

Mechanism of Uptake and Incorporation of the Non-human Sialic Acid *N*-Glycolylneuraminic Acid into Human Cells*[§]

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***N*-Glycolylneuraminic acid (Neu5Gc) is a widely expressed sialic acid in mammalian cells. Although humans are genetically deficient in producing Neu5Gc, small amounts are present in human cells *in vivo*. A dietary origin was suggested by human volunteer studies and by observing that free Neu5Gc is metabolically incorporated into cultured human carcinoma cells by unknown mechanisms. We now show that free Neu5Gc uptake also occurs in other human and mammalian cells. Inhibitors of certain non-clathrin-mediated endocytic pathways reduce Neu5Gc accumulation. Studies with human mutant cells show that the lysosomal sialic acid transporter is required for metabolic incorporation of free Neu5Gc. Incorporation of glycosidically bound Neu5Gc from exogenous glycoconjugates (relevant to human gut epithelial exposure to dietary Neu5Gc) requires the transporter as well as the lysosomal sialidase, which presumably acts to release free Neu5Gc. Thus, exogenous Neu5Gc reaches lysosomes via pinocytic/endocytic pathways and is exported in free form into the cytosol, becoming available for activation and transfer to glycoconjugates. In contrast, *N*-glycolylmannosamine (ManNGc) apparently traverses the plasma membrane by passive diffusion and becomes available for conversion to Neu5Gc in the cytosol. This mechanism can also explain the metabolic incorporation of chemically synthesized unnatural sialic acids, as reported by others. Finally, to our knowledge, this is the first example of delivery to the cytosol of an extracellular small molecule that cannot cross the plasma membrane, utilizing fluid pinocytosis and a specific lysosomal transporter. The approach could, thus, potentially be generalized to any small molecule that has a specific lysosomal transporter but not a plasma membrane transporter.**

Sialic acid (Sia)¹ is a generic name for a family of acidic nine carbon sugars typically found as the outermost units of glycan chains on the vertebrate cellular glycocalyx and on secreted

glycoproteins (1, 2). Their location and widespread occurrence on all vertebrate cells allow them to be involved in processes such as pathogen binding, inflammation, immune response, and tumor metastasis (3–8).

There are more than 50 kinds of Sias known in nature (2, 9, 10). Most are derived via biosynthetic modification of a Sia called *N*-acetylneuraminic acid (Neu5Ac) (3, 4, 9). The addition of a single oxygen atom to the *N*-acetyl group of Neu5Ac gives a very common variation called *N*-glycolylneuraminic acid (Neu5Gc). The surfaces of most primate cell types studied to date are dominated by two major Sias, which are Neu5Ac and Neu5Gc (11, 12).

For a Sia molecule to get attached to glycoconjugates it must first be activated by conversion to the sugar nucleotide derivative cytidine monophosphate-Sia (CMP-Sia). Thus, Sias are converted to CMP-Sias in the nucleus, which then return to the cytosol to be transported into the Golgi apparatus, where they serve as high energy donors for attaching Sias to newly synthesized glycoconjugates on their way to the cell surface (13). The biosynthetic transformation of Neu5Ac to Neu5Gc occurs at this sugar nucleotide level, wherein the CMP-Neu5Ac hydroxylase (CMAH) catalyzes the transfer of an oxygen atom to CMP-Neu5Ac, generating CMP-Neu5Gc (14–18). CMP-Neu5Gc can then be transported into the Golgi apparatus and used in the same manner as CMP-Neu5Ac to add Neu5Gc to newly synthesized glycoconjugates. Indeed, these two nucleotide sugars appear to be used interchangeably by the Golgi CMP-Sia transporter and by the mammalian sialyltransferases, which transfer Sia residues to cell surface and secreted glycoconjugates (16, 19–21). Neu5Ac or Neu5Gc molecules that are released from glycoconjugates during lysosomal degradation processes can also be exported back into the cytosolic compartment by a specific transporter (22). There, they are both available as substrates for reconversion to their respective CMP-Sia forms. Again, there appears to be no major difference in their conversion by CMP-Sia synthases (19, 23). In this manner, Neu5Gc can be “recycled” for repeated use in Golgi sialylation reactions (16).

Although Neu5Gc is a major Sia in most mammalian cells, it was long thought to be absent from healthy human tissues (1). Indeed, humans are genetically unable to synthesize Neu5Gc due to an exon deletion/frameshift mutation in the human *CMAH* gene (13, 24, 25). It has been estimated that this mutation occurred in the hominid lineage ~2.5–3 million years

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¹ The abbreviations used are: Sia, sialic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; ManNGc, *N*-glycolylmannosamine; DMB, 1,2-diamino-4,5-methylene dioxybenzene; WT,

Wild-type; LMW, cytosolic low molecular weight fraction; HMW, high molecular weight fraction which represents Sia bound to the membrane and soluble protein fractions; TBS, Tris-buffered saline; CMAH, CMP-Neu5Ac hydroxylase; TH, total homogenate; MFI, median fluorescence intensity; HPLC, high performance liquid chromatography; FCS, fetal calf serum; PBS, phosphate-buffered saline; MS, mass spectroscopy; RT, room temperature; FITC, fluorescein isothiocyanate.

ago (26). One dramatic consequence of this human-specific genetic defect appears to have been the sudden unmasking of the CD33-related Siglecs during human evolution, since the ancestral condition of these molecules was to recognize Neu5Gc (27).

Despite the absence of any known alternative pathway for the synthesis of Neu5Gc in humans, various groups have used antibodies to claim the expression of Neu5Gc in human tumors, particularly in various carcinomas (28–34). Recent studies from our laboratory re-explored this issue, confirming prior reports of Neu5Gc expression in human cancers and extending the finding to normal human tissues, detecting small amounts of Neu5Gc in epithelial and endothelial cells of normal humans (35). Definitive confirmation resulted from releasing and purifying sialic acids from such tissues and identifying a fluorescent derivatized form of Neu5Gc by HPLC and mass spectrometry analysis (35). Moreover, we showed that exogenously added free Neu5Gc could be incorporated into cultured human carcinoma cells *in vitro* by unknown mechanisms (35). We also carried out oral ingestion studies of Neu5Gc in human volunteers, providing evidence that the Neu5Gc found in human tissues could be originating from dietary sources, particularly red meat and milk products. It now becomes critical to understand the pathway(s) for uptake and metabolic incorporation of Neu5Gc and its potential precursor, *N*-glycolylmannosamine (ManNGc) into human cells.

EXPERIMENTAL PROCEDURES

Materials—Neu5Gc and Neu5Ac were, respectively, purchased from Inalco Spa (Milano, Italy) and Pfantstiehl Laboratories, Inc. (Waukegan, IL). 1,2-Diamino-4,5-methylene dioxybenzene (DMB), chlorpromazine, genistein, nystatin, amiloride, and saponin were from Sigma-Aldrich. Premium human serum type AB was from Irvine Scientific (Santa Ana, CA). Neu5Ac aldolase came from ICN (Costa Mesa, CA). All the reagents used were HPLC grade.

Cell Lines—Caco-2 cells (human epithelial cells isolated from a primary colon carcinoma), normal human skin fibroblasts (CCD-919-SK), and Chinese hamster ovary (CHO-K1) cells were purchased from ATCC (Manassas, VA). Mutant human skin fibroblasts (GM05520, GM08496, and GM01718) were obtained from the Coriell Institute for Medical Research (Camden, NJ). Chimpanzee and human Epstein-Barr virus-transformed lymphoblasts were a gift from Dr. Peter Parham, Stanford University.

Cell Culture—Caco-2 cells were propagated in α -minimal essential medium containing Glutamax™ and a mixture of ribonucleosides and deoxyribonucleosides (Invitrogen) supplemented with 20% FCS. All the fibroblast cell lines and CHO-K1 cells were cultured in the same media supplemented, respectively, with 15% non-heat inactivated FCS or 10% heat-inactivated FCS. Chimpanzee and human Epstein-Barr virus-transformed lymphoblasts were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FCS or 15% human serum. All the cultures were maintained at 37 °C in a 5% CO₂ atmosphere. To deplete any remaining Neu5Gc from FCS, the cells were split and cultured before Neu5Gc feeding experiments for at least 4 days in α -minimal essential medium supplemented with an adequate percentage of heat-inactivated premium human serum instead of FCS (40). The cells were then maintained under the same conditions during the whole feeding experiment. The human serum was heat-inactivated at 56 °C for 30 min before use.

