Metabolic Delivery of Ketone Groups to Sialic Acid Residues
APPLICATION TO CELL SURFACE GLYCOFORM ENGINEERING*

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The development of chemical strategies for decorating cells with defined carbohydrate epitopes would greatly facilitate studies of carbohydrate-mediated cell surface interactions. This report describes a general strategy for engineering the display of chemically defined oligosaccharides on cell surfaces that combines the concepts of metabolic engineering and selective chemical reactivity. Using a recently described method (Mahal, L. K., Yarema, K. J., and Bertozzi, C. R. (1997) Science 276, 1125–1128), we delivered a uniquely reactive ketone group to endogenous cell surface sialic acid residues by treating cells with the ketone-bearing metabolic precursor N-levulinoylmannosamine (ManLev). The ketone undergoes highly selective condensation reactions with complementary nucleophiles such as aminooxy and hydrazide groups. The detailed quantitative parameters of ManLev metabolism in human and non-human-derived cell lines were determined to establish a foundation for the modification of cell surfaces with novel epitopes at defined cell-surface densities. Ketones within the glycoconjugates on ManLev-treated cells were then reacted with synthetic aminooxy and hydrazide groups. The detailed quantitative parameters of ManLev metabolism in human and non-human-derived cell lines were determined to establish a foundation for the modification of cell surfaces with novel epitopes at defined cell-surface densities. Ketones within the glycoconjugates on ManLev-treated cells were then reacted with synthetic aminooxy and hydrazide-functionalized carbohydrates. The remodeled cells were endowed with novel lectin binding profiles as determined by flow cytometry analysis. The simplicity and generality of this method make it well suited for use in the study of carbohydrate-mediated cell surface interactions.

It has been known for several decades that the cell surface is richly decorated with a dense covering of complex oligosaccharides. Even before many of the specific biological functions of these carbohydrates were elucidated, it was apparent that remodeling the molecular landscape of the cell surface transformed the behavior of cells (1, 2). The discovery of their participation in cell-cell recognition events has brought cell surface glycoconjugates to the forefront of biological research in recent years. Numerous cell surface oligosaccharides have been sequenced and their interactions with receptors on opposing cells are understood in some molecular detail (3–7). Nevertheless, the interactions of cell surface oligosaccharides with protein receptors in solution and on opposing cells remain difficult to study at the molecular level in comparison to protein-protein interactions on cell surfaces.

The lag in carbohydrate research can be attributed, in part, to the difficulty in controlling the presentation of well defined carbohydrate epitopes on cell surfaces (for a perspective, see Ref. 8). While cell surface oligosaccharide structures can be conservatively altered by the introduction, overexpression, or deletion of genes encoding specific glycosyltransferases (9–17), the complexities of oligosaccharide biosynthesis impose some limitations on the use of genetic methods for modulating the structures of cell surface oligosaccharides. As a consequence, alternative methods for decorating cells with chemically defined oligosaccharides are the subject of much recent attention (18).

Owing to advances in methodology for the chemical and enzymatic synthesis of oligosaccharides during the last two decades, virtually any oligosaccharide structure is now accessible in chemical quantities and in homogeneous form. Therefore, methods for modulating the display of cell surface oligosaccharides that exploit the availability of synthetic molecules are increasingly valuable tools for biological research. An appealing strategy for controlling the display of oligosaccharide epitopes on cell surfaces is through the selective covalent attachment of chemically defined carbohydrate structures onto native glycoconjugates. One of the earliest examples of this approach, reported by Tolvanen and Gahmberg (19, 20), involved the treatment of cells with the mild oxidant sodium periodate to introduce aldehyde groups onto native oligosaccharides. The aldehydes were then condensed with glycosylhydrazines derived from treatment of free sugars with anhydrous hydrazine. Although the glycosylhydrazone products were released from the cells by glycosidic bond hydrolysis after several hours, the adducts were sustained long enough to perform lectin binding studies. With the aim of avoiding chemical reagents and oxidants that might harm cells, Palcic and co-workers (21, 22) reported an enzymatic method for transferring exogenous carbohydrate structures onto cells that exploits the substrate promiscuity of a fucosyltransferase. Synthetic analogs of GDP-fucose bearing additional carbohydrates appended to C-6 were enzymatically transferred by the enzyme onto cells expressing suitable glycosyl acceptor sites.

Here we report an alternative approach to cell surface glycoengineering that involves the introduction of a uniquely reactive functional group into cell surface glycoconjugates using endogenous metabolic processes (27–31). The unique functional group is then chemically elaborated with synthetic car-
bohydrazide epitopes of defined structure. The highly selective condensation reactions of aminooxy or hydrazide groups with ketones, affording the corresponding oximes or hydrazones, are well suited for this strategy as these functional groups are chemically orthogonal to native cell surface moieties (23–26). Accordingly, we exploited the intrinsic substrate promiscuity of the enzymes in the sialoside biosynthetic pathway (32–34) for the delivery of ketones into endogenous cell surface glycoconjugates. We have recently shown that N-levulinoylmannosamine (ManLev), an unnatural derivative of ManNAc, is converted to N-levulinoyl sialic acid (SiaLev) in human cell lines, resulting in the expression of chemically accessible ketones within cell surface glycoconjugates (Fig. 1) (35). The detailed quantitative parameters of ManLev metabolism are presented in this report, along with application to the cell surface glycoform engineering strategy depicted schematically in Fig. 1. Carbohydrates bearing either an aminooxy or hydrazide group were condensed with ketone groups endogenous to unnatural sialic acid residues on ManLev-treated cells, and the remodeled cells gained the ability to bind a lectin specific for the attached epitopes.

