Ubiquitous 9-O-Acetylation of Sialoglycoproteins Restricted to the Golgi Complex*

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9-O-Acetylation of sialic acid is known as a cell type-specific modification of secretory and plasma membrane glycoconjugates of higher vertebrates with important functions in modulating cell-cell recognition. Using a recombinant probe derived from influenza C virus hemagglutinin, we discovered 9-O-acetylated protein in the Golgi complex of various cell lines, most of which did not display 9-O-acetylated sialic acid on the cell surface. All cell lines expressed a sulfated glycoprotein of 50 kDa (sgp50) carrying 9-O-acetylated sialic acids, which was used as a model substrate. Like gp40, the major receptor for influenza C virus of Madin-Darby canine kidney I cells, sgp50 is 9-O-acetylated on O-linked glycans. However, gp40 was not 9-O-acetylated when expressed in Madin-Darby canine kidney II or COS-7 cells. The results demonstrate the existence of two 9-O-acetylation machineries for O-glycosylated proteins with distinct substrate specificities. The widespread occurrence of 9-O-acetylated protein in the Golgi further suggests an additional intracellular role for this modification.

Sialic acids constitute a family of acidic monosaccharides typically found at terminal positions of glycoconjugates. Their diversity is mainly the result of enzymatic acetylation of hydroxyl groups at carbons 4, 7, 8, and 9 (1). 9-O-Acetylation is the most prevalent modification of sialic acids of glycoproteins and gangliosides in higher vertebrates. It is a tissue-specific and tightly regulated modification involved in various important processes such as cell-cell interaction and development (2–4). For example, binding of the B-cell adhesion molecule Siglec-2 (CD22β) to sialylated ligands on the surface of thymocytes is inhibited by 9-O-acetylation (5). Furthermore, 9-O-acetylation was found to be crucial at the two-cell stage of murine development and later in the organization of the retina (6). 9-O-Acetyl sialic acid also serves as the receptor for influenza C virus (7), a group of corona viruses (8), and hemagglutinating encephalomyelitis virus (9) and thus appears to be a major determinant of their cell tropism (10).

O-Acetyl transferases for sialic acids have resisted purification or cloning thus far. However, their activity was localized to the trans side of the Golgi apparatus (11). By “freeze-frame” analysis with isolated membranes of rat hepatocytes, 9-O-acetylation was shown to occur in a compartment of the trans-Golgi network separate from that of sialylation and galactosylation (11). In this system, 9-O-acetylation was enriched on membrane-bound but not soluble cargo proteins leaving the trans-Golgi network.

9-O-Acetylation is specific for only a subset of sialoglycoproteins expressed in a given cell type, indicating that substrate recognition by 9-O-acetyl transferase(s) extends beyond the sialic acid itself. The 9-O-acetylation patterns of different cell types are thus likely to be the result of differential expression of substrates and/or transferase(s). Very few protein substrates for 9-O-acetylation of sialic acids have been identified at the molecular level, and all of them are sialomucins: bovine submaxillary mucin, CD43 and CD45RB of T lymphocytes (12), and gp40, the major receptor of influenza C virus in Madin-Darby canine kidney (MDCK1 type I cells (13, 14).

Influenza C virus was found to be extremely useful in detecting and characterizing 9-O-acetylated sialoglycoproteins and gangliosides. The viral 9-O-acetyl sialic acid binding activity (the hemagglutinin, H) is part of a multifunctional spike protein, HEF, which also contains a receptor destroying acetylerase activity (E) and a fusion function (F) (15). The entire virus has been used to detect 9-O-acetyl sialic acid in tissues and cell lines and on Western blots (13, 14, 16–19). In addition, a chimeric protein, CHE-Fc, consisting of the influenza C virus hemagglutinin-esterase domains fused to the Fc region of human IgG1, was constructed as a specific tool for the detection of 9-O-acetylated sialoglycoconjugates in histochemistry, Western blots, thin-layer chromatography overlays (20), enzyme-linked immunosorbent assay, flow cytometry (12), and even immunogold electron microscopy (11).

