in SDS-polyacrylamide gels, transferred to nitrocellulose, and analyzed by means of Western blots with an anti-pIgA-R, *Sambucus nigra* agglutinin (SNA lectin), and CHE-FcD, as described above. Protein loading was done in such a way that the amount studied from the starting material was the sum of both bound (~10-20% of total protein) and nonbound (~80-90% of total protein) fractions, as described (Saucan and Palade, 1994).

RESULTS

Rat Hepatocytes Express 9-O-Acetylated Sialic Acids at both Apical and Basolateral Plasma Membranes

We recently described a recombinant soluble probe (Klein *et al.*, 1994) derived by fusing the extracellular domain of the Influenza C-hemagglutinin esterase to the hinge and Fc portion of the human IgG heavy chain (CHE-Fc). This probe retains both the esterase and hemagglutinin functions of the viral glycoprotein, which is known to recognize and degrade O-acetyl groups on 9-O-acetylated sialic acids, regardless of their underlying linkage (Herrler *et al.*, 1985; Rogers *et al.*, 1986). Irreversible inactivation of the esterase with diisopropyl fluorophosphate (DFP) gives the derivative CHE-FcD, which retains the hemagglutinin activity. This molecule can be used to detect 9-O-acetylated sialic acids on tissue sections and Western blots of glycoproteins (Klein *et al.*, 1994). The unmodified CHE-Fc molecule can be used as a nonbinding control for the CHE-FcD probe, because the esterase activity is dominant at RT. Using the CHE-FcD probe for immunohistochemistry on cryostat sections at the light microscopic level, we previously showed that rat liver sections showed strong staining. However, the relative distribution of the 9-O-acetylation among the various cell types could not be determined precisely (Klein *et al.*, 1994). When semithin sections of rat liver were probed with CHE-FcD and a fluorescent secondary antibody, staining seemed to be concentrated primarily at the plasma membrane of hepatocytes, mainly at the level of basolateral (sinusoidal) domains (see examples in Figure 2, A and B). A weaker reactivity with CHE-FcD could also be seen

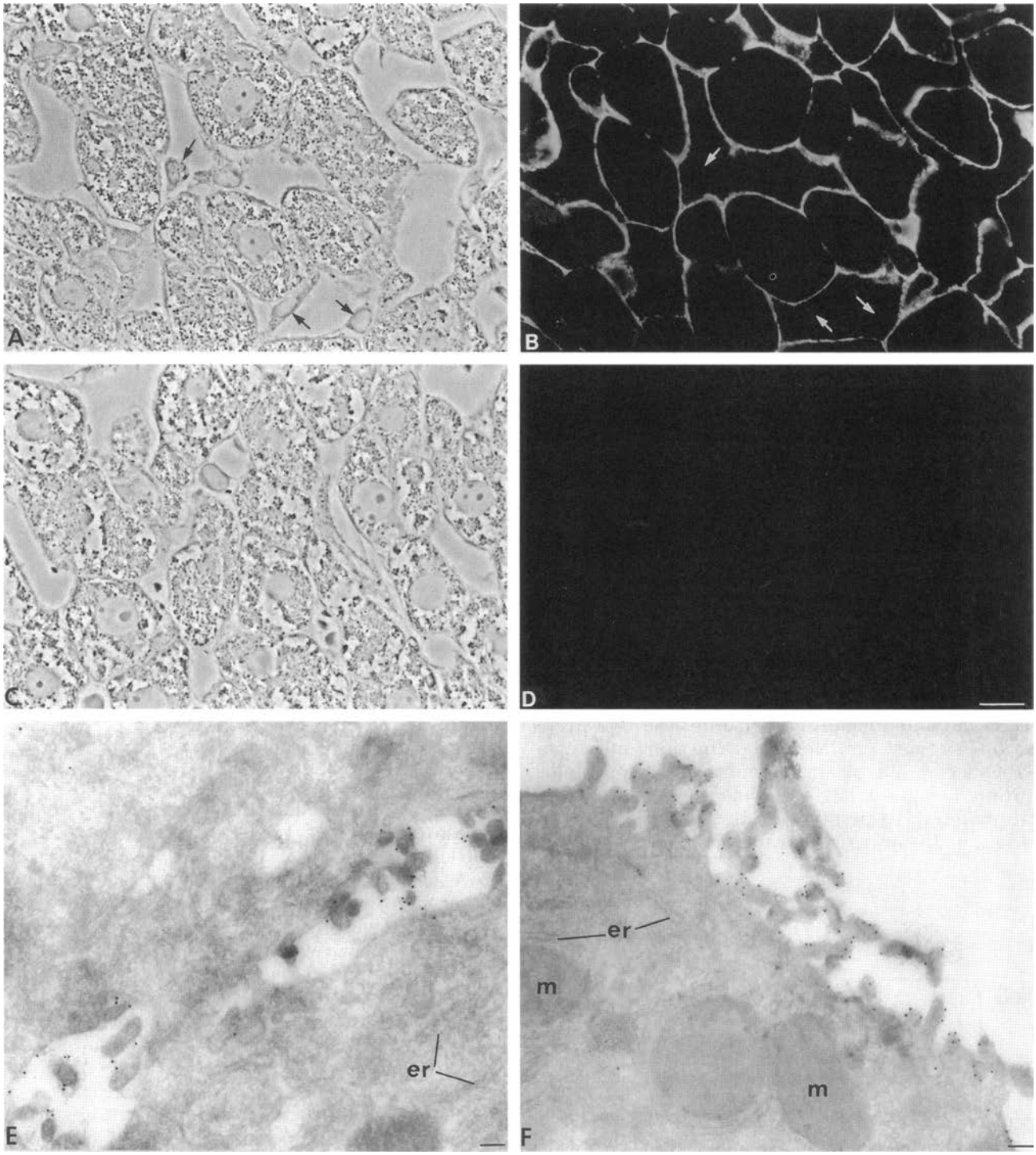

Figure 2. Rat hepatocytes express 9-O-acetylated sialic acids predominantly at both apical and basolateral plasma membrane domains. Immunofluorescence of semithin 0.5–1.0-μm cryosections of rat liver with a probe for 9-O-acetylated sialic acids (CHE-FcD) and its control (CHE-Fc) are shown in B and D, respectively. Phase contrasts of the fields analyzed are illustrated in A and C, respectively. Note that endothelial or Kupffer cells did not stain with CHE-FcD (arrows in A and B). Immunoelectron microscopy of ultrathin cryosections of rat liver that used CHE-FcD showed the reactivity at the membrane level on both apical, bile canalicular domains (E) and basolateral, sinusoidal domains (F). Scale bars, 10 μ (A–D) or 0.1 μ (E and F); er, endoplasmic reticulum; m, mitochondria.

at the level of apical (bile canalicular) domains by means of immunofluorescence. Endothelial and Kupffer cells were not reactive (arrows in Figure 2, A and B). The specificity of the labeling for 9-O-acetylated sialic acids was confirmed by the lack of signal with CHE-Fc (Figure 2, C and D). The presence of 9-O-acetylated sialoglycoconjugates in both apical (Figure 2E) and basolateral (Figure 2F) domains was confirmed by immunoelectron microscopy with gold-labeled secondary antibodies. Golgi elements within the cells were not easily visualized in most sections, presumably because of their relatively small fractional volume relative to cell size.