Preparation of ManNGc from Neu5Gc—ManNGc was prepared by incubating 73 μ mol of Neu5Gc with 624 units of lactate dehydrogenase, 30 μ mol of NADH, and 10 units of Neu5Ac aldolase, EC 4.1.3.3 (36), in 15 ml of 100 mM potassium phosphate buffer, pH 7.2. The incubation was carried out at 37 °C for 16 h. The ManNGc was separated from any unreacted Neu5Gc by passing the product serially over AG50WX-2 and AG1X-8 (Bio-Rad) ion-exchange resins as described previously (37). The run-through and five-column-volumes of water washes were collected and concentrated by freeze-drying. The reaction yield (91–98%) was followed by the disappearance of Neu5Gc using DMB derivatization of the reaction mixture and analysis by HPLC (see protocol described below).

Preparation and Purification of Sia from Bovine Submaxillary Mucin—A mixture of standard Sias were prepared from bovine submaxillary

lary mucin. Total mucins were extracted from frozen submaxillary glands as described previously (35). Sias then were released with mild acid, collected by dialysis (1000 daltons molecular mass cut-off), and purified on ion exchange columns under conditions determined to minimize loss of *O*-acetylation (37).

Neu5Gc and ManNGc Feeding Experiment—Neu5Ac, Neu5Gc, or ManNGc were dried, dissolved in the appropriate media supplemented with heat-inactivated human serum, sterilized using a Spin-X® (Corning Inc., Corning, NY), and then added to the cells. The pH of the media containing Sia was adjusted to neutrality using sterilized 1 M NaOH before starting the feeding experiment. Cells were cultured in the presence of up to 3 mM free Sia or ManNGc for 1 or 3 days at 37 °C. At the end of the feeding cells were washed with cold PBS, harvested either by scraping or with 2 mM EDTA for fibroblasts, and washed again with cold PBS before fractionation.

Fractionation of the Labeled Cells—Washed cell pellets were sonicated into 500 μ l of 20 mM sodium phosphate buffer or 20 mM Tris-HCl, pH 7.5, using 4 \times 15-s pulses of a sonicator cell disrupter, model Sonic Dismembrator (Fisher) at a probe setting of 3. The sonicate was centrifuged at 75 \times *g* for 15 min, and the pellet obtained consisted primarily of nuclei and unbroken cells. The pellet contained <5% of the incorporated sialic acid, as determined using a radioactive tracer (data not shown). The supernatant was, therefore, considered as the “total homogenate” fraction. A portion (20%) of the total homogenate fraction was taken for protein quantification and Sia analysis by DMB derivatization and HPLC analysis (see the protocol below). The remainder was centrifuged at 100,000 \times *g* for 1 h. The resulting pellet, called the “membrane” fraction, was then resuspended by sonication (15 s) in 200 μ l of sodium acetate buffer, pH 5.5. The 100,000 \times *g* supernatant, called the “soluble” fraction, was adjusted to 90% ethanol using absolute ice-cold ethanol and placed overnight at –20 °C. The flocculent precipitate, which represents the “soluble protein” fraction, was washed 3 times with 90% ice-cold ethanol and then resuspended in 200 μ l of water. The supernatant fluid representing the cytosolic low molecular weight (LMW) fraction was dried and brought up in 100 μ l with water before Sia analysis. All the Sias in these fractions were released with mild acid hydrolysis if necessary and then analyzed by HPLC after DMB derivatization. Protein quantification was performed on the total homogenate, membrane and soluble protein fractions by using the BCA protein assay kit from Pierce. In some experiments the resulting data obtained for the Sia bound to the membrane fraction and to the soluble proteins were pooled and presented here as a high molecular weight (HMW) fraction.

Sialic Acid Release, DMB Derivatization, and HPLC Analysis—The bound Sias from the total homogenate, membrane, and soluble protein fractions were released using 2 M acetic acid hydrolysis, 3 h at 80 °C (37). The released Sias or free Sias contained in the soluble LMW fraction were passed through a Microcon® YM-10 filter (Millipore, Bedford, MA) before DMB derivatization, performed according to Hara *et al.* (38). DMB-Sia derivatives from the different fractions were then analyzed by HPLC using a C18 column (Microsorb MV-TM 100 A, Varian). Isocratic elution was achieved using 7% methanol, 8% acetonitrile in water for 50 min at 0.9 ml/min flow. The eluant was monitored by fluorescence as described (38, 39).

Quantification of Sias—For all HPLC chromatograms, the quantification of Sias was done by comparison with known quantities of DMB-derivatized Neu5Gc and Neu5Ac used as standards and then reported in terms of pmol of Sia. For total homogenate, membrane, and cytosolic protein fractions, this number was expressed per mg of protein. Due to minor sample-to-sample variations in amounts and recoveries, we present the data in the figure as percent of Neu5Gc over total Sias rather than as absolute amounts. Full information on the actual amounts is presented in the Supplemental Material.

MS and MS/MS Analysis of DMB Derivatives—In some experiments the nature of the DMB derivatives of Sias was confirmed by mass spectrometry (39) on a Finnigan MAT HPLC with on-line mass spectrometry system using a model LCQ-Mass Spectrometer System A. A Varian C18 column was used and eluted in the isocratic mode with 8% acetonitrile, 7% methanol, 0.1% formic acid in water at 0.9 ml/min over 50 min. The eluant was simultaneously monitored by UV absorbance at 373 nm and by electrospray ionization mass spectrometry. The electrospray ionization settings used were capillary temperature of 210 °C, capillary voltage at 31 V, and lens offset voltage at 0 V. Spectra were acquired by scanning from *m/z* 150–2000 in the positive ion mode. In some instances, MS/MS was acquired by selecting the parent mass and using a 20% normalized collision energy. Data analysis was performed using the XCALIBUR data analysis program from the instrument manufacturer (26, 40).

Endocytosis Inhibition Experiments—Caco-2 or normal fibroblast cells were split and cultured in α -minimal essential medium supplemented, respectively, with 20 or 15% human serum for 4 days before starting the endocytosis drug inhibition experiments to deplete any Neu5Gc derived from FCS. Cells were then pretreated for 2 h with the specific inhibitors under the same culture conditions. Fresh media containing the same amount of inhibitor and 3 mM Neu5Gc was then added to the cells, which were incubated for 16 h or 3 days and finally harvested and fractionated as described above. Based on prior literature chlorpromazine, genistein, nystatin, and amiloride were used at final concentrations of 6 μ g/ml, 200 μ M, 25 μ g/ml, and 3 mM, respectively (41, 42).

Western Blot Analysis—Membrane proteins extracted from Neu5Gc-fed, ManNGc-fed, or non-fed human wild-type (WT) and mutant fibroblasts were separated by SDS-PAGE electrophoresis using an 8% polyacrylamide gel. The separated proteins were transferred onto nitrocellulose membrane, which was blocked overnight with Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Immunodetection was then performed by using our recently described anti-Neu5Gc antibody (35) (1:10,000 in TBS-T, 3 h, room temperature (RT)). Binding of the anti-Neu5Gc antibody was detected using a secondary horseradish peroxidase-conjugated donkey anti-chicken IgY antibody diluted at 1:30,000 in TBS-T for 45 min at RT (Jackson ImmunoResearch Laboratories, West Grove, PA). Final development of the blots was performed by using Supersignal West Pico ECL reagent (Pierce) and X-Omat Kodak film.