Here, we have used CHE-Fc as a probe to analyze the cellular distribution of 9-O-acetylated glycoproteins in a variety of cell lines by fluorescence microscopy. We discovered that all cell lines tested, including some that were previously shown to be resistant to infection by influenza C virus and considered to lack 9-O-acetyl sialic acid, stained positive intracellularly in the Golgi apparatus. A sulfated glycoprotein of 50 kDa could be precipitated by CHE-Fc in all cases and was useful as a model substrate of this ubiquitous, intracellular 9-O-acetylation. Our results indicate that differential 9-O-acetylation patterns in various cell types depend on differences in both the substrates and the acetylation machineries.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK I cells were obtained from G. Herrler (Tierärztliche Hochschule, Hannover, Germany), and calf primary aortic endothelial cells were obtained from K. Pfeider (Biozentrum, Universität Basel, Basel, Switzerland). These cell lines, as well as MDCK II, COS-1, CaCo2, HepG2, HEK-293, HeLa, NIH-3T3, BHK-21, CHO-K1, and CHO, Chinese hamster ovary; Ni2+-NTA, nickel-nitrioltriacetic acid; MAH, Maackia amurensis hemagglutinin; GalNAc, N-acetylgalactosamine.

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1 The abbreviations used are: MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; SNA, Sambucus nigra agglutinin; CHO, Chinese hamster ovary; Ni2+-NTA, nickel-nitrioltriacetic acid; MAH, Maackia amurensis hemagglutinin; GalNAc, N-acetylgalactosamine.

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CHO-Lecl cells, were grown in Eagle's minimal essential medium or Dulbecco's modified essential medium with 10% fetal calf serum at 37 °C with 7.5% CO₂. The media were supplemented with 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**CHE-Fc Production**—CHE-Fc, the chimera of influenza C virus hemagglutinin (HA) receptor binding site and the Fc region of mouse IgG, was produced in stably transfected HEK-293 cells provided by A. Varki (University of California, San Diego, CA). The cells were adapted to the protein-free medium CHO-S-SFM II (Invitrogen) and grown in a CELLine CL 350 device (Integra Bioscience). Conditioned medium was collected twice a week and kept at 4 °C until purification. CHE-Fc was isolated using protein A-Sepharose as described previously (20). The probe was concentrated by centrifugation in a Centricon-50 filter device (Amicon). To inactivate the esterase activity, CHE-Fc was incubated on ice for 30 min with 1 mM diisopropyl fluorophosphate, producing CHE-FcD. To improve the binding efficiency of the probe, inactivation was repeated, followed by dialysis against PBS. CHE-FcD was stable at 4 °C for several months.

**Immunofluorescence**—For indirect immunofluorescence staining, cells were grown on 14-mm glass coverslips, fixed with 3% paraformaldehyde for 20 min at room temperature, washed in PBS, and quenched with 50 mM NH₄Cl in PBS. To permeabilize the cells, the cells were then incubated with 0.1% Triton-X100 for 10 min at room temperature. Alternatively, cells were fixed with methanol and acetone according to Fudenberg and Futerman (19). The samples were then separated by SDS-PAGE, Trichloroacetic acid precipitation, and electrophoresis in a Centricon-50 filter device (Amicon). To inactivate the esterase activity, CHE-Fc was incubated on ice for 30 min with 1 mM diisopropyl fluorophosphate, producing CHE-FcD. To improve the binding efficiency of the probe, inactivation was repeated, followed by dialysis against PBS. CHE-FcD was stable at 4 °C for several months.