O-Acetylated Glycoproteins Are Also Present in Golgi-Enriched Preparations from Rat Liver

Because hepatocytes constitute most of the cell mass of the rat liver (~90%; Blouin *et al.*, 1977), Golgi preparations from this organ primarily represent the biosynthetic capabilities of this cell type. We studied preparations highly enriched in Golgi cisternae-like elements (confirmed by electron microscopy [EM]). Several glycoproteins from these preparations carried 9-O-acetylated sialic acids on N-linked oligosaccharides (Figure 3). The bands stained by the CHE-FcD probe are similar to those detected in two denser fractions from the same preparations, which are partially enriched in plasma membranes, as judged by 5′-nucleotidase activity. The qualitative similarities in the profile of 9-O-acetylated sialoglycoproteins from these two preparations suggest that the Golgi fractions may contain O-acetylated plasma membrane glycoproteins en route to the cell surface. Thus, these Golgi preparations seem to be appropriate model systems to study the biosynthetic intermediates in this process.

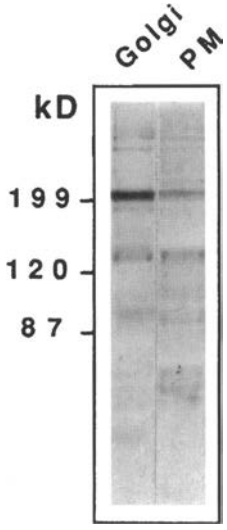

Figure 3. Golgi preparations from rat liver contain 9-O-acetylated sialic acids on proteins similar to those found in the plasma membranes. Rat liver crude smooth membranes were fractionated in a discontinuous sucrose gradient, as described in MATERIALS AND METHODS. Golgi membrane proteins (Golgi) and a heavier fraction enriched in plasma membrane proteins (PM) were separated in 7.5% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and probed with CHE-FcD to identify 9-O-acetylated sialoglycoproteins. Each lane contains 100 μg of protein from each fraction.

The question also arises whether the secretory glycoproteins produced by the same Golgi apparatus are also targets for O-acetylation.

FITC-labeled Sialic Acid Is Incorporated into Glycoconjugates in Sealed Golgi Compartments

In earlier studies, incubating identical Golgi preparations with CMP-[^{3}H]Sia or [^{3}H]Ac-CoA, the labeled products were found exclusively in sealed compartments (Hayes *et al.*, 1993). However, the resolution of tritium autoradiography is insufficient to determine morphologically whether both reactions had taken place in the same compartments. Substitutions at the 9- position of the CMP-Sia do not affect the ability of some sialyltransferases to use it as a donor for sialylation reactions (Gross and Brossmer, 1988). For example, the modified nucleotide CMP-9-fluoresceinyl-Neu5Ac (CMP-Sia-FITC) was shown to be capable of donating FITC to endogenous glycoproteins in permeabilized CHO cells (Gross, 1992). We reasoned that if CMP-Sia-FITC were transported correctly into the lumen of isolated rat liver Golgi compartments, it could act as a donor for sialylation of endogenous glycoprotein intermediates. The sites of incorporation of Sia-FITC could then be identified with a polyclonal monospecific antibody directed against FITC.

We first optimized conditions to allow for both immunocytochemical and biochemical studies. Golgi stacks were found by EM to be well-maintained at a sucrose concentration of 0.6 M, which is close to the initial concentration obtained when the fresh preparations are removed directly from the final sucrose step gradient. Golgi preparations were therefore adjusted to 0.6 M sucrose with maleate buffer for all assays. Under these conditions, uptake and incorporation of CMP-[^{3}H]Sia or [^{3}H]acetyl-CoA did not vary significantly from that obtained at lower sucrose concentrations (our unpublished results). The efficiency of [^{3}H]Sia incorporation into glycoproteins was typically ~4.0-5.0%. As shown in Figure 4, A and B, permeabilization of the Golgi compartments by previous exposure to Triton X-100 caused a decrease in sialylation reactions with either CMP-[^{3}H]Sia or CMP-Sia-FITC as donors. Thus, as with the [^{3}H]Sia donor, Sia-FITC incorporation occurs optimally in sealed Golgi compartments of correct topological orientation. In addition, incorporation could be inhibited in each case by an excess of CMP-Sia (10 μM; Figure 4). Thus, like CMP-[^{3}H]-Sia, CMP-Sia-FITC is efficiently transported across Golgi membranes and concentrated within intact compartments, and the Sia-FITC can be incorporated into nascent glycoconjugates by endogenous sialyltransferases. The internal pool of transported CMP-Sia-FITC is then washed away during the sectioning and processing of the samples, allowing immuno-

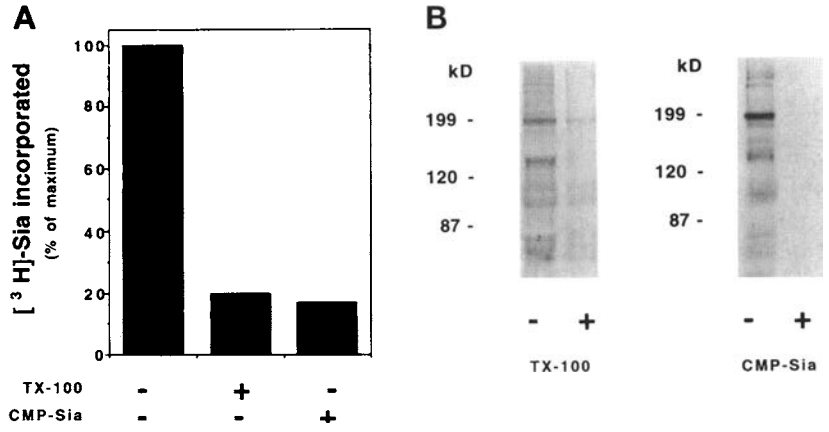

Figure 4. Sia-FITC is optimally incorporated into endogenous glycoproteins in intact Golgi preparations. Intact Golgi preparations were incubated with either CMP-[^{3}H]Sia (A) or CMP-Sia-FITC (B). Labeled sialoglycoproteins were analyzed either by liquid scintillation counting (A) or Western blotting with anti-FITC antibodies (B). Incubation of Golgi compartments in the presence (+) or absence (−) of TX-100 (0.1%) allowed analysis of Golgi intactedness on Sia incorporation. For both CMP-[^{3}H]Sia (A) or CMP-Sia-FITC (B), sialylation was optimal in intact Golgi compartments, indicating that both donors are transported into and concentrated in Golgi. Sialylation reactions with both donors were competed out by an excess of unlabeled CMP-Sia, indicating their specificity.

chemical detection of the products of the sialylation reaction (Sia-FITC covalently incorporated into glycoproteins) and their subcellular localization. This can be compared with other related reaction products, such as O-acetylation, with the use of appropriate probes.

