[33] Glycoprotein Sialate 7(9)-*O*-Acetyltransferase from Rat Liver Golgi Vesicles

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Introduction

Naturally occurring sialic acids can be O-acetylated at the 4-, 7-, 8-, or 9-hydroxyl positions (previously reviewed in this series by R. Schauer, Vol. 50, p. 64, and Vol. 138, p. 611). When [^{3}H]acetyl-coenzyme A (AcCoA) is incubated with isolated intact rat liver Golgi vesicles, the radioactivity is efficiently concentrated into the vesicles, and the acid-insoluble component of this label is found to be mainly in O-acetylated sialic acids.[1] These ^{3}H-labeled *O*-acetyl groups are found at both the 7- and 9-positions of *N*-acetylneuraminic acid residues that are $\alpha 2 \rightarrow$ 6-linked to N-linked oligosaccharides on endogenous glycoproteins.[2] Since 7-*O*-acetyl groups can undergo migration to the 9-position under physiological conditions, it had been previously hypothesized that all O-acetylation on the side chain might occur initially at the 7-position with subsequent migration to the 9-position.[3,4] However, our kinetic analyses using *O*-[^{14}C]acetyl internal and *O*-[^{3}H]acetyl external sialic acid standards or mild periodate oxidation of prelabeled vesicles indicate that *O*-acetyl esters are added directly to both the 7- and 9-positions of the sialic acids in the rat liver Golgi system.[2]

Double-label experiments with [^{3}H]acetyl-CoA and Ac[^{32}P]CoA indicate that the intact AcCoA molecule does not gain access to the lumen of the vesicles during the reaction (i.e., a true "transporter" is not involved). Furthermore, studies of prelabeled vesicles permeabilized with saponin demonstrate a [^{3}H]acetyl intermediate in the membrane that can transfer label to the 7- and 9-positions of exogenously added free *N*-acetylneuraminic acid. This second transfer reaction is not inhibited by coenzyme A, unlike the first step in the utilization of acetyl-CoA (the formation of the acetyl intermediate). Studies with amino acid-modifying reagents indicate that an active site histidine residue is involved in both steps of this acetylation reaction.[2] Thus, the O-acetylation of sialic acids in Golgi vesicles occurs via a transmembrane acetylation reaction, similar to that described

[1] A. Varki and S. Diaz, *J. Biol. Chem.* **260**, 6600 (1985).
[2] S. Diaz, H. H. Higa, B. K. Hayes and A. Varki, *J. Biol. Chem.*, in press (1990).
[3] J. P. Kamerling, R. Schauer, A. K. Shukla, S. Stoll, H. van Halbeek, and J. F. Vliegenthart, *Eur. J. Biochem.* **162**, 601 (1987).
[4] A. Varki and S. Diaz, *Anal. Biochem.* **137**, 236 (1984).

for the acetylation of glucosamine in lysosomes (see Bame and Rome, this series, Vol. 138, p. 607). However, several features of this Golgi reaction distinguish it from the lysosomal mechanism, including the nature and kinetics of the reaction.

Although the vesicles are impermeant to acetate, free [^{3}H]acetate also accumulates inside the vesicles during this labeling reaction. Our studies indicate that this occurs mainly as a by-product of the acetylation reaction itself, and not from the action of an acetyl-CoA hydrolase. A smaller proportion of the free [^{3}H]acetate arises from the action of an 9-*O*-acetylesterase activity present in the same vesicles. This enzyme is described in more detail elsewhere.[5]

Ester groups added to the 7-position can subsequently undergo migration to the 9-position, if it is not already acetylated. No evidence was found in rat liver for a mutase that could catalyze this migration reaction.[2] Thus, the rate of migration would depend on the pH and temperature conditions. At physiological pH (7.0) and temperature (37°), the $t_{\frac{1}{2}}$ for this migration was found to be 10 hr.[6] The various reactions described in this chapter are outlined in Fig. 1.

Assay Method

Reagents

Isolated intact rat liver Golgi vesicles (5–10 mg/ml)
[^{3}H]Acetyl-CoA (5–15 Ci/mmol), store in aliquots at −70°
PK buffer: 10 m*M* potassium phosphate, 150 m*M* sodium chloride, pH 6.5 (1× and 2× stocks, store at 4°)
Coenzyme A-SH (CoA), 10 m*M* in water, store frozen at −20°
8% Perchloric acid (PCA) in water, store at 4°
1 *N* Sodium hydroxide
2 *N* HCl
Scintillation counting cocktail for aqueous samples
Saponin (5% in water), store at 4° for up to 5 days
N-Acetylneuraminic acid (Neu5Ac), and glucuronic acid (GluA) 100 m*M* each, pH adjusted to 5–6 with NaOH, store frozen at −20°
Absolute ethanol, at −20°
Whatman 3 MM chromatography paper
95% Ethanol: 1 *M* ammonium acetate, pH 5.5 (7:3)
Diisopropylfluorophosphate (DFP) (Aldrich, Milwaukee, WI)

[5] H. H. Higa, A. Manzi and A. Varki, *J. Biol. Chem.*, in press (1990).
[6] R. Schauer, this series, Vol. 138, p. 611.