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**Western Blot Analysis**—Cells were split in two halves, one of which was incubated with 0.1% Triton-X100 for 15 min at 37 °C and neutralized with HCl. Both samples were then separated by SDS-PAGE and electrophoresed onto polyvinylidine difluoride membrane (Millipore). The membrane was blocked with 5% bovine serum albumin in PBS and incubated for 4°C with 3 μg/ml CHE-FcD in PBS/1% bovine serum albumin. After several washes in PBSB, PBS, and water, the coverslips were mounted in Mowiol 4–88 (Hoechst) containing 2.5% 1,4-diazobicyclo-(2,2,2)-octane and analyzed using a Zeiss Axioskop microscope. For double immunofluorescence, the fixed cells were also incubated with mouse monoclonal antibodies against giantin (a gift from H.-P. Hauri, Biozentrum) followed by incubation with Cy3-labeled anti-mouse IgG secondary antibodies.

**Results**

**Intracellular 9-O-Acetylated Sialic Acid Detected with CHE-FcD Fluorescence Microscopy**—CHE-Fc, the fusion protein of influenza C virus hemagglutinin-esterase with the constant region of human IgG, efficiently binds to 9-O-acetylated sialic acid if the esterase is inactivated by diisopropyl fluorophosphate treatment (20). We have used the resulting CHE-FcD as a probe for fluorescence microscopy in combination with a fluorescein isothiocyanate-labeled anti-human IgG antibody on MDCK I and II cells (Fig. 1). Previous studies using whole influenza C viruses have shown that MDCK I cells contain abundant 9-O-acetylated sialoglycoconjugates on their surface, mediating efficient infection, whereas the closely related MDCK II cells do not (13). As shown in Fig. 1A, unpermeabilized MDCK I cells were strongly labeled by CHE-FcD on the cell surface. Upon permeabilization, additional perinuclear staining became apparent (Fig. 1B), most likely corresponding to the trans-Golgi, whereas cell surface staining takes place. The specificity of staining for 9-O-acetylated sialic acid was demonstrated using CHE-Fc with the active esterase (Fig. 1C). In untreated cells, CHE-FcD hydrolyzed the 9-O-acetyl esters, staining was eliminated. Consistent with the experiments using viral infection, MDCK II cells were completely negative for CHE-FcD staining at the plasma membrane (Fig. 1E). In permeabilized cells, however, a strong perinuclear signal was discovered (Fig. 1F), which was specific for 9-O-acetylation because it was eliminated when
control for specificity, the cells in (E–H) were fixed with paraformaldehyde and permeabilized with Triton X-100 (B, C, F, and G) or not permeabilized (A and E). To detect 9-O-acetyl sialic acid, the cells were stained with CHE-FcD (A, B, E, and F) and a fluorescent antibody against the Fe portion of the probe. As a control for specificity, the cells in C and G were stained with CHE-Fc, in which the acetyl esterase was not inactivated by diisopropyl fluorophosphate treatment. In D and H, the cells were fixed with methanol and acetone to extract glycolipids and then stained with CHE-FcD. Bar, 20 μm.

untreated CHE-Fc was used (Fig. 1G). Even when the cells were fixed with methanol and acetone under conditions that have been shown to extract glycolipids (21), the labeling pattern was retained, indicating that the molecules detected by CHE-FcD are primarily glycoproteins.

A similar staining pattern was observed in a variety of different cell lines derived from different tissues and species (Table I and Fig. 2). None of these cell lines showed any detectable CHE-FcD labeling at the cell surface, yet all of them showed perinuclear staining. Intracellular 9-O-acetylation of sialoglycopolypeptides is thus a very common, if not ubiquitous, modification.

The intracellular staining pattern is reminiscent of the Golgi apparatus. To test this directly, calf primary aortic endothelial cells were colabeled with CHE-FcD and an antibody against the Golgi marker giantin (29). Both probes stained the same structures (Fig. 3, A and B). Even upon incubation with brefeldin A, which causes a dramatic redistribution of Golgi elements (30), the labeling pattern of both probes was affected in an identical manner (Fig. 3, C and D).