Functional Colocalization of β-Galactosyltransferase and Sialyltransferase, but not O-Acetyltransferase, in Rat Liver Golgi Fractions

The endogenous acceptors for sialylation in these Golgi preparations are known to be nascent glycoproteins with terminal β-linked Gal residues on N-linked chains (see Figure 1). When UDP-Gal is added to such preparations, it undergoes uptake and incorporation, resulting in the generation of additional β-Gal residues, presumably on endogenous glycoproteins carrying terminal GlcNAc residues (see Figure 1). If such residues are generated in Golgi compartments that are also competent for sialylation, this should result in an enhanced availability of acceptors. The addition of increasing concentrations of UDP-Gal to the Golgi preparation enhanced Sia-FITC incorporation from CMP-Sia-FITC (our unpublished results). We next analyzed the newly synthesized sialoglycoconjugates by immunoprecipitation with anti-FITC antibodies, followed by Western blot analysis with the same antibody. Addition of UDP-Gal also led to the appearance of some additional acceptors for Sia-FITC, as indicated by the comparison of immunoprecipitated sialoglycoproteins (Figure 5A, which depicts a single round of immunoprecipitation with anti-FITC antibodies). The efficiency of anti-FITC immunoprecipitation in the first round may depend not only on the presence of FITC but also on how it is presented in a particular molecule. This could

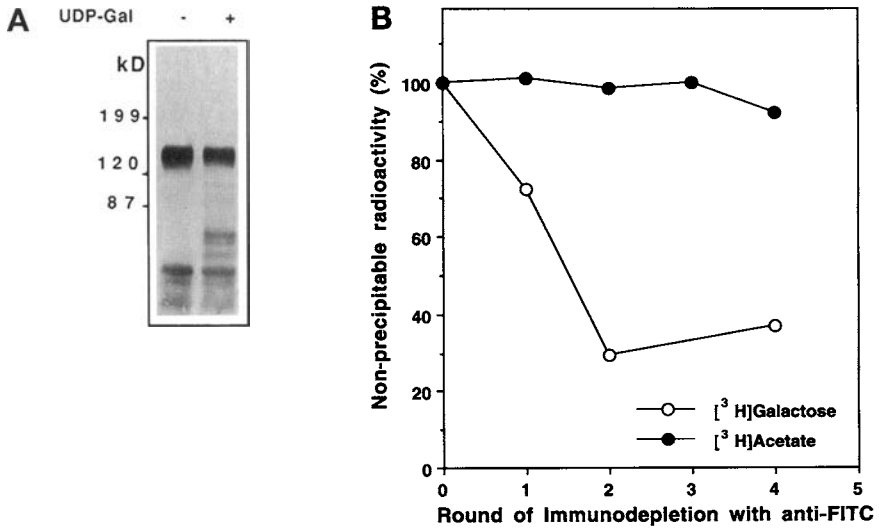

Figure 5. Sialyltransferase action overlaps functionally with galactosyltransferase but not with *O*-acetyltransferase. (A) Incorporation of CMP-Sia-FITC is enhanced when added simultaneously with UDP-Galactose (UDP-Gal), as detected by means of immunoprecipitation followed by Western blot of Golgi proteins with anti-FITC antibodies. Golgi preparations were either incubated in the absence of UDP-Gal (−) or in the presence of 0.5 μM of UDP-Gal (+). Material analyzed here was obtained after the first round of immunoprecipitation. (B) Golgi compartments were coincubated with CMP-Sia-FITC and either UDP-[^{3}H]Gal or [acetyl-^{3}H]acetyl-CoA. After chase, the compartments were solubilized, and serial immunoprecipitation of glycoproteins bearing covalently bound Sia-FITC was done with anti-FITC antibodies. Supernatants were analyzed to determine remaining radioactivity. The third immunodepletion sample from UDP-[^{3}H]Gal was lost in this particular experiment.

explain why not all bands present in Figure 4B are represented in Figure 5A.

Because these Golgi preparations are cytosol-free and therefore incapable of intercompartmental transport (Kagiwada *et al.*, 1993), these results provide evidence for the functional colocalization of β-galactosyltransferase and sialyltransferase activities. These same Golgi preparations contain sialoglycoproteins that are 9-O-acetylated (see Figure 3), and a functional O-acetylation reaction can be demonstrated within the sealed compartments by labeling with [^{3}H]acetyl-CoA, which gives incorporation of label almost exclusively into [^{3}H]O-acetyl-sialic acids (Diaz *et al.*, 1989; Hayes and Varki, 1993b). Furthermore, the pattern of bands obtained with [^{3}H]acetyl-CoA is very similar to that seen with [CMP-^{3}H]Sia (Diaz *et al.*, 1989). Despite this, when the Sia-FITC-labeled glycoconjugates were immunoprecipitated with anti-FITC, they did not show reactivity on a Western blot with CHE-FcD, even when unlabeled acetyl-CoA was added to the Golgi preparations in a concentration sufficient to saturate the acetylation reaction (our unpublished results). To confirm and quantitate the above finding, we simultaneously incubated the Golgi preparations with CMP-Sia-FITC and either [^{3}H]acetyl-CoA or UDP-[^{3}H]Gal. After the reaction, the preparations were lysed in detergent, and immunoprecipitation with anti-FITC antibodies was done to see whether [^{3}H]O-acetyl-groups or [^{3}H]Gal residues were attached to the same glycoprotein molecules that incorporated Sia-FITC. As shown in Figure 5B, up to 70% of the [^{3}H]Gal labeled in glycoconjugates could be immunoprecipitated by the anti-FITC antibodies, indicating that these newly galactosylated glycoconjugates could also be substituted by Sia-FITC in the same Golgi compartments. On the other hand, <5% of the [^{3}H]acetyl-CoA–labeled glycoconjugates were immunoprecipitated after four consecutive rounds of the immunoprecipitation procedure. This means either that the compartments of sialylation and O-acetylation are distinct or that the target molecules for the two reaction are different. However, as mentioned above, our previous work (Diaz *et al.*, 1989) has shown that a similar set of endogenous glycoprotein bands is subject to labeling by UDP-[^{3}H]gal, CMP-[^{3}H]sia, or [^{3}H]acetyl-CoA (Diaz *et al.*, 1989). Thus, although the same Golgi compartments contain the majority of the functional galactosylation and sialylation machineries, the compartment of O-acetylation seems to be relatively distinct.

O-Acetylated Sialoglycoconjugates Are Segregated from Newly Synthesized Sialoglycoconjugates in the Golgi Preparations

In parallel with the biochemical studies described above, paired samples were routinely prepared for immunoelectron microscopy. Immunoelectron microscopy studies were performed with an antibody against FITC and the CHE-FcD probe, each detected with appropriate gold conjugates of two different sizes. 9-O-Acetylated Sia residues (detected with 10 nm of gold goat anti-human IgG conjugates) tended to accumulate in vesicle-like structures in the TGN or in the rims of *trans*-most cisternae of the Golgi stacks (Figure 6A). The specificity of this result was confirmed by the absence of labeling with the unmodified CHE-Fc molecule (Figure 6B). The sites of incorporation of Sia-FITC were found to be discrete areas within and adjacent to the Golgi stacks (Figure 6, C and D).