EXPERIMENTAL PROCEDURES

Materials—Tunicamycin, benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (α-BnGalNAc), FITC-labeled avidin, biotin hydrazide (BH), demecolcine, colchicine, camptothecin, sodium azide, trypsin-EDTA, and sialidase (Clostridium perfringes, Type V) were purchased from Sigma Life Technologies, Inc. DME media was from CellGro, FCS and newborn calf serum were from Hyclone or Sigma. RPMI 1640 media was from Life Technologies, Inc., DME media was from BioWhittaker, and penicillin-streptomycin (P/S) was from Fisher. All reagents used in chemical syntheses were obtained from commercial suppliers and were used without further purification. NMR spectra were recorded on Bruker AMX-400 or AMX-300 spectrometers, mass spectral data were obtained at the University of California Berkeley Mass Spectrometry Facility, and combustion analyses were obtained at the University of California Berkeley Microanalytical Facility.

Synthesis of N-levulinoylmannosamine (ManLev, Mixture of Anomers)—To a solution of levulinic acid (2.85 ml, 27.8 mmol) in anhydrous tetrahydrofuran (77 ml) was added 4.2 ml of triethylamine (30.2 mmol). The mixture was stirred at room temperature under N₂. After 30 min, 3.11 ml (25.5 mmol) of isobutyl chloroformate were added dropwise by syringe. The reaction was stirred under N₂ for 3 h during which time a white precipitate formed. The crude levulinic acid isobutyl carbonic anhydride was used directly in the next step. To a solution of 5.00 g (32.3 mmol) of mannosamine hydrochloride in 154 ml of 5:4 H₂O/tetrahydrofuran was added 3.6 ml (25.5 mmol) of triethylamine. The mixture was stirred at room temperature for 5 min, after which the crude solution of levulinic acid isobutyl carbonic anhydride was added dropwise. After 24 h, the solvent was removed in vacuo. Chromatography on 400 ml of silica gel eluting with a gradient of 15:1 to 5:1 CHCl₃/MeOH, followed by passage down cation (Biorad AG50W-X8, pyridinium form) and anion (Biorad AG1-X2, acetate form) exchange columns eluting with H₂O, yielded 3.74 g (49%) of ManLev as a white amorphous foam. The compound was characterized as a mixture of anomers: Rₛ = 0.63 (5:3:2, BuOH/AcOH/H₂O); infrared spectrum (KBr pellet): 3404 (br), 2930, 1700, 1653, 1540, 1068 cm⁻¹; 1H NMR (300 MHz, D₂O, δ = 2.19 (s, 3), 2.20 (s, 3), 2.47–2.61 (m, 4), 2.75–2.90 (m, 4), 3.38 (dd, 1, J = 2.3, 3.4, 9.8), 3.49 (app t, 1, J = 9.8), 3.56–3.63 (m, 6), 4.01 (dd, 1, J = 4.7, 9.8), 4.26 (dd, 1, J = 1.5, 4.6), 4.41 (dd, 1, J = 1.5, 4.4), 4.98 (d, 1, J = 1.6), 5.07 (d, 1, J = 1.5); 13C NMR (100 MHz): δ = 29.2, 29.3, 29.4, 38.2, 38.3, 53.2, 54.1, 60.5, 66.6, 66.9, 68.9, 72.1, 72.1, 76.4, 93.1, 93.2, 175.9, 176.7, 214.1, 214.3; high resolution mass spectrum (FAB⁺), calculated for C₁₁H₂₀N₂O₇ (MH⁺): 278.1240, found 278.1238.

Chemically-defined oligosaccharide epitope

Cell Surface Glycoform Engineering

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N-Levulinoylmannosamine (ManLev), bearing an unnatural N-acyl side chain, is metabolized to the corresponding unnatural sialic acid (N-levulinoyl sialic acid or SiaLev) in cultured human cells. The ketone serves as a unique chemical handle for the selective attachment of defined oligosaccharides bearing a complementary nucleophile such as the aminooxy or hydrazide group (an example of the condensation of an aminooxy sugar with surface ketones to afford stable oxime-linked adducts is shown). The result is the introduction of a new oligosaccharide epitope onto cell surface glycoconjugates. Specific examples of an aminooxy sugar and an oligosaccharide hydrazide are shown in Fig. 10.

FIG. 1. A general approach to cell surface glycoform engineering achieved by the metabolic delivery of ketone groups to endogenous cell surface sialylglycoconjugates. N-Levulinoylmannosamine (ManLev), bearing an unnatural N-acyl side chain, is metabolized to the corresponding unnatural sialic acid (N-levulinoyl sialic acid or SiaLev) in cultured human cells. The ketone serves as a unique chemical handle for the selective attachment of defined oligosaccharides bearing a complementary nucleophile such as the aminooxy or hydrazide group (an example of the condensation of an aminooxy sugar with surface ketones to afford stable oxime-linked adducts is shown). The result is the introduction of a new oligosaccharide epitope onto cell surface glycoconjugates. Specific examples of an aminooxy sugar and an oligosaccharide hydrazide are shown in Fig. 10.
5 h during which time a white precipitate formed. The crude product was used directly in the next step. To a solution of 3.0 g (14 mmol) of glucosamine hydrochloride in 70 ml of 4.3 H2O/tetrahydrofuran was added 2.7 ml (15 mmol) of diisopropylethylamine. The reaction mixture was stirred at room temperature for 1 h, after which the crude solution of levulinic acid methyl ester was filtered and the ester was distilled off under vacuum.

After 22 h, the solvent was removed in vacuo. Chromatography on 250 ml of silica gel eluting with 5:1, CHCl3/MEOH, followed by passage down cation (Bio-Rad AG50W-X8, pyridinium form) and anion (Bio-Rad AG1-X2, acetate form) exchange columns eluting with H2O, yielded 1.0 g (3.9 mmol, 28%) of GlcLev as a white amorphous foam. The composition was characterized as a mixture of anomers; 1H NMR (300 MHz, D2O): 6 2.19 (s, 6), 2.50–2.54 (m, 4), 2.81–2.90 (m, 4), 3.40–3.59 (m, 12), 4.68 (d, 1, J = 8.2 Hz), 5.14 (d, 1, J = 3.4 Hz); 13C NMR (100 MHz): 6 29.2, 29.3, 29.6, 35.2, 54.0, 56.7, 60.8, 60.6, 70.1, 70.6, 71.6, 73.8, 75.9, 90.9, 94.9, 175.6, 213.9; high resolution mass spectrum (FAB+) calculated for C11H20NO7 (MH+): 152; found: 152.