Identification of a Sulfated 9-O-Acetylated Glycoprotein of 50 kDa—To visualize 9-O-acetylated glycoproteins, proteins in cell lysates were subjected to SDS-gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with CHE-FcD (Fig. 4). As a control for specificity, half the samples were treated with mild base (0.1 M NaOH for 15 min at 37 °C) before analysis, which is sufficient to hydrolyze the O-acetyl groups on sialic acid. Accordingly, bovine submaxillary mucin, an established 9-O-acetylated sialoglycoprotein, was recognized by CHE-FcD before but not after mild base treatment (Fig. 4, lanes 1 and 2). In MDCK I cells (Fig. 4, lanes 7 and 8), the major 9-O-acetylated protein has a molecular mass of ~40 kDa, corresponding to gp40, the major influenza C virus receptor at the cell surface (13, 14). In addition, a second specific band with an apparent molecular mass of ~50 kDa could be detected. Proteins with the same electrophoretic mobility were also observed in MDCK II and HEK-293 cells (Fig. 4).

Table I

| Cell line (origin) | Cell surface | Golgi 9-O-AcSia | 9-O-Acetylated sgp50 |
|------------------|--------------|----------------|---------------------|
| MDCK I (kidney, dog) | +            | +             | +                   |
| MDCK II (kidney, dog) | –           | +             | n.d.                |
| BHK-21 (kidney, hamster) | –           | +             | +                   |
| CaCo2 (intestine, human) | –           | +             | +                   |
| CHO-K1 (ovary, hamster) | –           | +             | +                   |
| COS-7 (kidney, monkey) | –           | +             | +                   |
| CPAE (endothelia, bovine) | –           | +             | +                   |
| HEK-293 (kidney, human) | –           | +             | +                   |
| HeLa (cervix, human) | –           | +             | +                   |
| HepG2 (liver, human) | –           | +             | +                   |
| NIH-3T3 (fibroblast, mouse) | –           | +             | +                   |

*Expression of a sulfated, 9-O-acetylated sialoglycoprotein (sgp50) as determined by precipitation from lysed, [35S]sulfate-labeled cells using CHE-FcD and protein A-Sepharose as in the experiments shown in Fig. 5.

n.d., not determined.
4, lanes 3–6), where they constitute the predominant 9-O-acetylated protein. Additional weak bands were not sensitive to mild base treatment and thus constitute unspecific signals.

As an alternative detection method, CHE-FcD coupled to protein A-Sepharose was used to directly precipitate 9-O-acetylated proteins from metabolically labeled cells. Using [35S]methionine for labeling led to considerable nonspecific background (data not shown). However, when cells were labeled with [35S]sulfate, the pattern of radioactive proteins specifically precipitated with CHE-FcD was strikingly simple (Fig. 5).

In all cell lines tested, a single major sulfated protein of ~50 kDa was detected (Fig. 5, lanes 1–12; Table I), which may correspond to the major 50-kDa protein detected by CHE-FcD on Western blots (Fig. 4). This protein was called sgp50 (for sulfated glycoprotein of 50 kDa).

To test whether sgp50 is a membrane protein, [35S]sulfate-labeled HEK-293 cells were extracted with 0.1% saponin (Fig. 5, lanes 15 and 16), which allows soluble polypeptides to be released into the medium, whereas the membranes remain sufficiently intact to retain integrated proteins (26). Alternatively, the labeled cells were subjected to alkaline extraction, which solubilizes even peripheral proteins, whereas integral membrane proteins remain pelletable with the membranes (Fig. 5, lanes 13 and 14). With both procedures, sgp50 remained with the membranes and was not released into the supernatant, indicating that it is an integral membrane protein.