Flow Cytometry—Human WT and mutant fibroblasts grown in media with 10% FCS plus 20% horse serum for 3 days were lightly trypsinized (0.04% trypsin, 0.53 mM EDTA for 5 min) to release cells from the flasks. The cells were washed with PBS and then fixed overnight with 1% paraformaldehyde in PBS. Fixed cells were permeabilized or not with 0.1% saponin in PBS at RT for 20 min. Chicken anti-Neu5Gc antibody was added to cells at a 1:200 dilution in PBS and incubated at RT for 30 min. Cells were then washed with PBS and resuspended in FITC-conjugated goat anti-chicken IgY (1 μ g/100 μ l) (Southern Biotechnology Associates, Birmingham, AB) and allowed to incubate for 30 min at RT. Labeled cells were washed with PBS and resuspended in 500 μ l of PBS for analysis of FITC fluorescence on a FACSCalibur (BD Biosciences).

Fluorescence Microscopy—Human WT and mutant fibroblasts were grown on poly-D-lysine-coated glass Chamber Slides (Nalge Nunc International, Naperville, IL) with media containing 10% FCS plus 20% horse serum for 4 days. Cells were fixed onto slides using 1% paraformaldehyde in PBS for 30 min at RT before permeabilizing with 0.1% saponin for 20 min at RT. Chicken anti-Neu5Gc antibody was then added at 1:50 dilution in PBS along with 1 μ g of mouse anti-LAMP-1 (clone H4A3, Pharmingen) and incubated at RT for 1 h. Bound antibodies were then detected with FITC-goat anti-chicken IgY and Cascade Blue-goat anti-mouse IgG (each at 1 μ g/100 μ l) at RT for 1 h. Cells were washed with PBS and covered with Gel/Mount (Biomedica, Foster City, CA) before fluorescence imaging with a Zeiss Microscope at 400 \times magnification with emission filters at 400 and 520 nm for Cascade Blue and FITC, respectively.

RESULTS

Free Neu5Gc Can Be Taken Up by Human Epithelial Cells from an Exogenous Source and Incorporated into Different Subcellular Fractions—We recently presented evidence suggesting that the small amounts of Neu5Gc found in some human tissues originated from dietary sources and showed that human Caco-2 cells (human epithelial cells from a primary colon carcinoma) in culture could metabolically incorporate free Neu5Gc (35), as determined by a Western blot of a total homogenate, using an anti-Neu5Gc antibody. We found increasing incorporation of Neu5Gc in the total homogenate fraction of the cells over time, with the highest level reached after incubation with 3 mM Neu5Gc for 3 days. Moreover, Western blotting with an anti-Neu5Gc antibody demonstrated metabolic incorporation of Neu5Gc into glycoproteins of these cells (35). We have now studied the partitioning of the exogenous Neu5Gc into different subcellular fractions of these cells. Before feeding, Caco-2 cells were split and cultured in human serum instead of FCS to eliminate traces of Neu5Gc in the cells (see "Experimental Procedures" for details). Culture was continued for 3 days in

the presence of 3 mM Neu5Gc using 3 mM ManNGc and Neu5Ac as positive controls. Indeed, it has already been shown that Neu5Ac and ManNGc can be incorporated into cells and that ManNGc or its peracetylated form can be metabolized into Neu5Gc (43–45). After the 3-day feeding, the cells were harvested, and the Neu5Gc content of the different subcellular fractions was analyzed by DMB derivatization, HPLC, MS, and MS/MS analysis. As shown in Fig. 1A, the DMB-HPLC profiles of Sias released from the membranes of Caco-2 cells fed with 3 mM ManNGc or Neu5Gc presented two peaks that correspond to Neu5Gc and Neu5Ac by comparison with the retention times of standards. Cells that were not fed or fed with Neu5Ac had only one peak corresponding to Neu5Ac. These results were confirmed by liquid chromatography-MS and MS/MS analysis, as described previously (39). DMB-Neu5Gc and DMB-Neu5Ac adducts gave signals at m/z 442/424 and 426/408, respectively, representing molecular ions of DMB-derivatized Neu5Gc and Neu5Ac and their dehydrated forms (35, 39). Liquid chromatography-MS and MS/MS data obtained on DMB-derivatized Sias released from the membranes of Caco-2 cells non-fed or fed with Neu5Ac gave only a single ion at m/z 426 that can be broken down to 408 by MS/MS, confirming the presence of Neu5Ac and the absence of Neu5Gc. The same analysis on Neu5Gc or ManNGc fed Caco-2 cell membrane Sias gave ions at m/z 442 and 426, which are, respectively, dehydrated to 424 and 408 in MS/MS analysis. These analyses confirmed the presence of Neu5Gc associated specifically within the glycoconjugates of the membranes of Caco-2 cells fed with ManNGc or Neu5Gc. All other subcellular fractions were also studied using the same DMB-HPLC approach. Due to sample-to-sample variations in amounts and recoveries, we present the data in this and subsequent figures as percent of Neu5Gc over total Sias rather than as absolute amounts (the latter information is presented in the Supplemental Material). Fig. 1B summarizes the results showing that the total homogenate (TH), HMW fraction (HMW is the combination of membrane and soluble protein fractions), and cytosolic LMW fraction contain 58, 46, and 70% Neu5Gc, respectively. In the experiment presented here, the % of Neu5Gc in the membrane fraction of cells fed with Neu5Gc was lower compared with the one obtained for cells fed with ManNGc. This was not always the case, as we observed in other feeding experiments that free Neu5Gc can be as efficient as ManNGc and sometimes even better. The relative percentages obtained for the other fractions (total homogenate, LMW, and soluble protein) were similar in several repeated ManNGc and Neu5Gc feeding experiments.

The Uptake Mechanism of Free Neu5Gc Is Not Specific for Human Epithelial Cells—The above experiments showed that free Neu5Gc can be taken up by human epithelial carcinoma cells from the media and incorporated into different subcellular fractions such as membrane-bound glycoconjugates, soluble proteins, and low molecular weight compounds present in the cytosol. To see if this is a specialized mechanism in human carcinoma cells, we did similar Neu5Gc feeding experiments on other human cell types such as normal skin fibroblasts and neuroblastomas. We found that fibroblast cells can also take up free Neu5Gc from the media, albeit in a less efficient manner. As presented in Fig. 2A, 28, 39, and 41% Neu5Gc are present in the TH, HMW, and cytosolic LMW fractions of the human normal fibroblasts after Neu5Gc feeding. Lower levels (4, 19, 12% Neu5Gc) were already present in the same fractions when fibroblast cells were not incubated in presence of 3 mM Neu5Gc. This Neu5Gc is assumed to be derived from Neu5Gc on FCS glycoproteins used for cell culture before feeding experiments (see below). Human neuroblastoma cells could also incorporate Neu5Gc with an efficiency comparable with the Caco-2 cells