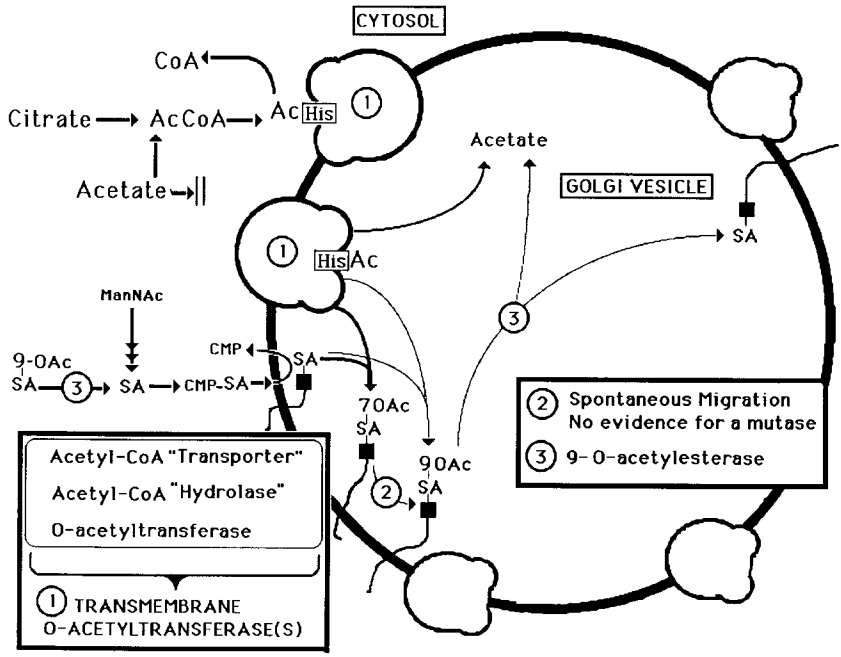

FIG. 1. Pathways of O-acetylation and de-O-acetylation of sialic acids in rat liver Golgi vesicles.

100 m*M* in 2-propanol, store in desiccator at −20°
Arthrobacter ureafaciens neuraminidase (Calbiochem, San Diego, CA)

Preparation and Storage of Golgi Vesicles

Isolated intact rat liver Golgi vesicles are prepared exactly as described by Leelavathi *et al.*[7] It is important to note that the quality of the Golgi preparations often depends on the age of the rats (3- to 4-months-old is optimal) and the care taken to obtain gentle but uniform homogenization. Typical preparations are enriched 50- to 100-fold for galactosyltransferase and are over 80% intact by the assay of Carey and Hirschberg.[8] Also, if cruder subcellular preparations are used, the assay must be modified accordingly (see below). Pretreatment of the Golgi vesicles with DFP immediately before use (final 1 m*M* DFP, 1% 2-propanol, on ice for 15 min) inactivates endogenous esterase activity and improves recovery of the

[7] D. E. Leelavathi, L. W. Estes, D. S. Feingold, and B. Lombardi, *Biochim. Biophys. Acta* **211**, 124 (1970).
[8] D. J. Carey and C. B. Hirschberg, *J. Biol. Chem.* **256**, 989 (1981).

product by 10–20%. Appropriate care must be taken in handling the stocks of the toxic inhibitor DFP.