gp40 and sgp50 Are 9-O-Acetylated by Different Machines—MDCK II cells and other cell lines may lack 9-O-acetylated sialic acids in the plasma membrane because they do not express plasma membrane substrates for 9-O-acetyl transferase. To test this possibility, gp40, the major 9-O-acetylated glycoprotein of MDCK I cells, was expressed in some of these cell lines. To allow simultaneous analysis of gp40 and endogenous sgp50 by 35S sulfation, we modified the amino terminus of gp40 on the DNA level by adding a triple tag sequence consisting of a His6 sequence for Ni2+/NTA-agarose precipitation (Fig. 6A), lanes 1–6). gp40HMY was expressed in both cell lines to a similar extent as shown by Ni2+-NTA-agarose precipitation (Fig. 6A, lanes 1 and 2). As expected, 35S-sulfated gp40HMY was 9-O-acetylated in MDCK I cells and thus isolated with CHE-FcD like sgp50 (Fig. 6B), lane 1–2). The indicated cell lines were labeled with [35S]sulfate, lysed, and treated with mild base (+ NaOH) to deacetylate sialic acids or mock-treated (−). The proteins were separated by SDS-gel electrophoresis, immobilized, and probed for 9-O-acetylated sialic acid using CHE-FcD, followed by horseradish peroxidase-conjugated antihuman IgG and chemiluminescence detection. Bovine submaxillary mucin (BSM) was used as a positive control. The positions of marker proteins are indicated with their molecular masses in kDa.

FIG. 4. Detection of 9-O-acetyl sialoglycoproteins by Western analysis using CHE-FcD. Cell lysates from HEK-293 and MDCK I and II cells were treated with mild base (+ NaOH) to deacetylate sialic acids or mock-treated (−). The proteins were separated by SDS-gel electrophoresis, immobilized, and probed for 9-O-acetylated sialic acid using CHE-FcD, followed by horseradish peroxidase-conjugated antihuman IgG and chemiluminescence detection. Bovine submaxillary mucin (BSM) was used as a positive control. The positions of marker proteins are indicated with their molecular masses in kDa.

FIG. 5. Identification of a sulfated 9-O-acetylated glycoprotein of 50 kDa. The indicated cell lines were labeled with [35S]sulfate, lysed, and treated with mild base (+ NaOH) to deacetylate sialic acids or mock-treated (−) lanes 1–12). 9-O-Acetylated sialoglycoproteins were then isolated using CHE-FcD and protein A-Sepharose and analyzed by SDS-gel electrophoresis and fluorography. To test for membrane integration, labeled HEK-293 cells were subjected to alkaline extraction (Alk.) and separation into a membrane pellet (P) and a supernatant (S) (lanes 13 and 14). Alternatively, the labeled cells were extracted with 0.1% saponine (Sap.) and separated into a soluble saponin extract (S) and the rest of the cells (C). The fractions were analyzed as described above. The arrowhead indicates the 50 kDa position.

FIG. 6. gp40HMY is 9-O-acetylated in MDCK I and CHO cells, but not in MDCK II or COS-7 cells. A. MDCK I and II, CHO-K1, and COS-7 cells expressing gp40HMY were labeled with [35S]sulfate, lysed, and incubated with Ni2+-NTA-agarose (Ni) or CHE-FcD/protein A-Sepharose (CHE) to isolate gp40HMY or glycoproteins with 9-O-acetylated sialic acids, respectively (lanes 1–6). In the case of COS-7 cells, Ni2+-NTA-agarose and CHE-FcD purification were performed successively. The positions of sgp50 and gp40 are indicated. The arrow points at the heterogeneous high molecular weight forms of gp40HMY. B, to analyze the nature of the high molecular weight forms of gp40HMY, [35S]sulfate-labeled gp40HMY from gp40HMY-expressing MDCK II cells was isolated with Ni2+-NTA-agarose (Ni) and then incubated with (Ch) or without (−) chondroitinase before SDS-gel electrophoresis and fluorography. The ~90-kDa band (asterisk) most likely represents covalent dimers of gp40HMY generated by oxidation during chondroitinase incubation. The positions of sgp50 and gp40 are indicated.