**Synthesis of Lactose Hydrazide (Lac-NHNH2)—** A solution of per-acetylated β-Ο-allyl lactose (1.0 g, 1.5 mmol) in 15 ml of acetonitrile was cooled to −78 °C and anhydrous hydrogen fluoride was bubbled through the solution until it was saturated. The solution was then purged with N2 for 5.0 min and Jones’ reagent (2.0 M CrO3/H2SO4 in H2O) was added until an orange color persisted. The reaction was warmed to room temperature, and after 1.5 h it was quenched with excess sodium bicarbonate. The reaction mixture was filtered and the filtrate was directly acetylated. The solution was washed with copious amounts of saturated NaHCO3. The aqueous layers were washed, acidified to pH 3.0, and extracted with CH2Cl2. The organic layer was dried over MgSO4 and the solvent was removed in vacuo to afford 0.54 g (52%) of the corresponding carboxylic acid. Without further purification, a solution of the acid (0.48 g, 0.692 mmol) in dry CH2Cl2 (6.9 ml) and anhydrous diisopropylethylamine (four drops) was treated with oxalyl chloride (66 µl, 0.761 mmol). The reaction mixture was stirred under N2 for 4.0 h at which time an additional 66 µl of oxalyl chloride was added. After 2.5 h, the solvent was removed in vacuo affording the crude acid chloride as a yellow foam. The acid chloride was dissolved in 7.0 ml of CH2Cl2 and hydrazine hydrate (0.325 ml, 10.4 mmol) was added to the solution. The reaction was stirred overnight and another 0.700 ml of hydrazine hydrate was added. The reaction was stirred for 2.0 days after which the solvent was removed in vacuo. The product, Lac-NHNH2, was purified by high performance liquid chromatography on an aminopropyl silica gel column eluting with a gradient of water (0–50% over 70 min) in acetonitrile to yield 0.185 g (65%) as a white solid; Rf = 0.28 (5:3.2 BuOH/AcOH/H2O); 1H NMR (400 MHz, D2O): 6 3.34–3.35 (m, 10, 3.88 (apparent d, 1, J = 3.2 Hz), 3.95 (dd, 1, J = 2.1, 13.3), 4.26 (d, 1, J = 15.3), 4.38 (d, 1, J = 15.3), 4.42 (d, 1, J = 7.8), 4.49 (d, 1, J = 7.9); 13C NMR (100 MHz): 6 80.0, 61.1, 67.8, 68.6, 71.0, 72.6, 72.7, 74.2, 75.0, 75.4, 78.2, 102.4, 103.0, 170.5. FAB-MS calculated for C14H27O12N2, 415; found: 415. 

**Tissue Culture/Cell Growth Conditions—** Unless otherwise specified, Jurkat cells were grown in RPMI 1640 media supplemented with 5% FCS, penicillin, and streptomycin. Jurkat cells, D1B, and S49 cells were grown in RPMI 1640 media supplemented with 10% FCS and PS, and COS-7 and HeLa cells were grown in DMEM media supplemented with 10% FCS and PS. In all cases, cells were incubated in a 5.0% carbon dioxide, water-saturated incubator at 37 °C.

Typically, cells were grown in 6-well polystyrene tissue culture plates, seeded at cell densities between 250,000 and 500,000 cells per well in 2.0 ml of media (cell quantification was by Coulter counter or by hemacytometer). When larger quantities of cells were required, 10-cm tissue culture plates, seeded at cell densities between 250,000 and 500,000 cells per plate, were used. The cells were incubated in a 5.0% carbon dioxide, water-saturated incubator at 37 °C.

Cells (2.0 × 106) were then digested with sialidase (C. perfringens, 0.2 units) and the supernatant containing the liberated sialic acid was analyzed without further purification by high-pH anion exchange chromatography on a Carbopac PA-1 column with pulsed amperometric detection using a Dionex high performance liquid chromatography system. The elution gradient was similar to that reported by Potvin et al. [38]: 20 µl NaOAc (in 100 µl NaOH) for 10 min followed by a gradient from 20 to 280 µl NaOAc (in 100 µl NaOH) over 20 min.

**Analysis of Cell Surface Ricin Binding Activity—** Cells were incubated with FITC-labeled Ricinus communis agglutinin (FITC-RCA120, Sigma, in avidin buffer). Cells were incubated on ice with FITC-RCA120 for 15 min, washed twice with avidin buffer, and analyzed by flow cytometry.

**Competitive Metabolism of ManLev and ManNAc—** ManNAc and ManLev (0.1 mmol in PBS) were premixed to give various concentrations of each monosaccharide. Specifically, either 20 or 80 µl of ManLev (to give a final concentration of 5.0 or 20 µM, respectively) was combined with one of various volumes of ManNAc (0 to 20 µl) to give a final concentration of ManNAc between 0 and 5.0 µM. The volume of the ManLev/ManNAc mixtures was adjusted to 100 µl with PBS and added to 250,000 HL-60 or Jurkat cells or to 100,000 HeLa cells, each suspended in 1.9 ml of fresh media. Cells were incubated for 2 days in 6-well tissue culture plates and analyzed by flow cytometry as described above. A similar experiment was also performed using Jurkat cells with a wider range of ManLev concentrations (1, 2, 4, 8, 12, 16, 20, and 30 µM).