Assay I: Transfer of Acetyl Groups to Endogenous Acceptors

Isolated intact Golgi vesicles (100–200 μg Golgi protein, pretreated with DFP) are incubated with [^{3}H]acetyl-CoA(1–5 μ*M*, 2–10 μCi) in 500 μl of PK buffer, pH 6.5, at 22° for 5–10 min, in an ultracentrifuge tube. The buffer and the label are first mixed at 22°, and the reaction is started by adding Golgi vesicles. The reaction is quenched by filling the tube with ice-cold PK buffer and centrifuged at 100,000 *g* for 30 min at 4°. The supernatant is aspirated, and the pellet is carefully surface-washed 3 times with 4 ml of ice-cold PK. The washed pellet is sonicated into 500 μl of ice-cold water, and transferred into 500 μl of 8% PCA in an Eppendorf tube. The mixture is allowed to stand on ice for 30–60 min and centrifuged at 10,000 *g* at 4°. Then 900 μl (90%) of the supernatant is counted with 10 ml of scintillation cocktail, and the remainder of the supernatant is aspirated off. The pellet is surface-washed once with 4% PCA and dissolved in 700 μl of 1*N* NaOH at 80° for 15 min. The tube is vortexed, and 350 μl of 2 *N* HCl is added. Then 945 μl (90%) of the mixture is counted with 10 ml of scintillation cocktail. Incorporation of label into the pellet is calculated based on the original specific activity. The acid-insoluble materials consist mainly of ^{3}H-labeled *O*-acetylsialic acids, and the acid-soluble component is almost exclusively in [^{3}H]acetate. In typical assays, the two components are approximately equal in amount. The accumulation of both components is completely inhibited by 0.5 m*M* CoA in the initial incubation. Such a reaction is therefore used to subtract the blank value.

It is emphasized that in cruder subcellular fractions, ^{3}H label from the donor [^{3}H]acetyl-CoA is transferred to a variety of other unknown acceptors that are not sialylated glycoproteins. Thus, if impure Golgi vesicles are used for assays, it is necessary to use specific release with neuraminidase (with appropriate buffer blanks) to quantitate accurately the proportion of the acid-insoluble product that is in ^{3}H-labeled *O*-acetylsialic acids.

Assay 2: Transfer of Acetyl Groups to Free Sialic Acids from Membrane Acetyl Intermediate

Golgi vesicles (1.5 mg) are first labeled with [^{3}H]acetyl-CoA in a scaled-up reaction (10×) with components identical to those described for Assay 1. A small portion (< 10%) of the membrane-associated radioactivity is in the form of a [^{3}H]acetyl intermediate that can be transferred to free sialic acid. The initial labeling reaction is quenched with 3.0 ml of PK buffer containing 0.1% saponin and centrifuged at 100,000 *g* for 30 min at

4°. The supernatant is aspirated off, and the pellet is carefully surface-washed twice with 4 ml of cold PK buffer containing 0.1% saponin. One milliliter of the same buffer is added, allowed to stand on ice for 3–5 min, and aspirated off carefully. The pellet is resuspended in 170 μl of PK buffer with very gentle sonication. Aliquots (40 μl) of the suspension are incubated in 100 μl reactions containing 10 m*M* Neu5Ac (sample) or 10 m*M* GluA (control) and PK buffer. The reactions (in ultracentrifuge tubes) are placed at 25° for 30 min and centrifuged at 100,000 *g* for 30 min. Then 100 μl of the supernatant is transferred into 900 μl of ice-cold 100% ethanol in Eppendorf tubes, mixed, and placed on ice for 1–2 hr. The tubes are centrifuged at 10,000 *g* for 15 min at 4°. The supernatants are transferred into fresh tubes and dried down on a Savant Speedvac. The dried samples are spotted on Whatman 3 MM paper and chromatographed in a descending system of 95% ethanol: 1 *M* ammonium acetate, pH 5.5 (7:3) for 9–12 hr. The lanes are cut into 1-cm strips and soaked in 0.5 ml of water, 5 ml of scintillation cocktail is added, and the radioactivity is determined. The product (a mixture of [*acetyl*-^{3}H]Neu5,9Ac$_2$ and [*acetyl*-^{3}H]Neu5,7Ac$_2$) is found in a single peak with an R_f of 0.7. The peak is not seen in the GluA control, and the background from this lane is subtracted. If desired, an internal standard of [^{14}C]Neu5Ac (2,000 cpm) can be added to each sample before spotting to monitor anomalies in chromatography in individual lanes. This standard migrates slower than the product, with an R_f of 0.55.

Properties

Acceptors. The transmembrane acetyltransferase can donate acetyl groups from [^{3}H]acetyl-CoA to endogenous glycoprotein acceptors, or to exogenously added free Neu5Ac. In the latter case, it is necessary to add saponin after the initial labeling reaction, to permit access of the free sugar to the inside of the vesicles. It has thus far not been possible to develop a reliable assay for transfer of acetate to exogenous glycoprotein acceptors.

Stability. The Golgi vesicles are gently resuspended into 10 mg/ml bovine serum albumin (BSA) soon after preparation and stored as aliquots in liquid nitrogen. Samples for use are snap-thawed on a water bath at room temperature and immediately placed on ice. The activity (Assay 1) is stable to the first cycle of freezing, and the frozen aliquots are stable for 3–6 months. However, they cannot be refrozen after thawing. At room temperature, activity is gradually lost (50% in 20 min). The activity is very labile to incubation at 37°. The prelabeled vesicles carrying the [^{3}H]acetyl intermediate (for Assay 2) can be stored frozen at −70° for at least 4 days.