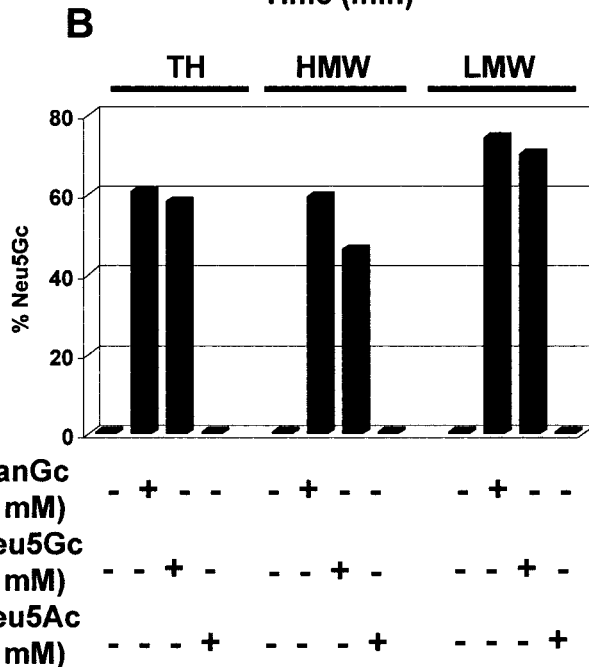
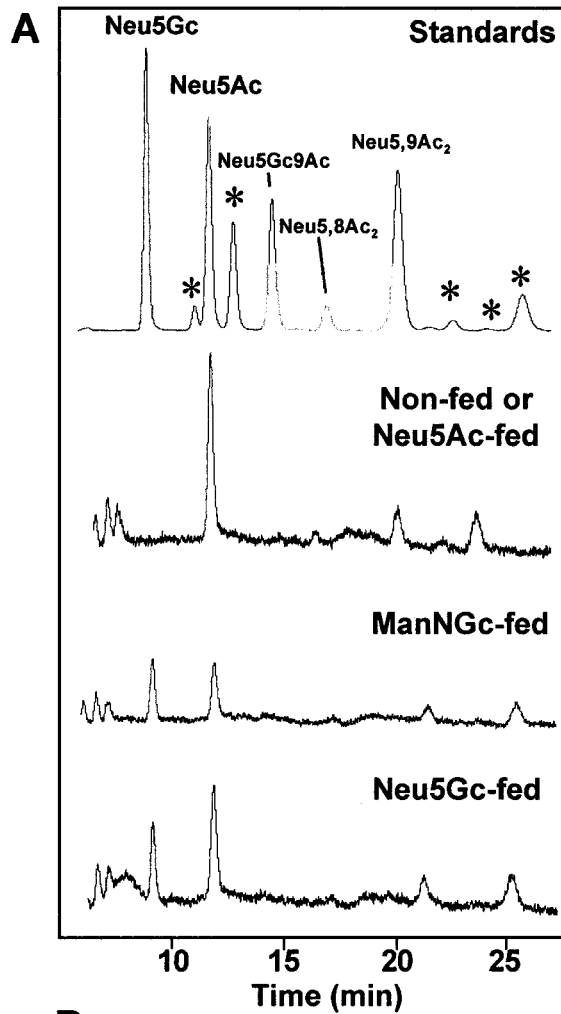


FIG. 1. Analysis of the incorporation of free Neu5Gc in human epithelial cells. Caco-2 cells were fed or not fed for 3 days with Neu5Gc, ManNGc, or Neu5Ac, each at 3 mM final concentration. The cells were harvested and fractionated, and the Sia content of the different fractions was analyzed by DMB derivatization and liquid chromatography analysis as described under "Experimental Procedures." **A**, DMB-HPLC profiles of Sia released from membrane fractions. Peaks indicated by an asterisk (*) correspond to Neu5Gc7Ac, Neu5,7Ac₂, Neu5,8,9Ac₃, Neu5Gc7,9Ac₂, Neu5,7,9Ac₃. MS and MS/MS data for

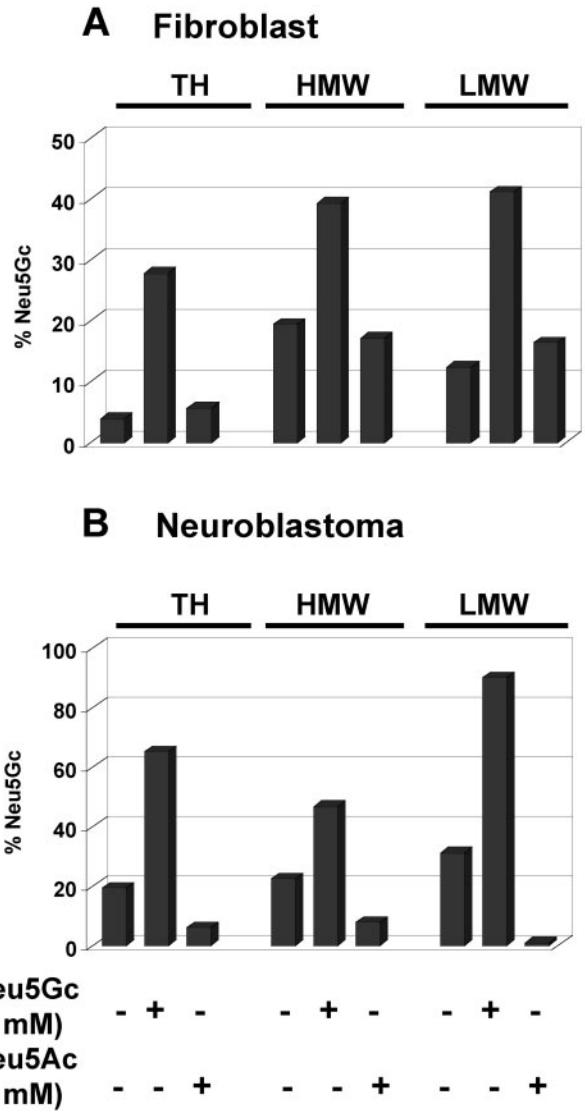


FIG. 2. Free Neu5Gc can be taken up and incorporated into other types of human cells. Human fibroblast and neuroblastoma cells were fed or not for 3 days with 3 mM Neu5Gc or Neu5Ac, and the cells then harvested and fractionated as described under "Experimental Procedures." The Sia content in the different fractions of the cells were analyzed by DMB derivatization followed by HPLC. The proportion of Neu5Gc (expressed as percent of total Sia) of the different fractions from human fibroblasts (**A**) or human neuroblastomas (**B**) is shown.

(Fig. 2B). These data indicate that the uptake mechanism of Neu5Gc can also occur in other human cell types, with varying efficiencies.

The Uptake Mechanism of Free Neu5Gc Is Not Specific for Human Cells—To ask if the uptake mechanism of free Neu5Gc is specific for human cells, we compared Neu5Gc feeding of human and chimpanzee lymphoblasts. Humans are evolutionarily most closely related to the chimpanzee, whose proteins are ~99% identical to those of humans (46). Of course, great apes such as chimpanzees are able to express Neu5Gc in large amounts because they have an active form of the CMP-Neu5Ac hydroxylase (12). Before the feeding experiment, both cell types were split and cultured in human serum instead of FCS for a

the peaks corresponding to DMB-Neu5Gc and DMB-Neu5Ac from the membrane-bound Sia of ManNGc and Neu5Gc-fed Caco-2 cells were obtained (not shown, see "Results"). **B**, proportion of Neu5Gc (expressed as percent of total Sia) in the different fractions of Caco-2 cells. Quantitative information (pmol of Sia/mg of protein) can be found in the Supplemental Material.

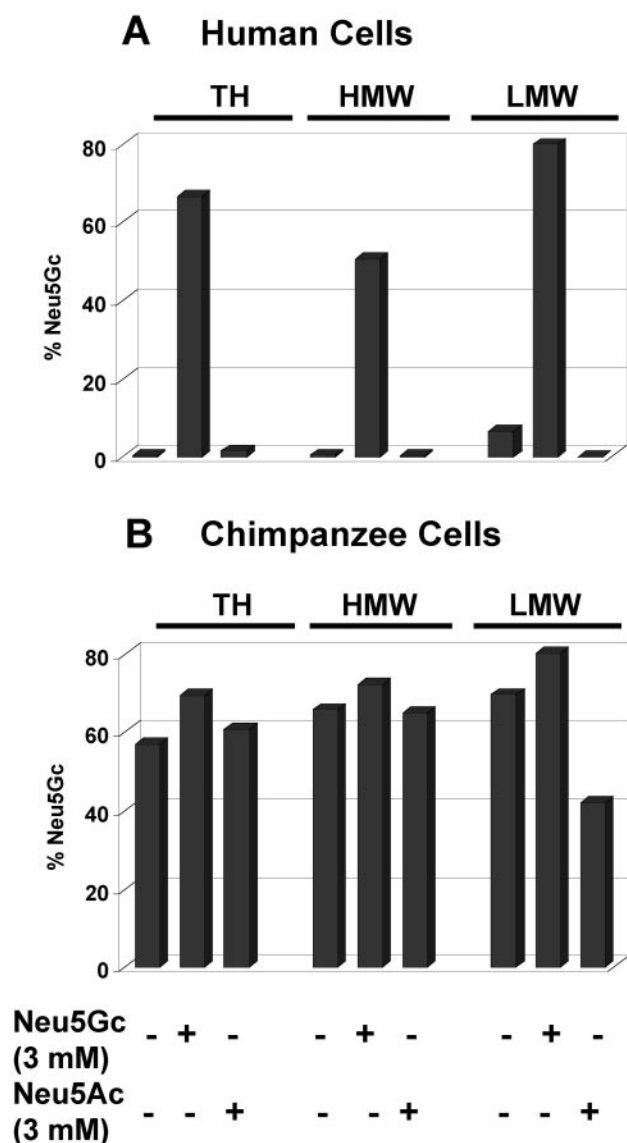


FIG. 3. Uptake of free Neu5Gc is not specific for human cells. Human and chimpanzee Epstein-Barr virus-transformed lymphoblasts were fed or not for 3 days with 3 mM Neu5Gc or Neu5Ac, and the cells were then harvested and fractionated, and the Sia content in the different fractions was analyzed by DMB derivatization followed by HPLC. The proportion of Neu5Gc (expressed as percent of total Sia) of the different fractions from human lymphoblasts (A) and chimpanzee lymphoblasts (B) is shown.