**Time/Dose-response Experiments—** ManLev (200 µl of 0 to 400 µmol solutions in PBS) was added to 1.8 ml of HL-60 or Jurkat cells in 6-well tissue culture plates to give ManLev concentrations ranging between 0 and 40 µM. At 24-h intervals, cells were counted and an aliquot was removed for subsequent steps. The cells were maintained continuously on ice to prevent membrane recycling (36, 37). BH-labeled cells were washed twice in avidin buffer (PBS, pH 7.4, with 0.1% newborn calf serum and 0.1% sodium azide). The cells were resuspended in 100 µl of avidin buffer and added to 100 µl of 11.2 µg/ml FITC-labeled avidin (generally, 200 µg/ml avidin) at a final concentration of 5.8 µg/ml was used to be used for subsequent steps and the cells were maintained continuously on ice to prevent membrane recycling (36, 37). BH-labeled cells were washed twice in avidin buffer (PBS, pH 7.4, with 0.1% newborn calf serum and 0.1% sodium azide).

**Analysis of Cell Surface Ketones by Flow Cytometry—** Jurkat, HL-60, or HeLa cells (1.0 × 107 to 2.0 × 107, depending on the experiment) were washed two times in 1.0 to 10 ml of biotin buffer (PBS, pH 6.5 with 0.1% newborn calf serum) and then resuspended in 0.4–1.6 ml of biotin buffer. The washed cells were added to 100–400 µl of 5.0 mM biotin hydrazide (BH, dissolved in PBS) to give a final concentration of 1.0 mM BH. Cell/BH mixtures were incubated at room temperature for 1.5 to 2.0 h, during which time the BH label was attached to the cell surface ketones and the BH label was washed out. The BH-labeled cells were washed twice in avidin buffer (PBS, pH 7.4, with 0.1% newborn calf serum and 0.1% sodium azide). The cells were resuspended in 100 µl of avidin buffer and added to 100 µl of 11.2 µg/ml FITC-labeled avidin (generally, 200 µg/ml avidin) at a final concentration of 5.8 µg/ml was used to be used for subsequent steps and the cells were maintained continuously on ice to prevent membrane recycling (36, 37). BH-labeled cells were washed twice in avidin buffer (PBS, pH 7.4, with 0.1% newborn calf serum and 0.1% sodium azide)
after addition of ManLev by trypsinizing the cells, resuspending in 2.0 ml of media, removing 1.5 ml of cells (and adding back 1.5 ml of media with the appropriate ManLev concentration) for analysis by flow cytometry. The culture containing 150,000 cells was likewise analyzed, but on the second, fourth, and sixth days of incubation.

Experimental Manipulation of Cell Surface Ketones and the Effect of Growth Inhibitory Agents—The time-dependent depletion of cell surface ketones and the effect of cell growth inhibitors were explored using Jurkat cells that had been incubated with 5 mM ManLev for 6 days to achieve high levels of ketone expression. ManLev was removed from 5.0 ml of cells (2 × 10^6) at t = 0 and 12 h and the cells were resuspended in 5.0 ml of fresh media. Aliquots of cells (0.5 ml) from the 0 and 24 h time points were removed and analyzed by flow cytometry at 24-h intervals for 4 days. In parallel, Jurkat cells were exposed to cytostatic agents. Demecolcine, colchicine, and camptothecin were found to cause cell death before the ketone-specific signal diminished to background levels in control cells, and were therefore deemed unsuitable for the experiment. However, inhibition of cell growth with minimal toxicity (<15% cell death as determined by trypsin blue staining) was achieved by adding 50 μg/ml sodium azide to the growth media subsequent to removal of ManLev (39). At the end of 4 days the remaining cells (~1.0 ml) were tested for viability by trypsin blue staining. The rate of ketone depletion resulting from simple dilution due to cell division was predicted by determining cell growth rates experimentally and assuming that cell surface ketone density was proportional to total membrane surface area, and therefore, total cell number.

Quantification of Cell Surface Ketones by Flow Cytometry and Scatchard Analysis—The total number of ketones per cell was estimated by quantifying the number of cell surface-associated avidin molecules by Scatchard analysis with 75S-labeled avidin. HL-60, HeLa, and Jurkat cells were grown in 2.0 ml of media containing 80, 100,000 for HeLa and COS-7, and 200,000 for Jurkat (44, 45). Thus, competition of unnatural substrates with these endogenous sugars can result in a cell surface ketone density that is reduced in proportion to total cell surface sialic acid.

RESULTS

Metabolic Conversion of ManLev to Cell Surface SiaLev and Detection of Cell Surface Ketones by Flow Cytometry—The biosynthesis of sialylglycocomponents begins with the precursor N-acetylmannosamine (ManNAc) which can be obtained from the extracellular milieu by passive transport or biosynthesized from either GlcNAc or UDP-GlcNAc within the cytoplasm (42). ManNAc is phosphorylated by a cytosolic kinase and then condensed with phosphoenolpyruvate to form sialic acid 9-phosphate in a reaction catalyzed by sialic acid 9-phosphate synthase. Subsequent dephosphorylation by a phosphatase affords free sialic acid, which is converted to CMP-sialic acid by a synthetase within the nuclear compartment. CMP-sialic acid is then transported into the Golgi compartment and the sialic acid residue is transferred onto the terminus of an oligosaccharide chain by a sialyltransferase.

Since the majority of intracellular ManNAc derives from GlcNAc, one might consider GlcNAc analogs as alternative precursors to unnatural sialosides. There are a number of factors that disfavor this approach. First, GlcNAc serves as a precursor for at least three glycoprotein components (GlcNAc, GalNAc, and sialic acid), whereas ManNAc is transphosphorylated primarily into sialic acid (45). Second, endogenous GlcNAc and UDP-GlcNAc cannot be sustained at high glycosylations concentrations (44, 45). Thus, competition of unnatural substrates with these abundant native metabolites would be severe. By contrast, endogenous ManNAc is maintained at very low cytosolic concentrations as part of the tight regulation imposed on sialic acid biosynthesis (46, 47). Consequently, exogenous mannosamine analogs can accumulate at competitive levels and
readily intercept the sialoside metabolic pathway. As an additional advantage, ManNAc analogs are neutral and therefore more readily taken up by cells than downstream, anionic metabolites such as free sialic acid analogs.