Inhibitors. The transfer to endogenous acceptors and the accumulation of free acetate are both inhibited by CoA ($K_i \times 0.74\ \mu M$) and by the histidine-modifying reagents diethyl pyrocarbonate (DEP) and *N*-bromosuccinimide (NBS) (each at 0.5 m*M*). Both are not significantly affected by reducing agents, divalent cations, EDTA, CMPNeu5Ac, UDPGlcNAc, tunicamycin, or adenosine 3′-phosphate 5′-phosphosulfate (PAPS). They are also not affected by sulfhydryl poisons, ion channel inhibitors [atractylosides and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS)], proton pump inhibitors (oligomycin, sodium vanadate), weak bases (ammonium sulfate), or carboxyl ionophores (monensin). Uptake and incorporation are completely abolished by the nonionic detergent Triton X-100 above its critical micellar concentration (0.02%). Transfer from the acetyl intermediate to free Neu5Ac is blocked by DEP, but not by CoA (at up to 5 m*M*).

Catalytic Properties. The uptake and incorporation of acetyl groups into endogenous glycoproteins show an apparent K_m of 2.8 μM for AcCoA, with a V_{max} of 15.4 pmol/min/mg protein. The kinetics of concomitant accumulation of free acetate inside the vesicles are very similar (K_m 2.9 μM, V_{max} 30.6 pmol/min/mg). All other data (see above) suggest that the transfer to endogenous acceptors and the accumulation of free acetate represent the same process. The combined process has a K_m of 2.9 μM for AcCoA, with a V_{max} of 45 pmol/min/mg. The transfer from the acetyl intermediate to free sialic acid shows a K_m of 13.9 m*M* for Neu5Ac, with a V_{max} of 0.57 pmol/min/mg protein. Thus, the transfer to endogenous glycoprotein acceptors appears to be far more efficient than that to the exogenously added free sugar.

Subcellular Localization. Since a reliable assay of the *O*-acetyltransferase using an exogenous acceptor has not been developed, we could not study its subcellular distribution directly. We therefore used the endogenous acceptor assay (Assay 1). Crude and purified vesicles obtained during the Golgi purification were incubated with [^{3}H]acetyl-CoA and CMP[^{14}C]Neu5Ac, and the labeled membranes were solubilized and treated with neuraminidase to determine incorporation into endogenous glycoproteins in each case. In comparison to the uptake and incorporation of CMP[^{14}C]Neu5Ac, the utilization of [^{3}H]acetyl-CoA to acetylate endogenous sialic acids showed a similar enrichment.

A previous chapter in this series suggested that the *O*-acetyltransferase might be equally distributed between the membrane and cytosol fractions.[9] For the reasons indicated above, we could not directly assay for a transfer-

[9] R. Schauer, this series, Vol. 50, p. 374.

ase activity in the cytosol. However, in view of the novel transmembrane mechanism of the Golgi acetyltransferase, it can be assumed that if a cytosolic transferase exists, it must be a completely different enzyme.

Acknowledgments

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[34] Microsomal Sulfation of Proteochondroitin, Chondroitin, and Chondroitin Oligosaccharides

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Chondroitin sulfate, a polymer of glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc), can be sulfated on either or both the 4 and 6 positions of the GalNAc and on the 2 position of the GlcA. Accordingly there are separate sulfotransferases that transfer the sulfate from PAPS[1] (the active sulfate donor) to these residues. Chondroitin sulfate is found in most, if not all, tissues and cells, and chondroitin sulfotransferase activities have been observed from multiple sources. The highest activities have been found in microsomal fractions,[2-7] although activity has also been found in soluble fractions,[3,8-13] indicating that the enzymes can be solubilized fairly

[1] PAPS, 3′-Phosphoadenylylphosphosulfate; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-OS, 2-acetamido-2-deoxy-3-*O*-(β-D-Glc-4-enepyranosyluronic acid)-D-galactose; MES, 2-(*N*-morpholino)ethanesulfonic acid.

[2] Chondroitin polymerization and sulfation activity appear to reside in the Golgi. However, detailed examinations of Golgi fractions have not been performed.

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[7] D. M. Delfert and H. E. Conrad, *J. Biol. Chem.* **260**, 14446 (1985).

[8] F. D'Abramo and F. Lipmann, *Biochim. Biophys. Acta* **25**, 211 (1957).

[9] S. Suzuki, this series, Vol. 8, p. 496.

[10] Y. Nakanishi, M. Shimizu, K. Otsu, S. Kato, M. Tsuji, and S. Suzuki, *J. Biol. Chem.* **256**, 5443 (1981).

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