couple of weeks. As expected, the Neu5Gc content of chimpanzee lymphoblasts could not be eliminated completely because of the endogenous production of Neu5Gc. After a 3-day feeding of 3 mM Neu5Gc or Neu5Ac, the cells were harvested and fractionated, and the Neu5Gc content of the different subcellular fractions were analyzed. As shown in Fig. 3A, the human cells fed with 3 mM Neu5Gc contained 67% Neu5Gc in the TH, 51% in the HMW fraction, and 80% in the LMW cytosolic compounds. In contrast, the same cells had almost no detectable Neu5Gc when they were non-fed or fed with Neu5Ac (Fig. 3A). With chimpanzee cells, we measured base-line levels at 57% Neu5Gc in the TH, 66% in the HMW fraction, and 70% in the LMW fraction of non-fed cells (Fig. 3B), representing the endogenous production of Neu5Gc by these cells. When the chimpanzee lymphoblasts were fed with 3 mM Neu5Ac, the percentages of Neu5Gc present in the different fractions changed only minimally (Fig. 3B), presumably because of biosynthetic transformation of Neu5Ac to Neu5Gc occurring at the sugar nucle-

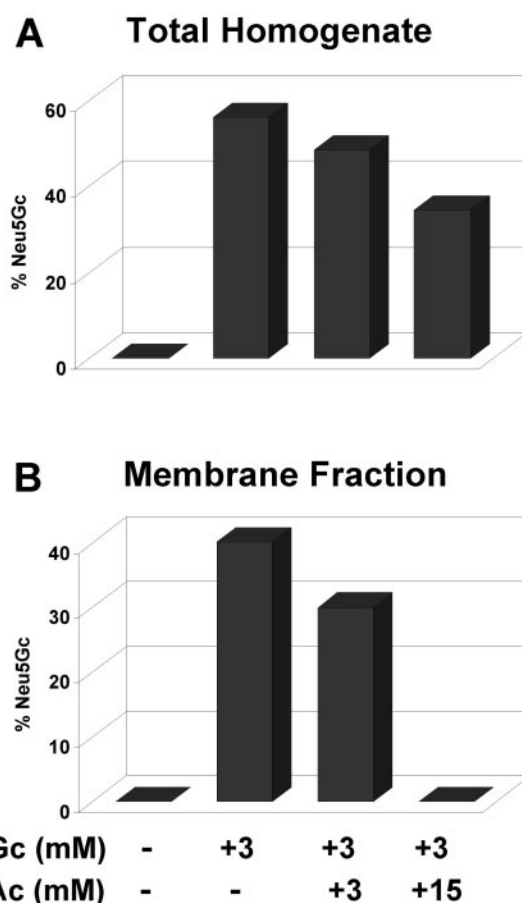


FIG. 4. Free Neu5Ac can compete with the incorporation of free Neu5Gc. Caco-2 cells grown in human serum were fed for 3 days with 3 mM Neu5Gc with or without addition in the media of Neu5Ac at 3 or 15 mM final concentration. The cells were then harvested and fractionated, and the Sia content in the different fractions was analyzed by DMB derivatization followed by HPLC. The proportion of Neu5Gc (expressed as percent of total Sia) of total homogenate fraction (A) and membrane fraction (B) is shown.

otide level (14–18). When the chimpanzee lymphoblasts were fed with 3 mM Neu5Gc, we observed an increase above the base-line levels to 70% for the TH, 72% for the HMW fraction, and 84% for the LMW fraction (Fig. 3B). Similar experiments have been done with CHO-K1 cells and with epithelial cells isolated from a spontaneous tumor from a *CMAH* gene knock out mouse.² Because all these experiments gave similar results, we can conclude that the uptake mechanism of Neu5Gc is not specific for human cells but can also take place in other mammalian cells. However, because non-human cells often have large endogenous amounts of Neu5Gc, the consequences are more dramatic in human cells.

Free Neu5Ac and Neu5Gc Are Taken Up and Incorporated by the Same Pathways—From these data and from other recent reports in the literature (43), we conclude that Neu5Ac and Neu5Gc can be taken up by many kinds of cells from an exogenous source and incorporated into endogenous glycoconjugates. Several prior studies indicate that Neu5Gc and Neu5Ac are used interchangeably by essentially all of the steps leading to their final incorporation into glycoconjugates. Higa and Paulson (19) showed that CMP-Sia synthetases from calf brain and from bovine and equine submaxillary glands both converted Neu5Ac and Neu5Gc to their CMP derivatives efficiently. They also studied six mammalian sialyltransferases

² M. Bardor, S. Diaz, and A. Varki, unpublished observations.

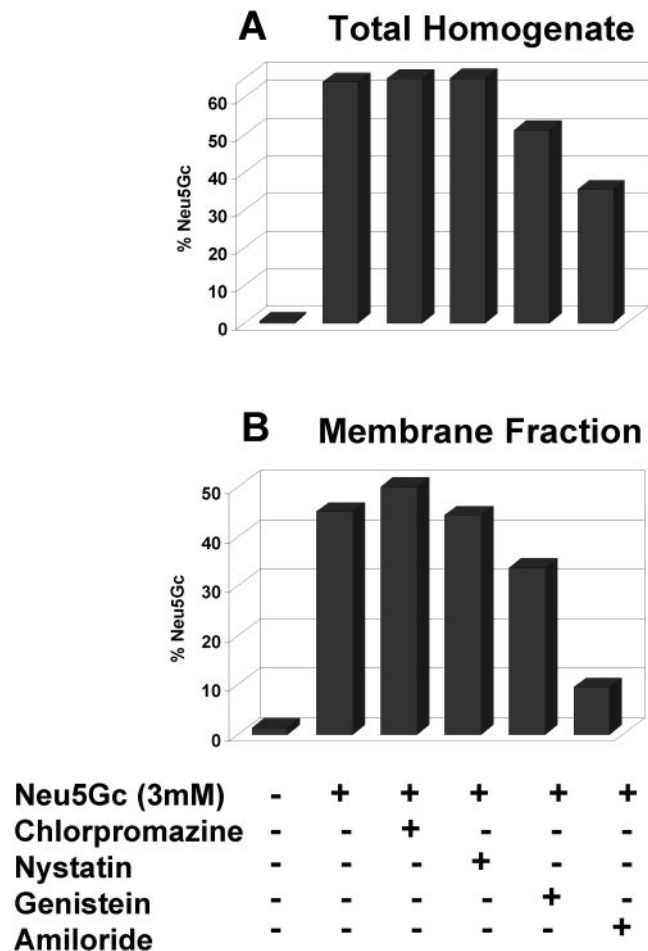


FIG. 5. Free Neu5Gc enters cells *via* endocytic processes. Caco-2 cells grown in human serum were fed for 3 days with 3 mM Neu5Gc in the presence or absence of various inhibitors of endocytic pathways. The cells were then harvested and fractionated, and the Sia content in the different fractions was analyzed by DMB derivatization followed by HPLC. The proportion of Neu5Gc (as percent of total Sia) of total homogenate fraction (A) and membrane fraction (B) is shown.