We synthesized the unnatural analog ManLev by condensation of the isobutyl carbonic anhydride derivative of levulinic acid with mannosamine. The metabolic conversion of ManLev to cell surface SiaLev was evaluated in three human cell lines, Jurkat (T-cell lymphoma), HL-60 (promyeloid leukemia), and HeLa (cervical epithelial cell carcinoma). Quantification of cell surface SiaLev residues was achieved as described previously (35). Briefly, the cells were first incubated with the ketone-specific probe biotin hydrazide (BH) (Fig. 2 A). The hydrazide forms a stable (under assay conditions) covalent adduct with ketones, thereby selectively biotinylating cells expressing SiaLev. Second, the biotinylated cells were stained with FITC-labeled avidin and the fluorescence was quantified by flow cytometry. The chemoselective ligation reaction between BH and SiaLev was complete after a period of 2 h and proceeded fastest at lower pH, in accordance with the established rates and pH dependence of traditional hydrazone-forming reactions (Fig. 2 B) (48, 49). In order to maintain optimal cell viability in subsequent experiments, cell surface chemoselective ligation reactions were performed at pH 6.5. Jurkat, HL-60, and HeLa cells incubated with ManLev all showed a large increase in fluorescence compared with control cells, indicating that ManLev is metabolized to cell surface SiaLev in human cells (Fig. 2 C).

**SiaLev Is the Major Metabolic Product of ManLev**—Although we predicted that ManLev would be converted into the corresponding sialoside, we addressed the possibility that ketone expression resulted from conversion of ManLev to N-levulinoylglucosamine (GlcLev) (Fig. 3 A). Since there is facile interconversion of UDP-GlcNAc and UDP-GalNAc (46), and either GlcNAc or GalNAc is incorporated into virtually all glycoproteins, we were concerned that GlcLev might have many avenues for metabolism to glycoconjugates, especially if the enzymes involved in formation of GlcNAc-derived glycoconjugates were as permissive for unnatural substitution as those comprising the sialic acid biosynthetic pathway. Therefore, we synthesized GlcLev and incubated Jurkat cells with this compound under conditions identical to ManLev (5.0 mM, 48 h). Flow cytometry analysis after BH labeling and FITC-avidin staining showed a slight increase in fluorescence above background (less than 2-fold), in contrast to the large (greater than 30-fold) increase in fluorescence resulting from ManLev treatment (Fig. 3 B). The low levels of ketone expression associated with GlcLev treatment may result from direct incorporation into cell surface glycoconjugates through UDP-GlcLev, or from conversion of GlcLev to ManLev followed by metabolism to SiaLev. The pathways open to glucosamine derivatives may be significantly less tolerant of non-native N-acyl substitutions than the enzymes in the sialoside pathway. Alternatively, the high concentrations of endogenous GlcNAc and UDP-GlcNAc may preclude competition by non-preferred, unnatural substrates. We conclude from these results that SiaLev, and not GlcLev, is the major cell surface-associated biosynthetic product of ManLev, and accounts for the majority of cell surface ketones.

The most straightforward method for the direct demonstration of SiaLev on the surface of ManLev-treated cells would be to abrogate the ketone-dependent fluorescence signal by treatment with sialidases, enzymes that liberate sialic acids from glycoconjugates. Unfortunately, the activity of sialidases on substrates with unnatural N-acyl substituents is known to be dramatically reduced, precluding such analysis (50, 35). Instead, we evaluated the effects of ManLev exposure on the
amount of normal sialic acid on Jurkat cells. Free sialic acid was released from ManNAc- and ManLev-treated cells by sialidase, and then quantified by HPAEC (32, 38). Compared with ManNAc treatment, treatment with ManLev caused a significant (4.0–10-fold) depression in the amount of normal sialic acid liberated upon sialidase digestion (Fig. 3C). This result is consistent with the replacement of native sialic acids with unnatural SiaLev on cell surface glycoconjugates.

To assess the similarity between ManNAc and ManLev metabolism, we evaluated changes in the cell surface binding activity of the galactose-specific lectin ricin (51, 52) in response to prolonged (6 day) treatment with both substrates. Our study was motivated by the prior observation that exposure of cells to excess ManNAc for greater than 5 days increases flux in the sialic acid metabolic pathway, resulting in greater numbers of cell surface sialic acids and a concomitant decrease in exposed galactose residues (53, 54). Thus, under normal conditions where free sialic acid acceptor sites exist on the cell surface due to subsaturating levels of sialoside precursors, the addition of exogenous ManNAc results in an increase in cell surface sialylation. Presumably, it is this “excess capacity” in the sialoside biosynthetic pathway that allows alternate substrates such as ManLev to be utilized at high levels. We treated Jurkat cells with various concentrations of ManNAc or ManLev for 6 days and observed a decrease in ricin binding activity that correlated with the concentration of the substrate in the media (Fig. 3D). The response was similar for ManNAc and ManLev, indicating that both substrates increase metabolic flux in the sialoside pathway to a similar extent. These results further substantiate the conclusion that ManLev is metabolized to SiaLev similarly to the metabolism of ManNAc to sialic acid.

ManNAc and ManLev Compete in the Same Biosynthetic Pathway—If ManLev is used as an alternate substrate by the enzymes that biosynthesize cell surface sialosides, then ManNAc should competitively inhibit the display of ketones during co-incubation with ManLev. Accordingly, addition of ManNAc to the culture media concurrently with ManLev inhibited ketone expression with IC₅₀ values of 1–4 mM depending on the cell line (Fig. 4A). The difference in IC₅₀ values for HL-60 cells compared with Jurkat and HeLa cells may reflect differences in the substrate preferences of their respective enzymes. There are a large number of cell and tissue-specific sialyltransferases that transfer CMP-sialic acid, and presumably CMP-SiaLev, onto glycoconjugates (55). Thus heterogeneity among sialyltransferases in the various cell lines may account for the apparent differences in IC₅₀ for ManNAc, a possibility we are currently exploring.