We next performed a series of double-labeling studies with gold particles of different sizes. Sites of O-acetylated sialoglycoproteins were labeled with 10 nm of gold goat anti-human IgG conjugates, whereas sites of Sia-FITC incorporation were labeled with 5 nm of gold goat anti-rabbit conjugates. Some representative examples of the results are shown in Figure 7. A clear segregation of O-acetylated sialoglycoconjugates (detected by CHE-FcD) from the compartment of sialylation (sites of Sia-FITC incorporation) was observed (Figure 7, A and B). The extent of segregation was quantitated by counting ~4500 beads on several independent sections. In 85% of membranous structures studied, there was complete separation of the different-sized gold particles. Among the remaining 15% of the structures, more than one-half represent situations showing a substantial enrichment of only one size or another of the gold particles. This segregation persisted even when both O-acetylation and sialylation were driven to a maximum extent possible by preincubation with saturating UDP-Gal and acetyl-CoA (final 10 μM and 30 μM, respectively) before adding CMP-Sia-FITC.

The orientation of the Golgi stacks could be determined by double-labeling with antibodies directed either against α2-6sialyltransferase (Figure 7C), which has been previously localized to the *trans*-Golgi compartment and the TGN in rat hepatocytes (Roth *et al.*, 1985), or against α-mannosidase II (Figure 7D), which is concentrated in the *medial* Golgi stacks of the same

Figure 6 (on facing page). Immunoelectron microscopic detection of sites of CMP-Sia-FITC incorporation and of sialic acid O-acetylation in Golgi preparations. (A) Indirect immunogold labeling (10-nm diameter gold particles) of ultrathin cryosections of Golgi-enriched fractions of rat liver demonstrating the localization of CHE-FcD–reacting molecules (9-O-acetylated sialoglycoconjugates), primarily across the TGN, and some discrete, *medial* to *trans*, cisternal labeling. *Cis* and *trans*-portions of Golgi complex (Gc) are indicated. (B) Negative control for CHE-FcD; fractions were incubated with CHE-Fc as primary probe. (C) Indirect immunogold labeling (5-nm diameter gold particles, arrowheads), showing the relative Sia-FITC distribution in isolated Golgi. Labeling is concentrated in the TGN; no labeling is seen on the cisternae. (D) Detail in higher magnification of the boxed area in Figure 6C. Scale bar, 0.1 μm.

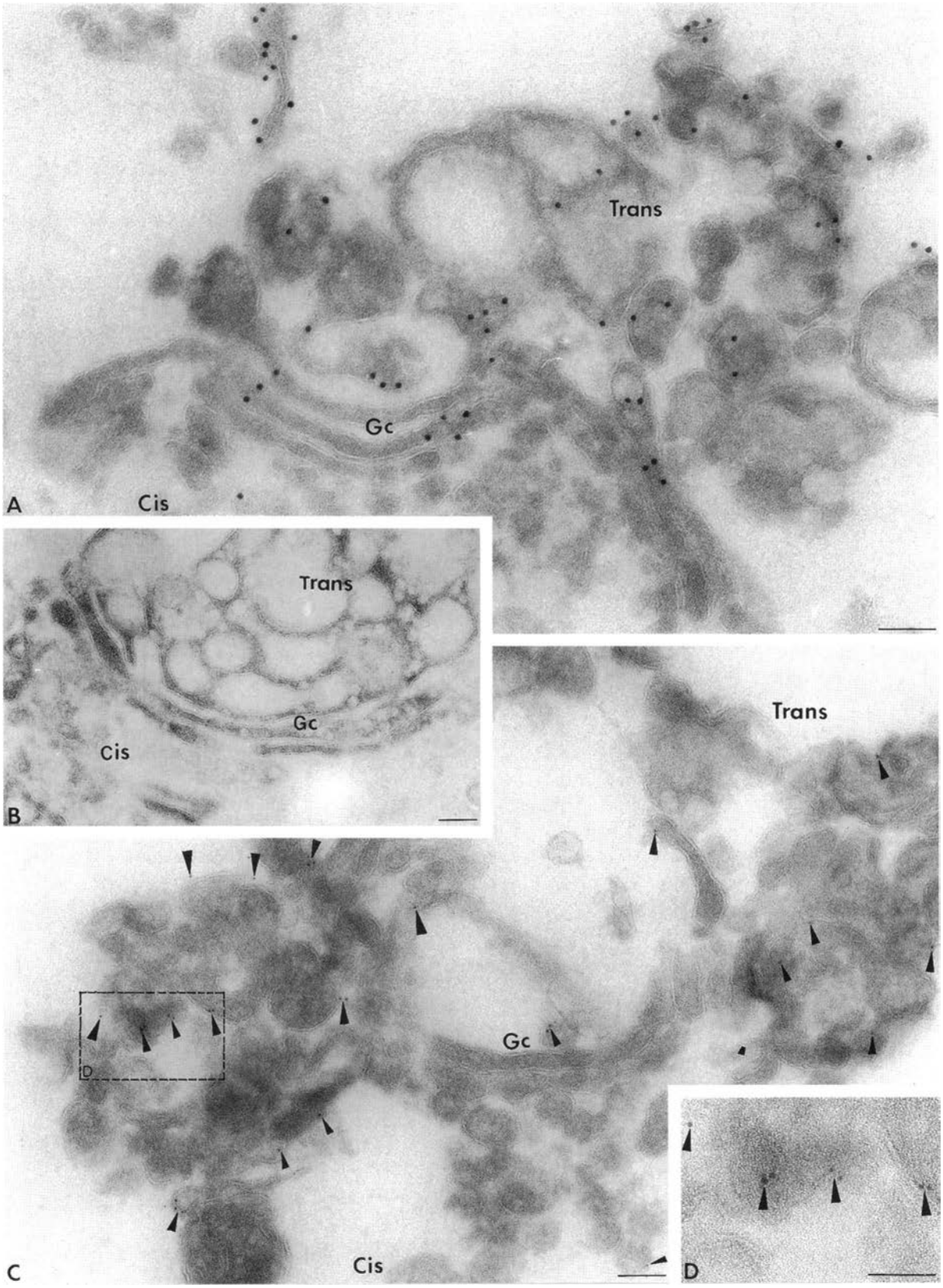

Figure 6.