purified from porcine, rat, and bovine tissues and concluded that CMP-NeuAc and CMP-NeuGc were equally good donor substrates for all the enzymes. Schauer *et al.* (23) showed that the frog liver CMP-Sia synthetases had very similar K_m values for Neu5Ac and Neu5Gc. In our own prior work (16) we concluded that CMP-Neu5Gc and CMP-Neu5Ac could be taken up by Golgi vesicles and incorporated into the endogenous glycoproteins at an approximately equal rate. Similar observations were made by Lepers *et al.* (20, 21) in rat and mouse liver Golgi. Thus, we could ask whether Neu5Gc and Neu5Ac are taken up and incorporated via the same pathways by doing competition experiments in Caco-2 and human normal fibroblast cells. Both cell lines gave similar results, and only the results for Caco-2 cells are presented in Fig. 4. Feeding was done for 3 days with 3 mM Neu5Gc in the absence or presence (3 or 15 mM) of Neu5Ac in the media. The base-line incorporation of 56% Neu5Gc in the TH was reduced to 48% in the presence of 3 mM Neu5Ac and further decreased to 35% in the presence of added 15 mM Neu5Ac (Fig. 4A). The percentage of Neu5Gc was even more affected in the membrane-bound fraction, reducing from 41 to 29.9% with 3 mM Neu5Ac and almost to zero in the presence of 15 mM Neu5Ac (Fig. 4B). Because a 5-fold excess of Neu5Ac was enough to abolish the incorporation of Neu5Gc into the membrane fraction of the cells, we can conclude that both molecules likely use the same pathways to enter into

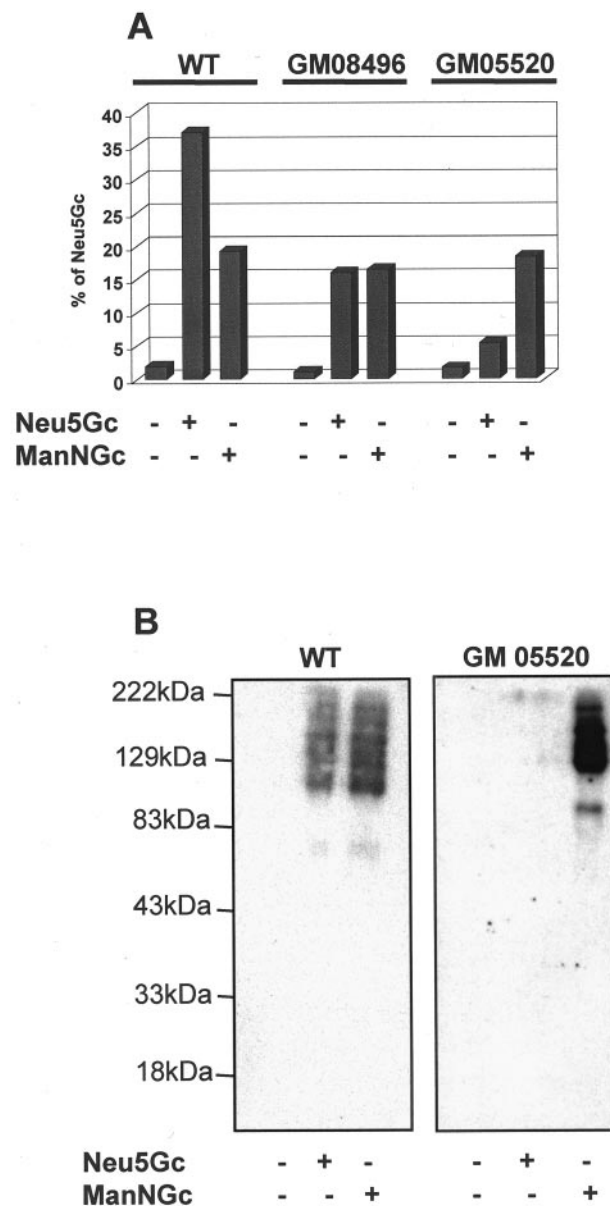


FIG. 6. The lysosomal sialic acid transporter is involved in the metabolic incorporation of free Neu5Gc. WT and lysosomal sialic acid transporter mutant human fibroblasts (GM08496 and GM05520) grown in human serum and fed for 3 days with 3 mM Neu5Gc, ManNGc, or Neu5Ac. The cells were then harvested and fractionated, and the Sia content in the different fractions was analyzed by DMB derivatization followed by HPLC. A, the proportion of Neu5Gc (expressed as percent of total Sia) in the membrane fraction is shown. B, Western blot analysis of proteins extracted from the membrane fractions of wild-type and GM05520 mutant human fibroblasts, using an anti-Neu5Gc antibody, after Neu5Gc or ManNGc feeding.

human cells and become available for metabolic incorporation. It is of course possible that there are minor differences in utilization of Neu5Gc and Neu5Ac by various enzymes and transporters in the pathways.

Free Neu5Gc Enters into Cells via Pathways of Endocytosis—Negatively charged hydrophilic molecules like sialic acids usually do not cross membranes (47–49). To understand how free Neu5Gc enters into cells, we explored the hypothesis that it does so via endocytic pathways. Thus, Neu5Gc feeding experiments on Caco-2 cells were done in the presence of drugs that are known to inhibit various endocytic pathways common to most cell types (50–53). Based on literature, we decided to use chlorpromazine for blocking the clathrin-dependent pathway