Interestingly, the IC₅₀ value of ManNAc was found to be independent of the ManLev concentration. Cells simultaneously treated with various doses of ManNAc and either 5.0 or 20 mM ManLev showed identical ketone expression levels (Fig. 4A). We confirmed this result by measuring the IC₅₀ for ManNAc at a series of ManLev concentrations up to 30 mM using Jurkat cells (Fig. 4B), and saw no change in the apparent IC₅₀. One possible explanation is that one or more enzymes in the pathway demonstrate a preference for ManNAc over ManLev that is evident only as the ManNAc concentration increases. There are several scenarios that might produce this outcome. For example, the Kₘ values for N-levulinoyl substrates might be lower than those for N-acetyl substrates for one or more of
the enzymes in the biosynthetic pathway, while the catalytic efficiencies of the enzymes with N-acetyl substrates might be higher than with N-levulinoyl substrates, as reflected in $k_{cat}$ values. At lower concentrations, the N-levulinoyl substrates would dominate the pathway, but at higher concentrations the N-acetyl substrates would dominate irrespective of the concentration of competing N-levulinoyl analogs. Additional experiments with individual enzymes in the pathway will be required to directly address this possibility. Although ManNAc is the preferred substrate when both mannosamine derivatives are added to the cells in excess, it should be noted that under normal conditions, the intracellular concentration of ManNAc is maintained at micromolar levels (46, 47) and exogenous ManLev can compete effectively in the biosynthetic pathway.

Time and Dose Dependence of the Metabolic Conversion of ManLev to Cell Surface SiaLev.—The expression of cell surface ketones was found to be dependent on both the concentration of ManLev present in the growth media and the length of time that cells were exposed to ManLev. The dose dependence of ketone display saturated at ManLev concentrations of 25 to 30 mM for Jurkat cells and the fluorescence increased for 5 to 6 days (120–144 h) before reaching a steady-state level (Fig. 5A). This level could be sustained indefinitely provided that ManLev was maintained in the growth media.

A detail of early time points is shown in Fig. 5B for Jurkat cells treated with ManLev or ManNAc (similar results were observed for HL-60 and HeLa cells, data not shown). These data indicate that SiaLev on cell surface glycoconjugates cannot be detected until almost 15–20 h after the monosaccharide precursor is added to the media. By contrast, in vivo pulse-chase experiments have demonstrated the appearance of radiolabeled cell surface sialic acids within 8 min after injection of radiolabeled ManNAc into laboratory rats (56). Similar studies in chickens and mice showed conversion of radiolabeled ManNAc into protein-bound sialic acid between 10 min and 4 h (57, 58). One explanation for the apparent slower kinetics of ManLev metabolism in the present work is that the biosynthetic pathway for sialoglycoconjugates is rendered less efficient by the unnatural N-acetyl side chain. Another possibility is that our fluorescence assay, which has a lower limit of detection corresponding to $\sim 10^4$ ketones per cell, may not have the sensitivity required to monitor the low levels of ketones that could be accumulating at early time points. Nevertheless, once expression of ketones increases above background, the fluorescence signal corresponding to SiaLev labeling follows a kinetic profile which is similar to that reported for the expression of native cell surface-bound sialic acid following exogenous ManNAc supplementation (54, 59).

In addition to the human cell lines Jurkat, HL-60, and HeLa, we evaluated the quantitative parameters of ManLev expression in cells derived from non-human sources. The efficiency of the conversion of ManLev to cell surface SiaLev was found to depend strongly on the species origin of the cells. Most strikingly, we observed that murine cell lines convert ManLev to SiaLev inefficiently compared with similar human cell lines. In the murine-derived erythroleukemia cell line D1B and T-cell lymphoma S49, levels of SiaLev expression were approximately 10% of that displayed by the human T-cell lymphoma Jurkat (Fig. 5C). These differences in the numbers of SiaLev residues per cell cannot be accounted for by differences in cell size or total levels of cell surface sialic acid under normal conditions. The murine D1B and S49 cells are similar to Jurkat cells in both size and total cell surface sialic content (data not shown), yet express 90% fewer ketones when incubated with ManLev. The Green Monkey kidney cell line COS-7 expressed SiaLev at levels approximately 50% lower than observed with similar human cells. COS-7 cells are comparable to HeLa cells in size and total cell surface sialic acid content, yet their ketone expression levels are roughly half of that observed with HeLa cells. The disparity between ManLev metabolism in human and non-human cell lines may reflect differences in substrate promiscuity of the biosynthetic enzymes, a possibility we are currently exploring.

Quantitation of Cell Surface Ketone Levels by Scatchard Analysis.—The absolute number of chemically accessible ketone groups on the surface ManLev-treated cells is of significant interest for cell surface remodeling experiments as it provides a measure of the abundance and density of chemical attachment sites. To quantify the number of ketones per cell, we performed a Scatchard analysis on ManLev-treated, BH-labeled cells using $^{35}$S-labeled streptavidin, and then further related these values to the data obtained by flow cytometry. Scatchard plots for Jurkat, HL-60, and HeLa cells are shown in Fig. 6A. The number of $^{35}$S-labeled streptavidin molecules bound to ManLev-treated, BH-labeled cells is plotted against flow cytometry data in Fig. 6B. The slope of the line derived from the data from all three cell lines is $1.3 \times 10^3$. Using the simplified assumption that each streptavidin molecule binds only one cell-surface biotin moiety, each “arbitrary unit” from flow cytometry represents the signal from $1.3 \times 10^3$ labeled cell surface ketones. We used this figure to convert the maximum level of ketone expression into absolute numbers. We calculate that there are at least $2.0 \times 10^6$ ketones per cell expressed on ManLev-saturated Jurkat and HL-60 cells, and over $10^7$ ketones per cell on saturated HeLa cells. The actual number of ketones per cell is probably higher since not all SiaLev residues are labeled with BH at the concentration used for cell surface staining, and since not all BH moieties might be accessible to FITC-avidin or $^{35}$S-labeled streptavidin binding.