In CHO cells, however, only sgp50, and not gp40HMY, was recognized by CHE-FcD (Fig. 6A, lane 4). The machinery for 9-O-acetylation of sgp50 therefore does not recognize gp40. The same negative result was also obtained in transfected COS-7 cells (Fig 6A, lanes 5 and 6), where gp40HMY
runs with slightly lower mobility, probably due to different glycosylation.

Does differential 9-O-acetylation of gp40 and sgp50 correlate with a major difference in glycosylation of the two substrates? To answer this question, we analyzed the two model substrates with respect to the presence of N- or O-glycosylation and the linkages of their sialic acids. gp40 is a mucin-like protein with extensive O-linked glycosylation, but without N-linked glycans (13, 14). On our gels, we always observed a diffuse signal around 70–120 kDa (arrow in Fig. 6, A and B) in addition to the compact band of 40 kDa when gp40 or gp40\(^{\text{HMY}}\) was isolated. When gp40\(^{\text{HMY}}\) expressed in MDCK I cells was labeled with \(^{[35S]}\)sulfate, purified using Ni\(^{2+}\)-NTA-agarose, and incubated with chondroitinase, the diffuse band collapsed into the 40-kDa band (Fig. 6B, lane 2), indicating that a fraction of the proteins carried chondroitin sulfate chains. However, 9-O-acetylation of gp40\(^{\text{HMY}}\) was obviously independent of glycosaminoglycan addition (Fig. 6A, lane 3).

To test sgp50 for O-linked glycans, sgp50 was first precipitated with CHE-FcD from \(^{[35S]}\)sulfate-labeled MDCK I cells (Fig. 7A, lane 1), desialylated (lane 2), and then incubated with O-glycosidase (lane 3). A shift in mobility upon SDS-gel electrophoresis indicated the presence of O-glycans. Similarly, labeled sgp50 precipitated with CHE-FcD was incubated with or without endoglycosidase F and analyzed either directly (Fig. 7A, lanes 4 and 5) or after a second CHE-FcD precipitation (lanes 6 and 7). Endoglycosidase F digestion produced two forms with increased electrophoretic mobility, indicating the presence of at least two N-linked glycans in sgp50. Even the lowest of the two forms was still recognized by CHE-FcD and thus carried 9-O-acetyl sialic acid (Fig. 7A, lane 7). As an alternative approach, MDCK II cells were labeled with \(^{[35S]}\)sulfate in the presence of tunicamycin under conditions that completely inhibit N-glycosylation. Using CHE-FcD, a 9-O-acetylated form of sgp50 was precipitated with a mobility identical to that of the lowest endoglycosidase F digestion product (Fig. 7A, lane 9). These results show that sgp50 carries both N- and O-linked glycans and suggest that the O-linked glycans contain 9-O-acetylated sialic acid.

To analyze gp40 and sgp50 with respect to the most common linkages of sialic acids, MDCK I and MDCK II cells expressing gp40\(^{\text{HMY}}\) were labeled with \(^{[35S]}\)sulfate, lysed, and subjected to precipitation with CHE-FcD/protein A-Sepharose, SNA-agarose, or MAH-agarose (lanes 8–13) before SDS-gel electrophoresis and fluorography. A small amount of full-size sgp50 is often detected in the tunicamycin-treated sample, most likely corresponding to molecules that were synthesized before incubation with the inhibitor and incorporated \(^{[35S]}\)sulfate during the labeling period. In MDCK I and II cells expressing gp40\(^{\text{HMY}}\) were labeled with \(^{[35S]}\)sulfate, lysed, and incubated with MAH-agarose or SNA-agarose (lanes 1–4). From other MDCK I lysates, 9-O-acetylated proteins were first isolated with CHE-FcD/protein A-Sepharose and then released by mild base treatment and subjected to a second isolation with MAH-agarose or SNA-agarose or to no further treatment (lanes 5–7). The positions of sgp50 and gp40 are indicated. The circles indicate unrelated, sulfated proteins with a2–6-linked sialic acids.