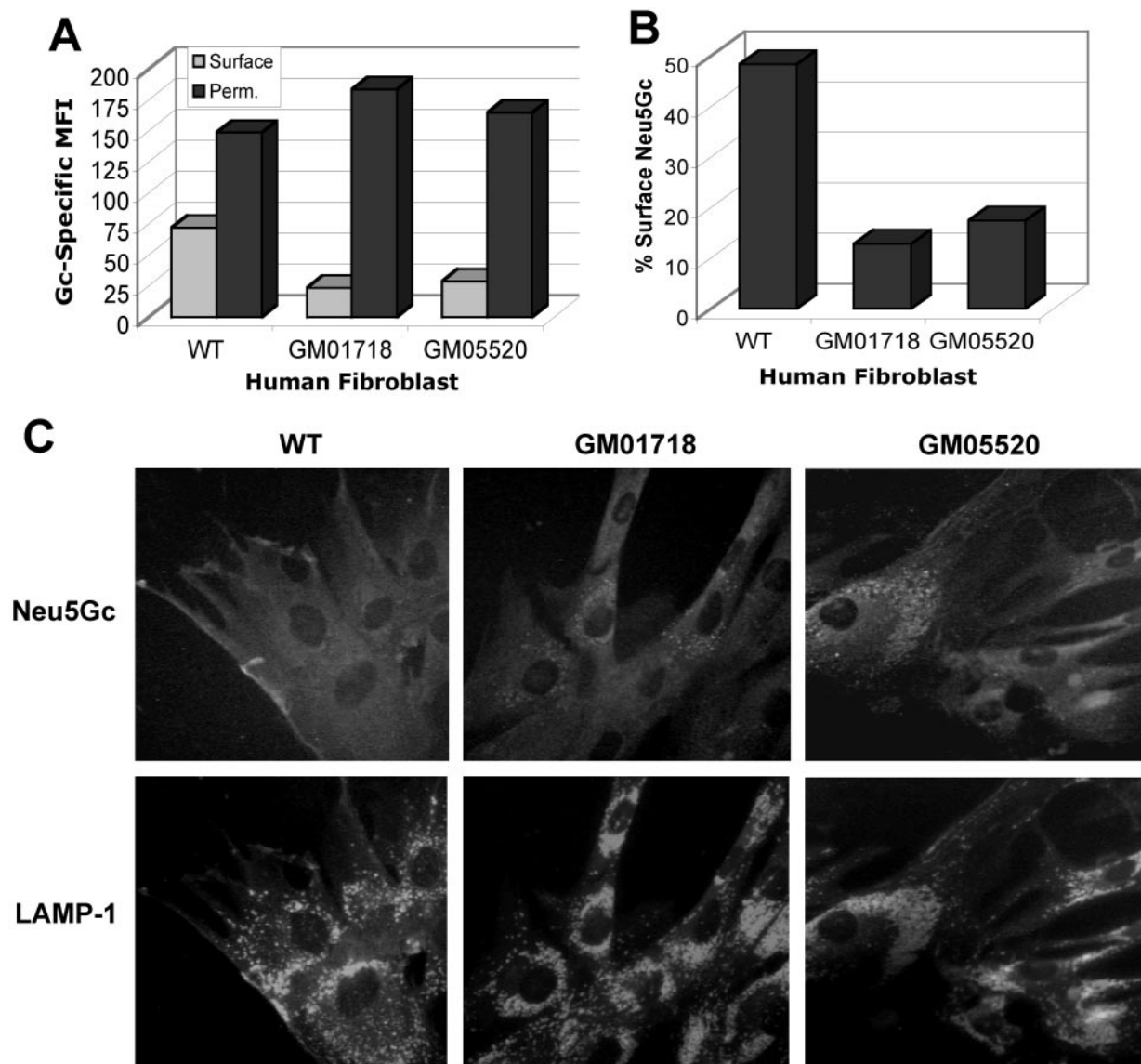


FIG. 7. The lysosomal sialidase and sialic acid transporter are required for metabolic incorporation of Neu5Gc from serum glycoconjugates. WT fibroblasts, lysosomal sialidase mutant fibroblasts (GM01718), and Sia transporter mutant fibroblasts (GM05520) were incubated with 10% FCS plus 20% horse serum (rich sources of glycosidically bound Neu5Gc) for 3 days. *A*, cells were then released from the flasks, fixed, and analyzed for binding of a chicken anti-Neu5Gc antibody with and without permeabilization by flow cytometry. Neu5Gc-specific median fluorescence intensity (MFI) is the MFI of the chicken anti-Neu5Gc staining with the background secondary alone MFI subtracted out. At least 5000 cells were counted for each staining. *B*, percent surface Neu5Gc was calculated by dividing the non-permeabilized Neu5Gc-specific MFI by the permeabilized Neu5Gc-specific MFI. *C*, WT, GM01718, and GM05520 fibroblasts were grown on slides for 4 days, fixed, permeabilized, and stained with chicken anti-Neu5Gc IgY and mouse anti-LAMP-1 IgG1. Antibody binding was detected with FITC-goat anti-chicken IgY and Cascade Blue-goat anti-mouse IgG.

(54, 55) and nystatin and genistein for the clathrin-independent pathways (with an additional specific action of nystatin on caveolar uptake) (56). Amiloride was used as an inhibitor of fluid phase pinocytosis (57). All these drugs were used at concentrations described under “Experimental Procedures” and based on prior literature (41, 42). As before, the Caco-2 cells were incubated in an appropriate media containing human serum instead of FCS and pretreated with the drug for 2 h followed by the addition of 3 mM Neu5Gc for 16 h or 3 days. As shown in Fig. 5A, incorporation of Neu5Gc in the TH fraction in the presence of chlorpromazine and nystatin (~65% in both cases) was about the same as for the non-treated Caco-2 cells. In contrast, Neu5Gc incorporation into cells was decreased in the presence of genistein (51%) and much further by amiloride (35.4% Neu5Gc). Analysis of incorporation into membrane-bound glycoconjugates gave similar results; although there was no obvious difference in the Neu5Gc incorporation for cells

cultured without (45%) or with chlorpromazine (50%) or with nystatin (44%), genistein and amiloride caused marked reduction of incorporation to 34 and 10%, respectively. These results indicate that exogenous free Neu5Gc enters cells via clathrin-independent endocytic pathways with a major contribution from fluid phase pinocytosis.

The Lysosomal Sialic Acid Transporter Is Required for Export of Free NeuGc from the Lysosome to the Cytosol—Free Neu5Gc molecules entering the cell via endocytic pathways would still be restricted from passively diffusing out of endosomes into the cytosol. We hypothesized that they would eventually reach the lysosome, where they would have the opportunity to utilize the previously known lysosomal sialic acid transporter (58–60) to reach the cytosol. To test this hypothesis, we made use of fibroblasts from a patient (GM05520) with a severe infantile form of sialic acid storage disease (ISSD) (61), a disease that is caused by a genetic defect in this transporter

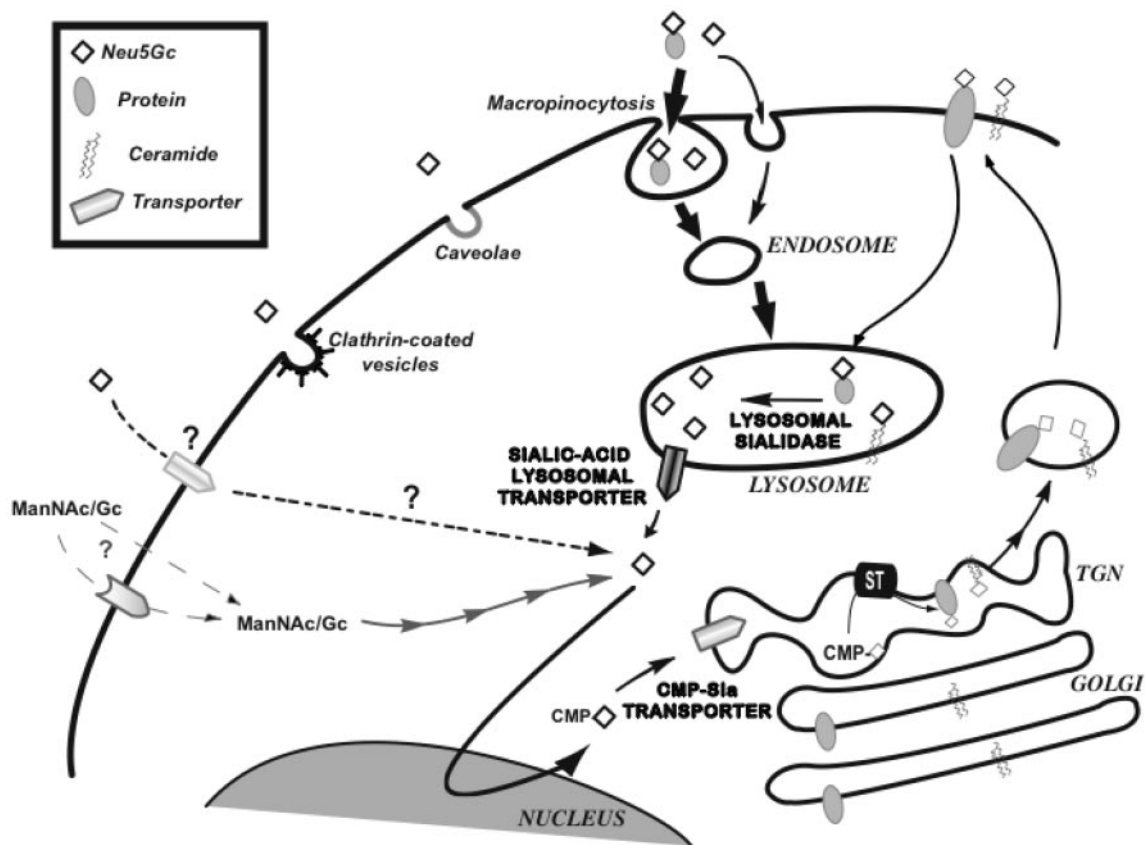


FIG. 8. **Proposed pathways for the uptake and incorporation of Neu5Gc in human cells.** The model proposed is based on the data presented in this study and upon prior literature. The *diamond shape* represents a Neu5Gc molecule. The *thickness* of the various *arrows* suggests the relative importance of various pathways in delivering Neu5Gc into the cell. See "Discussion." ST, sialyltransferase; TGN, Trans-Golgi network.