Time-dependent Depletion of Cell Surface Ketones.—Having introduced an unnatural monosaccharide, SiaLev, into cell surface glycoconjugates, we were interested in studying how the
cells would turnover and degrade the unnatural glycoconjugate. We therefore treated cells with 5.0 mM ManLev for 6 days to achieve steady state levels of ketone expression, and then removed ManLev from the media to monitor any changes in the number of ketones per cell as a function of time. Upon removal of ManLev from the growth media of Jurkat, HL-60, and HeLa cells, the fluorescence signal diminished to 50% of its maximal level in under 48 h (Fig. 7 shows the data for Jurkat cells; HeLa and HL-60 cells showed a similar time-dependent decrease in the number of ketones per cell). For each of the cell lines, fluorescence returned to background levels in approximately 5 days. Interestingly, the number of ketones on the cell surface decreased at rates similar to those observed for native sialic acid (54) and a variety of glycoconjugates (60) following removal of exogenous monosaccharide precursors. The simplest explanation for the loss of fluorescence signal is that normal cell growth resulted in dilution of the levulinoyl-sialoglycoconjugates on the plasma membrane. Indeed, there is excellent agreement between the fluorescence signal predicted by considering the effects of cell division (solid line) and that observed experimentally. The contribution of cell division to ketone depletion and loss of signal was confirmed by the addition of the cell growth inhibitory agent sodium azide to the media, after which the fluorescence signal decreased at a significantly lower rate (Fig. 7). Although cell growth can account for the diminishing fluorescence signal, we cannot rule out the possibility that SiaLev is actively removed from cell surface glycoconjugates during constitutive membrane recycling processes. None of the commercially available sialidases cleave the unnatural derivative (35), but there may be enzymes in human cell lines that can hydrolyze SiaLev residues. Still, the rate of cell division and concomitant SiaLev dilution is apparently faster than any active turnover mechanisms and the latter are not discernible using our flow cytometry assay.

**Long-term Ketone Expression Has no Adverse Effects on Cell Viability**—In previous studies, unnatural ManNAc derivatives have been designed with the specific purpose of repressing sialic acid biosynthesis and effecting cell death (29, 31). By contrast, exposure of cells to ManLev does not adversely affect cell viability. All three cell lines appeared normal by light microscopy even when grown with concentrations of ManLev up to 40 mM. To confirm this important result, we monitored the doubling time of Jurkat cells during prolonged exposure to 5.0 mM ManLev, followed by prolonged incubation after removal of ManLev from the media (Fig. 8A). The doubling time of the cells remained unaltered throughout the duration of the experiment. In a complementary experiment, we incubated Jurkat cells with ManLev for 300 h, and then added ManNAc to the media to suppress ketone expression while ManLev was still present (Fig. 8B). After another 300 h, ManNAc was removed from the media containing both monosaccharides, causing the fluorescence signal to reappear following the kinetics and quantitative levels ascribed in Fig. 5. Throughout the duration of this 900-h experiment, cell doubling times remained normal and unaltered. Furthermore, transmission electron micrographs of sectioned Jurkat cells grown in the presence of 20 mM ManLev for 3 days (expressing >10⁵ ketones per cell) showed no obvious cellular abnormalities (not shown).

We saw no evidence for lysosomal storage dysfunction, indicating that these cells possess a mechanism for disposing of SiaLev-modified glycoconjugates although we have not yet identified the details of turnover and/or recycling. The conclusion from this series of experiments is that the presence of cell surface ketones has no apparent adverse effects on normal cellular processes. Furthermore, ketone expression can be modulated in a predictable and reproducible way by simply modulating the concentrations of ManLev and ManNAc in the media.

**SiaLev Is Found in Both N-Linked and O-Linked Glycoproteins**—The distribution of SiaLev residues among the many classes of cell surface glycoconjugates is a matter of significant interest as it pertains to the generality of substrate promiscuity.
The curve expected from dilution of cell surface ketones by cell division (decreased the rate of ketone depletion) was determined by quantifying the extent of cell growth at each time point and normalizing the initial ketone-de- pendent signal accordingly. Inhibition of cell growth by sodium azide cell growth at each time point and normalizing the initial ketone-de- pendent experiments).

Fig. 6. Quantitation of the absolute numbers of accessible ketones per cell by Scatchard analysis. A, typical Scatchard plots are shown for HL-60, HeLa, and Jurkat cells. The number of bound streptavidin molecules per cell is $3.81 \times 10^6$, $1.24 \times 10^6$, and $5.89 \times 10^6$ for Jurkat, HL-60, and HeLa cells, respectively, while the corresponding mean fluorescence intensities obtained by flow cytometry are 41.2, 86.7, and 419, respectively. B, the number of $^{35}$S-labeled streptavidin molecules was plotted against mean fluorescence intensity from several experiments to correlate numbers obtained by flow cytometry to absolute numbers of cell surface bound $^{35}$S-labeled streptavidin molecules.