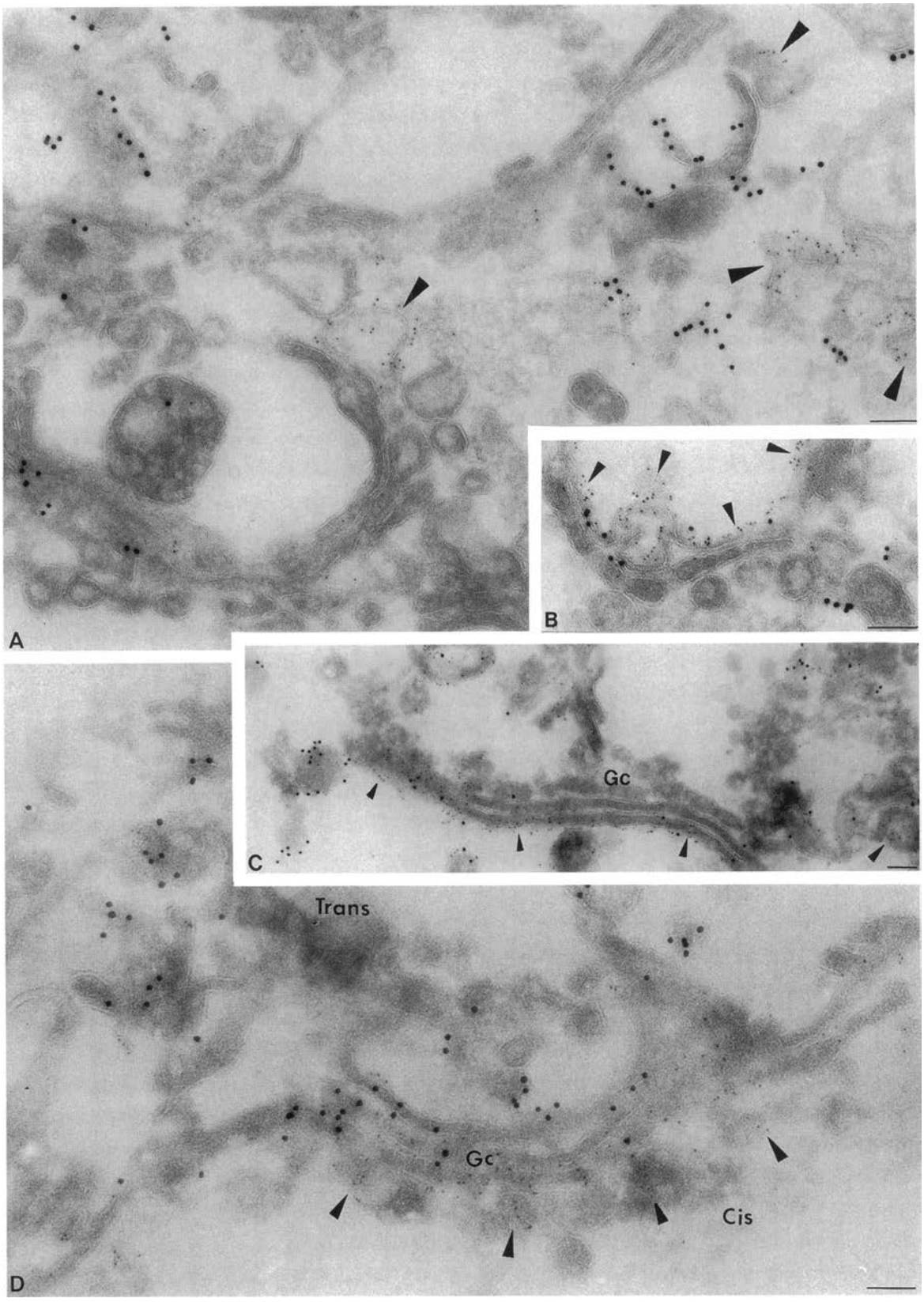

Figure 7.

cells (Velasco *et al.*, 1993). Approximately 42% of CHE-FcD–positive areas (large gold particles) colocalized with sialyltransferase-positive areas (small gold particles, Figure 7C). In contrast, there was little overlap of α-mannosidase II labeling with the actual sites of CHE-FcD labeling (~12%, see Figure 7D) or with Sia-FITC incorporation (our unpublished results). Thus, distribution of sialyltransferase was broader than the distribution of Sia-FITC incorporation. This supports the concept that the presence of the enzyme per se does not necessarily define a functional Golgi compartment (Hayes *et al.*, 1993). Taken together, both biochemical and ultrastructural data indicate that the functional compartments of sialylation (sites of Sia-FITC labeling) and O-acetylation (both pre-existing endogenous residues and those generated by the addition of excess acetyl-CoA) are substantially, if not completely, distinct.

Lactose-4 MU Is an Exogenous Acceptor for Sialyltransferase Action in Intact Golgi Compartments

Because the CMP-Sia-FITC donor has FITC linked to C9 of Sia, such residues cannot be 9-O-acetylated. However, the endogenous internal pool of CMP-Sia in fresh Golgi preparations (Hirschberg and Snider, 1987) will compete with exogenously added CMP-Sia-FITC. Thus, only a proportion of the endogenous glycans is substituted by Sia-FITC; the rest are modified by Sia from endogenous CMP-Sia. Indeed, on coincubating Golgi preparations with equimolar amounts of CMP-Sia-FITC and CMP-[^{3}H]Sia, we could immunoprecipitate radiolabeled sialoglycoconjugates with anti-FITC antibodies ([^{3}H]Sia incorporation was reduced approximately twofold). This indicates that endogenous glycans can be modified simultaneously by Sia-FITC and Sia. The latter should be freely available for 9-O-acetylation if this reaction mechanism coexisted in the same compartment. We approached the latter issue by coincubating both Sia donors with [^{3}H]acetyl-coA (in this case CMP-[^{14}C]Sia, instead of CMP-[^{3}H]Sia, was used). After immunoprecipitation, Sia was released and analyzed by paper chromatography. No O-acetylated sialic acid was observed under these conditions (our unpublished results). It could be questioned whether the presence of the 9-FITC groups on some Sia residues might somehow restrict O-acetylation on other Sia residues. However, such steric hindrance is unlikely, because the different antennae of multiantennary N-linked oligosaccharides have considerable range of mobility in solution (Carver *et al.*, 1989).

Regardless, we decided to take one more independent approach to this issue, studying the possibility of the O-acetylation reaction occurring on an exogenous acceptor present in all compartments of sialylation. This acceptor must be introduced without disrupting compartment integrity. Such an approach recently had been presented (Etchison *et al.*, 1995), wherein similar rat liver Golgi preparations were incubated with the diffusible derivative 4-methylumbelliferylβ-xyloside. This exogenous acceptor crossed the Golgi membrane and was elongated by endogenous glycosyltransferases; a sialylated subset was found to be trapped inside the compartments of synthesis (Etchison *et al.*, 1995). We chose 4-methylumbelliferylβ-lactoside (Galβ1-4Glc-4-MU) to function as an acceptor for the sialyltransferases of rat liver. As shown in Figure 8A, this molecule did undergo addition of radiolabeled Sia residues from CMP-[^{3}H]Sia, giving a product that was trapped inside the compartments and, after release, was bound to a hydrophobic C_{18} cartridge. This sialylation of 4-MU-lactose was saturable (Figure 8A), reaching a maximum with a 5 mM exogenous concentration.