(22, 62). As shown in Fig. 6, the percent of Neu5Gc incorporation into membrane-bound glycoconjugates was reduced from 37% in normal WT fibroblasts to 5% in these mutant cells. As a control we also studied the metabolic conversion of ManNGc into Neu5Gc in these cells, which presumably occurs after passive diffusion through the plasma membrane and does not require the lysosomal sialic acid transporter. As predicted, we found that there was essentially no difference between normal (19% Neu5Gc) versus mutant fibroblasts (18% Neu5Gc) after feeding with 3 mM ManNGc. We also studied another similar mutant human fibroblast cell line (GM08496) with a partial inhibition of function of the lysosomal sialic acid transporter (63). This cell line was isolated from a patient suffering from Salla disease, a milder adult form of sialic acid storage disease (64). Neu5Gc feeding of these cells resulted in 16% Neu5Gc in membrane-bound glycoconjugates in comparison to the 37% seen in normal WT fibroblasts. Again, feeding with ManNGc gave no obvious change from the control (17% Neu5Gc). To further confirm that there was a difference in incorporation into glycoproteins, we carried out a Western blot analysis of proteins extracted from the membranes of wild-type and GM05520 mutant human fibroblasts using an anti-Neu5Gc antibody with or without prior Neu5Gc or ManNGc feeding. As shown in Fig. 6B, the mutant fibroblasts could not incorporate Neu5Gc into glycoproteins but could in fact convert it from ManNGc. Taken together, our data confirm the hypothesis that the lysosomal sialic acid transporter plays a crucial role in delivering free sialic acids that enter into cells via endocytosis to the cytosol for activation and incorporation into glycoconjugates.

Both the Lysosomal Sialidase and the Lysosomal Sialic Acid Transporter Are Required for Incorporation of Glycoprotein-bound NeuGc into Human Cells—Several studies (including

this one) have shown that when human cells are transferred from conventional media containing FCS into serum-free media or human serum the small amounts of endogenous Neu5Gc in these cells gradually disappear. It has always been assumed that this is because FCS contains many glycoproteins with attached Neu5Gc. However, the pathway by which these glycosidically bound Neu5Gc molecules enter the cell and eventually become incorporated into endogenous glycoproteins has never been defined. This question is also of direct relevance to human gut epithelial cells, which would be exposed to glycoprotein-bound Neu5Gc of dietary origin (red meat, milk products for example). Based on the above findings, it is reasonable to hypothesize that the Neu5Gc-carrying serum glycoproteins enter the cell via fluid phase pinocytosis, eventually reaching the lysosome, where they are exposed to the lysosomal sialidase. The resulting free Neu5Gc in the lysosome would then have the opportunity to use the lysosomal sialic acid transporter to reach the cytosol to be salvaged and eventually converted to CMP-Neu5Gc.

To test this hypothesis we again made use of the GM05520 mutant human fibroblasts, which are completely deficient in the lysosomal sialic acid transporter as well as GM01718 mutant human fibroblasts, which have less than 1% lysosomal sialidase activity compared with normal fibroblasts (65). For these studies, it was important to differentiate between cell surface and internal Neu5Gc. Thus, instead of subcellular fractionation, we turned to the method of flow cytometry, using our previously described affinity purified Neu5Gc-specific chicken antibody (35). As shown in Fig. 7A, after 3 days of feeding with 10% FCS plus 20% horse serum (both rich sources of glycoprotein-bound Neu5Gc), the total surface expression of Neu5Gc was significantly lower in both mutant fibroblasts compared with WT fibroblasts. Permeabilization of cells revealed similar

levels of total Neu5Gc glycoconjugates (Fig. 7A), but the majority in the two mutants was internal (Fig. 7B). To confirm trapping of Neu5Gc glycoconjugates in lysosomes, we performed fluorescence microscopy analysis of permeabilized fibroblasts, co-labeling cells with a known marker for lysosomes, LAMP-1 (66). We found even distribution of Neu5Gc staining on WT normal fibroblasts with little co-localization with lysosomes (Fig. 7C). On the other hand, both the lysosomal sialidase and the transporter mutants demonstrated significant accumulation of Neu5Gc glycoconjugates in the lysosomes (Fig. 7C).

The results with the sialidase-deficient fibroblasts confirm our hypothesis that this enzyme must act to release free Neu5Gc from glycoproteins and to make it available for metabolic incorporation. The accumulation of Neu5Gc glycoconjugates in the lysosomal transporter mutant was unexpected. A likely explanation is that accumulation of free Sia at a high concentration in the lysosomes inhibits the action of the lysosomal sialidase, resulting in accumulation of glycosidically bound Neu5Gc. The residual levels of Neu5Gc detected on the surface of both mutant cells might be explained by direct incorporation of gangliosides and glycosylphosphatidylinositol-anchored proteins bearing Neu5Gc from the serum. Taken together these data indicate that bound Neu5Gc molecules that enter into human cells via pinocytosis are released by the lysosomal sialidase and are then transported by the lysosomal sialic acid transporter to the cytosol, where they are available for activation and incorporated into glycoconjugates (Fig. 8). Of course depending on the type of glycoprotein involved, bound Neu5Gc could also be delivered to lysosomes via other pathways of endocytosis, e.g. receptor-mediated endocytosis via clathrin-coated vesicles.

DISCUSSION

It has long been assumed that free sialic acids could not be efficiently incorporated into cells because of their negative charge and hydrophilic nature (47–49). Thus, neutral *N*-acetylmannosamine has traditionally been used as a precursor to feed cells, for conversion into Neu5Ac. The same concept has been applied to various unnatural mannosamine derivatives (67), and the addition of *O*-acetyl esters to the hydroxyl groups of mannosamine derivatives has been used to enhance delivery across the plasma membrane (44, 68, 69). In fact, one early study (70) suggested that radioactive sialic acids could be incorporated into cells, and more recent work of others has shown “efficient” uptake of a variety of kinds of sialic acids into cells (43, 71). However, the kinetics of incorporation showed no evidence of saturation even at >10 mM concentrations (43), suggesting that the uptake was not due to a high efficiency cell surface transporter for sialic acids. Our own more recent work using a natural sialic acid (Neu5Gc) gave similar results (35).

The present study resolves all these discrepancies by showing that free sialic acids from the medium can be taken up into cells via non-clathrin-mediated mechanisms, mostly amiloride-sensitive fluid-phase pinocytosis. The content of the resulting pinocytotic vesicles and endosomes would eventually be delivered to the lysosome, where the described previously sialic acid transporter then delivers the molecules into the cytosol. We also show that the incorporation of glycosidically bound Neu5Gc from exogenous glycoproteins occurs by similar delivery to the lysosome and release by the lysosomal sialidase followed by export into the cytosol (Figs. 7 and 8). Once activated to CMP-Neu5Gc, molecules from both sources (free and originally bound) would be indistinguishable from those that were endogenously synthesized by the cells.

Most recently, we have shown that human embryonic stem cells can incorporate Neu5Gc from medium glycoconjugates,

making them targets for the naturally occurring antibodies that circulate in most humans.³ Our preliminary data also suggest that these antibodies could also be related to diseases in intact humans. Thus, the mechanism by which Neu5Gc is incorporated into human cells is of potentially great importance. Further studies of this process are also relevant both to the ongoing attempts by various groups to incorporate different kinds of unnatural sialic acids into cultured cells (43, 71–73) and also to our efforts to understand how exogenous dietary Neu5Gc gain entry into normal human tissues. In this regard it is of note that Neu5Gc accumulation appears to be enhanced in naturally occurring tumors and in fetal tissues. We suggest that this may be explained by the fact that fluid-phase macropinocytosis is enhanced by growth factors (50–52, 57, 74), which are expected to be very prominent in these two situations.

Finally, to our knowledge this is the first reported example in which an extracellular small molecule that cannot cross the plasma membrane is delivered efficiently to the cytosol utilizing fluid pinocytosis and a specific lysosomal transporter. The approach could, thus, potentially be generalized to any small molecule that has a specific lysosomal transporter but not a plasma membrane transporter. For example, one could envisage that the neutral sugars GlcNAc and GalNAc, which do not have a high efficiency plasma membrane transporter (75), could nevertheless be delivered to the cytosol via the lysosomal GlcNAc/GalNAc transporter (76). The prediction is that adding millimolar concentrations of these sugars into the medium would result in significant delivery to the cytosol.

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