Fig. 7. Characterization of glycans on gp40\(^{\text{HMY}}\) and sgp50 in MDCK cells. A, 9-O-acetylated proteins of \(^{[35S]}\)sulfate-labeled MDCK I cells were isolated with CHE-FcD/protein A-Sepharose, desialylated by incubation with neuraminidase (Neu), and incubated with O-glycosidase (OG, lanes 1–3). Alternatively, CHE-FcD-precipitated labeled proteins were boiled in SDS, incubated without (−) or with endoglycosidase F (EF), and analyzed immediately (lanes 4 and 5) or after a second CHE-FcD precipitation (lanes 6 and 7). To block N-glycosylation, MDCK II cells were treated with (Tu) or without (−) tunicamycin, labeled with \(^{[35S]}\)sulfate, lysed, and subjected to precipitation with CHE-FcD/protein A-Sepharose, MAH-agarose, or SNA-agarose (lanes 8–13) before SDS-gel electrophoresis and fluorography. A small amount of full-size sgp50 is often detected in the tunicamycin-treated sample, most likely corresponding to molecules that were synthesized before incubation with the inhibitor and incorporated \(^{[35S]}\)sulfate during the labeling period. B, MDCK I and II cells expressing gp40\(^{\text{HMY}}\) were labeled with \(^{[35S]}\)sulfate, lysed, and incubated with MAH-agarose or SNA-agarose (lanes 1–4). From other MDCK I lysates, 9-O-acetylated proteins were first isolated with CHE-FcD/protein A-Sepharose and then released by mild base treatment and subjected to a second isolation with MAH-agarose or SNA-agarose or to no further treatment (lanes 5–7). The positions of sgp50 and gp40 are indicated. The circles indicate unrelated, sulfated proteins with a2–6-linked sialic acids.

To show more directly that 9-O-acetylation occurred on O-glycans in both gp40 and sgp50, cells were treated with either benzyl-GalNAc to inhibit sialylation on O-glycans (27) or \(\beta-D\)-xyloside to block glycosaminoglycan synthesis on proteins (28). Treatment of MDCK I cells expressing gp40\(^{\text{HMY}}\) with benzyl-GalNAc did not affect synthesis of gp40\(^{\text{HMY}}\) or its mobility upon gel electrophoresis, as judged by precipitation with Ni\(^{2+}\)-NTA-agarose (Fig. 9, lanes 1 and 2). Removal of sialic acid has previously been observed not to alter its electrophoretic mobil-
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**Fig. 8.** Glycosylation of sgp50 in CHO cells. CHO-K1 cells and CHO-Lec1 cells, which lack sialic acids on N-glycans, were labeled with [35S]sulfate, lysed, and subjected to precipitation with CHE-FcD/protein A-Sepharose, MAH-agarose, or SNA-agarose. Samples were analyzed by SDS-gel electrophoresis and fluorography. The positions of sgp50 and gp40 are indicated. The open circle indicates a second sulfated 9-O-acetylated glycoprotein of ~38 kDa in CHO cells. The closed circles indicate two unrelated MAH-positive sulfated glycoproteins.

**Fig. 9.** 9-O-Acetylation of O-linked sialic acid in both gp40 and sgp50. MDCK I cells expressing gp40(LMY) (lanes 1–8) or normal MDCK I cells (lanes 9–12) were incubated for 18 h with or without benzyl-GalNAc (G) or 4-methylumbelliferyl xyloside (X) to inhibit sialylation of O-glycans or synthesis of glycosaminoglycans, respectively. The cells were then labeled with [35S]sulfate, lysed, and subjected to precipitation with CHE-FcD/protein A-Sepharose or N2F–NTA-agarose. To test the effect on sgp50 in the absence of X-glycans (sgp50-N), the cells in lanes 9–12 were treated with tunicamycin (Tu) before and during labeling.