The newly sialylated 4-MU-lactoside molecules trapped inside the Golgi compartments were collected by centrifugation, released by ethanol extraction, and purified by reverse-phase chromatography with the use of either C_{18} cartridges or a C_{18} HPLC column. As shown in Figure 8B, the sialylated material gave a single major peak on the latter column. Differential sensitivity to sialidases from *A. ureafaciens* (cleaves sialic acids in all linkages) and Newcastle disease virus (cleaves only α2, 3 and α2, 8 linkages) indicated that 85% was [^{3}H]Siaα2-6Galβ1-4Glc-4-MU. Because most, if not all, of the 9-O-acetylation in the native rat liver is found on Siaα2-6-lactosamine units (Diaz *et al.*, 1989), this sialylated product should be an acceptor for the *O*-acetyltransferase (we had shown previously in Higa *et al.* [1989] that even free sialic acid can act as an acceptor for the rat liver *O*-acetyltransferase). Moreover, this [^{3}H]Siaα2-6Galβ1-4Glc-4-MU would accumulate in all compartments that have both the α2-

Figure 7 (cont). Sites of CMP-Sia-FITC incorporation and of sialic acid O-acetylation are physically segregated—comparison with the distribution of enzyme markers. (A and B) Immunogold localization of CHE-FcD–reacting glycoconjugates (10-nm gold particles) and Sia-FIT–labeled glycoconjugates (5-nm gold particles) on ultrathin cryosections of Golgi-enriched rat liver fractions demonstrating the nearly complete segregation of O-acetylated sialoglycoproteins (large gold) and newly sialylated glycoconjugates (small gold, arrowheads). (C) Immunogold localization of CHE-FcD–reacting glycoconjugates (large gold) and α2-6sialyltransferase (small gold). Note the relatively broad distribution of the α2-6sialyltransferase (small gold), as compared with A and B, showing more restricted distribution of newly sialylated glycoconjugates. 9-O-Acetylated sialoglycoconjugates detected by CHE-FcD are found discretely on the cisternae and in the TGN (large gold). (D) Localization of Golgi α-mannosidase II (α-Man II; small gold) and 9-O-acetylated sialoglycoconjugates (large gold). Although α-Man II is found only on the *cis* and *medial* cisternae, 9-O-acetylated sialoglycoconjugates are broadly distributed across the TGN and more discretely on the cisternae. Gc, Golgi complex. Scale bar, 0.1 μm.

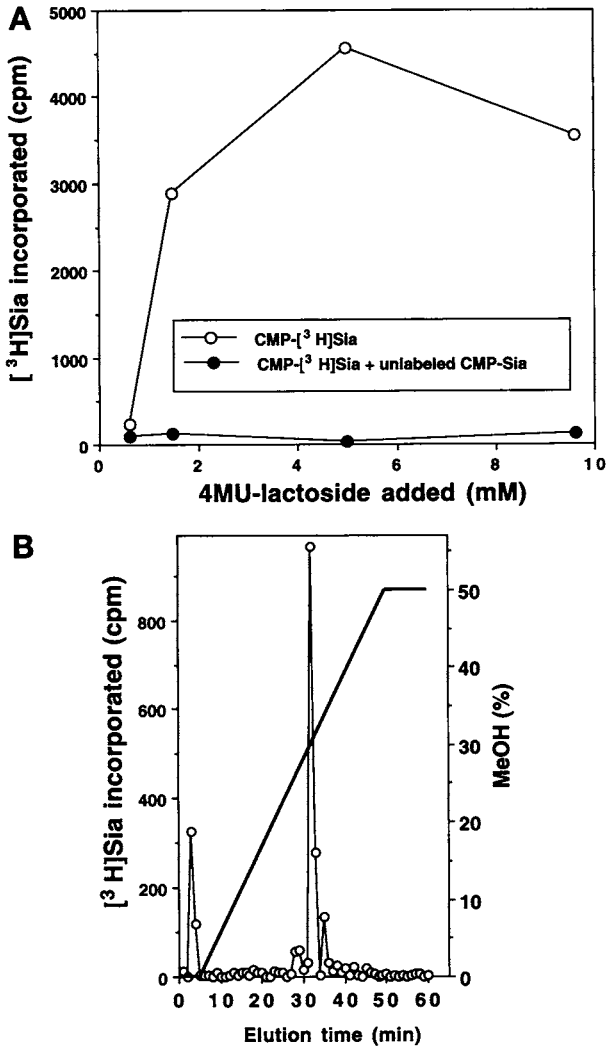

Figure 8. Sialylation of 4-MU-lactoside within intact Golgi compartments. (A) Golgi compartments were incubated with varying concentrations of 4-MU-lactoside and 1 μM of CMP-[^{3}H]Sia in the presence or absence of an excess of CMP-Sia. After incubation, the preparation was centrifuged, the pellet that contains the Golgi compartments was extracted with 70% ethanol, and 4-MU-derivatives were purified by reverse-phase chromatography. [^{3}H]Sia-substituted 4-MU-lactosides were measured by liquid scintillation counting. A saturation curve was obtained, indicating that 4-MU-lactosides were concentrated within Golgi compartments and modified further. (B) [^{3}H]Sia-substituted 4-MU-lactosides obtained as above were then separated by reverse-phase HPLC with a gradient of methanol, as described under MATERIALS AND METHODS. A single major peak was identified as Siaα2-6(3)Galβ1-4Glcβ1-4-MU.

6sialyltransferase and the CMP-Sia transporter. We therefore coincubated Golgi compartments with saturating amounts of 4-MU-lactoside (5 mM), CMP-[^{3}H]Sia (1 μM), and excess unlabeled acetyl-CoA (10–30 μM), isolated the sialylated 4 MU-derived products by reverse-phase chromatography, and analyzed them for the presence of O-acetylated [^{3}H]Sia residues. After release by *A. ureafaciens* sialidase, the [^{3}H]Sias were purified and studied. As shown in Figure 9, ~85% of the ^{3}H-label had the same mobility of unmodified Sia, whereas a second peak comprising ~10-15% of the material had the mobility of 9-O-acetylated-Sias. However, this peak was also seen in the absence of added unlabeled acetyl-CoA. Furthermore, it is completely resistant to base treatment (Figure 9), indicating that it is not a 9-O-acetylated species. Similar base-resistant sialic acids of unknown identity were previously noted in the endogenous acceptors labeled by CMP-[^{3}H]Sia in the same type of Golgi preparations (Hayes *et al.*, 1993). Regardless, these data indicate that sialylation and O-acetylation of Sia residues are topographically segregated events in the rat liver Golgi apparatus independent of the origin of the acceptor (endogenous or exogenous).

Vesicular Carriers Carrying Segregated Membrane Proteins Are Selectively Enriched in 9-O-Acetylated Sialic Acids

Because hepatocyte cell surfaces are heavily stained by CHE-FcD, plasma membrane proteins are predicted as major substrates for the *O*-acetyltransferase machinery. Golgi compartments can be permeabilized with

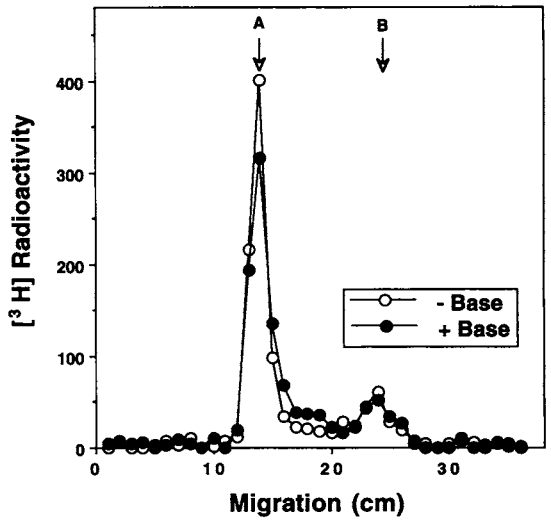

Figure 9. The sialylated-4-MU-lactoside within intact Golgi compartments does not acquire O-acetyl groups. Golgi compartments were coincubated with 4-MU-lactose (5 mM), CMP-[^{3}H]Sia (1 μM), and acetyl-CoA (10–30 μM); the sialylated-4-MU-lactoside was purified and treated with *Arthrobacter ureafaciens* sialidase, and the released Sia was separated in a paper chromatography system, as described under MATERIALS AND METHODS. In this system, Neu5Ac (A) is separated from O-acetylated forms and other Sia-derivatives, which migrate faster (B). Positive identification of 9-O-acetylated forms is then possible because of its sensitivity to base treatment. As shown, no base-sensitive Sias were released from sialylated-4-MU-lactoside, indicating that O-acetylation is not present.

saponin, allowing centrifugal separation of the soluble internal content from membrane-bound sialoglycoproteins. Under these conditions, >95% of the [^{3}H]O-acetyl-Sia residues labeled in vitro with [^{3}H]-acetyl-CoA were found on membrane-associated sialoglycoproteins (Diaz and Varki, unpublished observations). Thus, although even free sialic acid can act as an O-acetylation acceptor in vitro, membrane proteins seem to be selectively targeted in the intact Golgi preparations, suggesting that they are segregated into the compartment of O-acetylation. In this regard, Saucan and Palade (1994) recently demonstrated that membrane-bound and secretory proteins in rat liver hepatocytes are transported from the Golgi apparatus to the cell surface via sets of different carriers. In their studies, the two types of vesicular carriers could be defined by immunoisolation with an antibody directed against the cytosolic tail of the polymeric IgA receptor (pIgA-R), immobilized on magnetic beads. Membrane-bound proteins were segregated in pIgA-R–positive vesicles, whereas secretory proteins were primarily present in pIgA-R–negative vesicles (Saucan and Palade, 1994). In these studies, the majority of proteins (80–90%) is found in pIgA-R–negative fraction.

We tested whether O-acetylated sialoglycoproteins were differentially distributed between these two distinct populations of carrier vesicles. The vesicles were prepared from rat liver and subjected to immunoisolation exactly as described earlier (Saucan and Palade, 1994). Proteins of the starting material (SM), nonbound vesicles (NB), and bound (B) vesicles were separated by SDS-PAGE and then transferred to nitrocellulose. Figure 10, left panel, shows a Western blot with an anti-pIgA-R antibody as a control for the immunoisolation procedure, confirming the enrichment of pIgA-R in the bound fraction. Figure 10, middle panel, shows the distribution of total sialylated proteins in the fractions probed with the *Sambucus nigra* agglutinin, which recognizes Sia in α2,6-linkages (Shibuya *et al.*, 1989). Sialylated molecules were noted in both fractions, although some enrichment in the bound fraction was observed. As shown in Figure 10 (right panel), 9-O-acetylated sialoglycoproteins were selectively segregated into the pIgA-R–positive fraction despite the fact that it contained only 10–20% of the total protein. Given the incompleteness of this immunofractionation protocol (Figure 10, left panel), it is impossible to define whether complete segregation occurs. Regardless, it is likely that O-acetylation occurs primarily after the completion of sialylation, only on the subset of proteins destined for the plasma membrane. It remains to be determined whether this O-acetylation event occurs before or after the segregation of this subset of proteins from others destined for secretion.

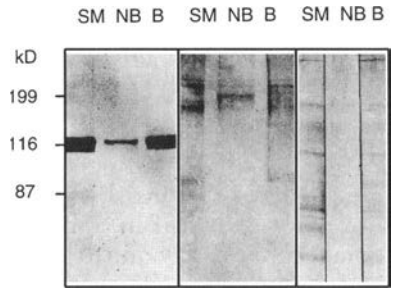

Figure 10. Vesicular carriers carrying segregated membrane proteins are selectively enriched in 9-O-acetylated sialic acids. Hepatocyte membrane and secretory proteins are transported from Golgi to the plasma membrane by distinct carriers. Membrane proteins are carried by polymeric-IgA receptor (pIgA-R)-containing vesicles (Saucan and Palade, 1994). Golgi light vesicles were fractioned by immunoisolation on the basis of the expression of the polymeric-IgA receptor. Proteins of the starting material (SM), nonbound vesicles (NB), and bound vesicles (B) were separated in SDS-PAGE and then transferred to nitrocellulose. Protein loading was done in such a way that the amount studied from the starting material was the sum of both bound and unbound fractions. Western blot of vesicular carrier proteins with anti-pIgA-R shows enrichment of pIgA-R in the B fraction (left panel). Western blot of vesicular carrier proteins with lectin from *Sambucus nigra*, which recognizes α2,6-linked Sias, indicates that sialylated proteins are present in both NB and B fractions (middle panel). Western blot of vesicular carrier proteins with CHE-FcD shows a substantial enrichment of 9-O-acetylated sialoglycoproteins in the B fraction (right panel).

DISCUSSION

Sequential modifications of N-linked sugar chains occurring on newly synthesized glycoproteins have long been used as signposts for the organization of the Golgi apparatus. There is little doubt that the enzymes that work early in the pathway are found mainly in the earlier (*cis* and *medial*) regions of the Golgi, whereas those acting later tend to occur in the *trans* stacks and the *trans*-Golgi network. The original model, in which each major step of the pathway was confined to a discrete stack of the Golgi (Goldberg and Kornfeld, 1983; Quinn *et al.*, 1983; Dunphy and Rothman, 1985; Griffiths and Simons, 1986), has given way to the realization that there can be some overlap in the distribution of the enzymes in a cell-type–dependent way (Roth *et al.*, 1986; Mellman and Simons, 1992; Hayes *et al.*, 1993; Hayes and Varki, 1993b; Nilsson *et al.*, 1993; Velasco *et al.*, 1993). Furthermore, the presence of a given enzyme in a particular Golgi stack does not mean that it actually functions in that location (Hayes *et al.*, 1993). Given the cytosolic location of sugar nucleotide donor synthesis and the rapid rate of transport through the stacks, several other factors are relevant, including the specific membrane antiporters that translocate donors into the lumen of the compartments (Hirschberg and Snider, 1987; Milla and Hirschberg, 1989), the presence of enzymes that degrade these donors (Martina *et al.*, 1995), and the availability

of the correct biosynthetic intermediate as an acceptor (Hayes *et al.*, 1993).