It is not surprising per se that 9-O-acetylated proteins can be detected in the Golgi because this modification has previously been shown to occur in Golgi membranes of rat liver (38, 39), more precisely in a compartment of the trans-Golgi network separate from the compartment of sialylation (11). Secretory and plasma membrane proteins thus pass through this compartment and may acquire 9-O-acetylation as one of the last modifications before arrival at the cell surface. The known functions of 9-O-acetylation in cell-cell and host-pathogen interactions as well as the relative abundance of 9-O-acetyl sialic acids in secretory proteins (20) pointed to a predominantly extracellular role of this modification. The striking presence of 9-O-acetylated proteins in the Golgi and their absence from the cell surface also suggest an intracellular function.

Western analysis revealed a major 9-O-acetylated glycoprotein of ~50 kDa in HEK-293 and MDCK II cells, which was also prominently present in MDCK I cells besides gp40. Using sulfate labeling, a sulfated membrane protein of the same size, sgp50, could be precipitated with CHE-FcD in all cell lines analyzed. Because sulfation yielded good sensitivity and low background, sgp50 and gp40 tagged with a sialylation site (gp40(LMY)) were useful tools to analyze the glycosylation characteristics of these two 9-O-acetylation substrates.

The simplest explanation for the absence of 9-O-acetylated proteins on the surface of a cell line is that the cells do not express substrates that are transported to the plasma membrane. However, gp40(LMY), which is modified in MDCK I cells, was not acetylated when expressed in MDCK II and COS-7 cells. The 9-O-acetyl transferase responsible for acetylation of the Golgi-resident substrate(s) apparently does not recognize gp40 as a substrate. There are thus two distinct 9-O-acetylation machineries: a ubiquitous one with mainly intracellular substrates including sgp50, and a second one present in MDCK I cells that recognizes gp40. In MDCK II and COS-7 cells, this latter machinery and its substrate gp40 are missing.

Previously, it has been shown that 9-O-acetyl transferases can have clear linkage specificity. CHO-K1 cells, which lack α2–6 linked sialic acids on N-linked glycans (34) and do not bind CHE-FcD at the plasma membrane, presented 9-O-acetyl sialic acid on the cell surface upon expression of the α2–6 sialyltransferase for N-glycans (ST6Gal I), but not when the competing α2–3 sialyltransferase (ST3Gal III) was transfected (35). The parental CHO cells thus have a 9-O-acetyl transferase for N-linked Siaα2–6Gal but essentially no corresponding substrates. This transferase is therefore not a candidate en-
zyme for the 9-O-acetylation of sgp50 or gp40. There are thus at least three distinct 9-O-acetylation machineries for glycoproteins, modifying gp40, sgp50, or N-linked Siaα2→6Gal, respectively.

gp40 and sgp50 are 9-O-acetylated on O-linked glycans. Because both proteins are recognized by MAH, they positively contain sialylated core 1 structures, i.e. Siaα2→3Galβ1→3GalNAc and/or Siaα2→3Galβ1→3(Siaα2→6)GalNAc, most probably in addition to other structures for which there is no direct evidence at present. Considering the high specificity of 9-O-acetylation as observed for example in MDCK I and II cells, substrate recognition of 9-O-acetyl transferases must reach beyond the sialic acid and its linkage and must include more extended, possibly rare glycan structures and/or contributions from the protein core. Our findings indicate that 9-O-acetylation is more widespread than previously known and may also have intracellular functions.

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37. mathematical model. The analysis of this model shows that the 9-O-acetylation of sialic acid residues is mediated by two distinct transferases, one acting on the Golgi apparatus and the other in the endoplasmic reticulum.

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