One way to dissect the relative importance of these factors is to prevent or inhibit transport and then examine the biosynthetic capabilities of the individual compartments. If these compartments are prepared directly from chilled fresh tissues, they should contain endogenous acceptors arrested in transit, in compartments where they could receive further modifications. Adding labeled donor nucleotides to these preparations reinitiates the process of uptake and incorporation in a cytosol-free situation in which intercompartmental transport cannot occur. Indeed, fresh Golgi preparations from rat liver (Hayes *et al.*, 1993; Etchison *et al.*, 1995), melanoma cells (Sjoberg and Varki, 1993), and rat retina (Maxzúd *et al.*, 1995) do allow such a "freeze-frame" analysis. The radioactive sugar nucleotide donors are concentrated within intact compartments whose biosynthetic capabilities are far superior to any broken ones present in the same preparation. This is proven by the observations that 1) detergents decrease rather than increase the efficiency of labeling, and 2) endogenous products are protected from added proteases (Hayes *et al.*, 1993). Studies using this approach have shown both the expected biosynthetic intermediates as well as several unexpected products (Hayes *et al.*, 1993; Hayes and Varki, 1993a,b). Also, in Golgi fractions from rat liver, evidence was found for functional overlap of GlcNAc-transferases, galactosyltransferases, and sialyltransferases (Hayes and Varki, 1993b). In those papers, we also indicated why potential mixing of compartments during homogenization is not a major confounding factor. A disadvantage of this approach is that the Golgi compartments are studied as a mixture and cannot be physically separated. Although each compartment defines itself functionally (making the study of the products valid), it was impossible to visualize the location of the products. Here we present some approaches to circumvent this deficiency and show that the O-acetylation of Sias occurs primarily in a compartment distal to, and distinct from, the compartments where Sia addition itself takes place.

Using a modified sugar donor (CMP-Sia-FITC) recognized by both the CMP-sialic acid transporter (Gross, 1992) and the major rat liver sialyltransferases (Gross and Brossmer, 1988), we visualize the *functional compartments* of isolated Golgi stacks where sialylation takes place; these are mostly distinct from sites where O-acetylation of sialic acids is found. This principle could be extended to any nucleotide donor in which a detectable sugar modification does not cause loss of recognition by the nucleotide transporter and/or the relevant sugar transferases.

The sites of sialylation in the Golgi apparatus (mainly *trans*-Golgi and TGN) are generally considered the last stations of the Golgi apparatus through which a glycoprotein must pass. However, O-acetylation of sialic acids occurs after the transfer of sialic acids to glycoproteins (Diaz *et al.*, 1989), and previous indirect evidence suggested it might occur in a distinct compartment (Hayes and Varki, 1993b). Until now, it was not possible to address this matter directly because of the lack of a reliable probe for detecting the *O*-acetyltransferase or its product. The latter can now be detected with an Influenza C hemagglutinin-esterase–derived recombinant probe (Klein *et al.*, 1994) called CHE-FcD. Here we show that, although there is some overlap between the distribution of the α2-6sialyltransferase antigen and the endogenous 9-O-acetylated sialic acids, there is very little overlap between the products of the sialylation and O-acetylation reactions, even when both reactions are driven to completion with an in vitro incubation. It is unlikely that this result is an artifact of the system used. First, the rates of sialylation and O-acetylation in this system were previously shown to be relatively similar (Diaz *et al.*, 1989), indicating that the availability of endogenous acceptors is not limiting. Furthermore, we show a clear overlap between the two immediately earlier steps in the pathway—galactosylation and sialylation. Finally, a similar result was obtained with an exogenous acceptor capable of penetrating into all of the stacks.

Some subtleties of O-acetylation deserve comment. We have noted that, in the rat liver Golgi, O-acetylation occurs at both the 7- and the 9- positions of endogenous sialic acids (Diaz *et al.*, 1989; Butor *et al.*, 1993). The 7-O-acetyl groups can then migrate to the 9-position under mildly alkaline conditions. It is currently not known whether the 7-O-acetylated product can be recognized by the CHE-FcD probe. If not, a discrete 7-*O*-acetyltransferase reaction that overlaps with the sialylation could have been missed in this study. Another possibility is that the same *O*-acetyltransferase transfers to both positions and that the detection of the 9-O-acetylated product therefore localizes both reactions. Indirect evidence also suggests that there may be multiple sialate/*O*-acetyltransferases with differing specificities, depending on the nature of the glycoconjugate and/or the linkage of the Sia residue (reviewed in references of Varki, 1992). Thus, the results obtained here should not be extrapolated to other cell types in which sialic acids are 9(7)-O-acetylated. Indeed, our earlier work suggests that O-acetylation of the terminal Sia residue of ganglioside G_{D3} is not segregated from the compartment of sialylation in human melanoma cells (Sjoberg and Varki, 1993).

The data presented here suggest discrete compartments for sialylation and O-acetylation reactions in rat liver Golgi. It is possible that distinct subdomains within the *trans*-Golgi stacks and TGN might selectively harbor functional complexes of all the compo-

nents required for each reaction. Although such functional complexes have been detected in the medial Golgi (Nilsson *et al.*, 1994), none have been convincingly demonstrated to date in the *trans* Golgi. Perhaps the *O*-acetyltransferase machinery is selectively located at the exit point from the *trans*-Golgi network and is functionally expressed only in a subset of budding vesicles. This fits our observation that O-acetylated glycoproteins are selectively found in the subfraction of vesicular carriers enriched in plasma membrane proteins. Further work is needed to know whether O-acetylation is the cause or the consequence of the formation of these carriers.

The use of glycosides as alternate acceptors for glycosylation reactions is well established for intact cells (Lugemwa and Esko, 1991; Freeze *et al.*, 1993). In such studies, derivatives such as 4-methylumbelliferyl (4-MU) β-glycosides added to the culture medium must penetrate two barriers: the plasma membrane and the membrane of the Golgi compartments. However, Freeze and colleagues (Etchison *et al.*, 1995) have shown that monosaccharide glycosides can also be added directly to intact Golgi compartments. Here we have used a disaccharide glycoside as an exogenous acceptor that successfully penetrated the Golgi membrane. Interestingly, Esko and colleagues have recently shown that, for disaccharide glycosides to enter the Golgi of intact cells, it is necessary to modify them to increase their hydrophobicity (Sarkar *et al.*, 1995). This implies that the Golgi membrane may be relatively more permeable to small saccharides than the plasma membrane. In keeping with this, when a β-xyloside was added to intact Golgi, one of the products (Galβ1-4xyloside) was found to diffuse back out of the Golgi compartments at a significant rate (Etchison *et al.*, 1995). However, the further addition of sialic acid caused the molecule to be irreversibly trapped in the Golgi lumen (Etchison *et al.*, 1995).

Because the exogenous lactoside acceptor can enter all compartments in excess, the overlap between the sialylation and O-acetylation compartments should have been seen, if it existed. Although no O-acetylation was seen, we did observe an unusual base-resistant modification of the sialic acids that were transferred to the lactoside derivatives. The nature of this modification, which occurs during the short in vitro incubation, remains unknown. It does serve to emphasize that O-acetylation is only one of many possible modifications of sialic acids observed in biological systems (Varki, 1992). Identification of the subcellular sites at which these modifications are synthesized should provide useful signposts for understanding the organization and function of the Golgi apparatus and the exocytic pathway.

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