Fig. 7. Time-dependent depletion of SiaLev residues on Jurkat cells (■, ○, and ♦ represent data from three independent experiments). The curve expected from dilution of cell surface ketones by cell division (solid line) was determined by quantifying the extent of cell growth at each time point and normalizing the initial ketone-de- pendent signal accordingly. Inhibition of cell growth by sodium azide decreased the rate of ketone depletion (□ and ○ represent two independent experiments).

among members within the families of sialoside biosynthetic enzymes. We have begun to address this issue by searching for SiaLev residues in the two most prevalent classes of cell surface glycoproteins, N-linked and O-linked. The three human cell lines used in this study were chosen for their differential patterns of expression of N-linked and O-linked glycans. Jurkat cells are deficient in the core 2 N-acetylglucosaminyltras- ferase, resulting in a deficiency in mature (i.e. sialylated) O- linked glycoproteins (61). As a result, the majority of their sialylated cell surface glycans are N-linked. We treated Jurkat cells with various concentrations of tunicamycin, an inhibitor of N-linked glycosylation (62), prior to incubation with Man- Lev, and observed a dramatic reduction in the ketone-specific fluorescence signal (Fig. 9A). Thus, we conclude that SiaLev residues are presented within the context of N-linked glycoproteins.

Unlike Jurkat cells, HL-60 cells are characterized by a preponderance of O-linked mucin-like cell surface glycoproteins (63). We evaluated the effects of α-BnGalNAc, an inhibitor of the elongation of O-linked oligosaccharides (64, 65), on cell surface ketone expression in these cells. Prior treatment with 4.0 mM α-BnGalNAc abrogated the fluorescence signal accompanying ManLev exposure by >75% (Fig. 9B), indicating that SiaLev resides within O-linked glycoproteins as well. As expected, SiaLev expression on Jurkat cells was only mildly af- fected by treatment with α-BnGalNAc (~25% reduction). Sia- Lev expression on HeLa cells, which possess both sialylated N- and O-linked glycans in abundance, was moderately affected by α-BnGalNAc (~40% reduction). We were unable to perform reciprocal experiments using tunicamycin to inhibit N-linked oligosaccharide biosynthesis in HL-60 and HeLa cells due to side effects that compromised cell viability.

Cell Surface Glycoform Remodeling with Ricin-binding Epitopes—Finally, we exploited cell surface ketones as chemical handles for the display of chemically defined carbohydrate epitopes on the surface of cells. We prepared two synthetic derivatives, aminooxy galactose (Gal-ONH$_2$) (66) and a lactose hydrazide derivative (Lac-NHNH$_2$), which possess suitable nucleophilic carbohydrate functional groups for condensation with cell surface ketones (Fig. 10A). Both analogs were chosen for their potential binding activity with ricin, providing a mechanism for detecting and quantifying their presence on modified cell surfaces.

The reaction of Gal-ONH$_2$ and Lac-NHNH$_2$ with ketone groups on ManLev-treated cells was investigated using two methods. First, we evaluated the ability of both analogs to inhibit the reaction of a small molecule probe, BH, with ketones on the surface of the human T-cell line Jurkat. Jurkat cells were incubated with 20 mM ManLev to incorporate high levels of ketones into cell surface sialosides (approximately $2.5 \times 10^6$ ketones/cell as assessed by flow cytometry). The cells were then reacted with BH and various concentrations of Gal-ONH$_2$ or Lac-NHNH$_2$, and the biotin moieties attached to the cell surface were quantified by staining the cells with fluorescein (FITC)-labeled avidin followed by flow cytometry analysis. As shown in Fig. 10B, the reaction of BH with cell surface ketones was inhibited in a dose-dependent fashion by both synthetic carbohydrate analogs, indicating that the nucleophilic carbohydrates were competing for reaction with cell surface ketone groups. Gal-ONH$_2$ appears to be a more potent competitor than Lac-NHNH$_2$ by a factor of about 2-fold, consistent with the greater nucleophilicity of the aminooxy group compared with the hydrazide at the pH of the reaction (pH 6.5) (48, 49).

The second method used to monitor the cell surface condensa- tion reaction involved the direct detection of the new carbo-
hydrate epitopes using FITC-labeled ricin. In addition to Jurkat cells, we also investigated glycoform remodeling on the human cervical epithelial cancer-derived cell line HeLa. Jurkat or HeLa cells were treated with 20 mM ManLev resulting in the expression of approximately $2.5 \times 10^6$ or $8.0 \times 10^6$ ketones on the cell surface, respectively. Next, the cells were incubated with either Gal-ONH$_2$ or Lac-NHNH$_2$ (50 mM, pH 6.5) to introduce the synthetic epitopes onto the cell surface. In parallel, a control population of cells was treated with N-acetylmannosamine (ManNAc, 20 mM) instead of ManLev, and then exposed to the synthetic carbohydrates under identical conditions. Finally, the cells were stained with FITC-labeled ricin and analyzed by flow cytometry. As shown in Fig. 10C, the ManLev-treated Jurkat and HeLa cells exposed to either Gal-ONH$_2$ or Lac-NHNH$_2$ gained significant ricin binding activity compared with ManNAc-treated controls. ManLev- and ManNAc-treated cells that were not exposed to the synthetic carbohydrates showed similar background levels of ricin binding activity. These results demonstrate that endogenous glycoconjugates expressing levulinoyl groups can be remodeled with

**FIG. 8.** Experimental manipulation of cell surface ketone expression levels and long-term cell viability. A, SiaLev expression in Jurkat cells was maintained indefinitely at a constant level in the presence of 5.0 mM ManLev (■, , and ● represent data from independent experiments). When ManLev was removed from the media at $t = 300$ h, the fluorescence signal decreased to the background level (□, ○, and △ represent data from independent experiments). B, Jurkat cells were incubated continuously with ManLev (5.0 mM), and at $t = 300$ h, ManNAc (5.0 mM) was introduced into the media. ManNAc was then removed at 600 h while maintaining ManLev in the media. Data from two separate experiments are presented (■ and □). In both panels, the doubling time (in hours) of the cells is represented by the solid line and remains essentially unchanged throughout the duration of the experiments.

**FIG. 9.** SiaLev is resident in both O-linked and N-linked glycans, as determined by abrogation of ketone-dependent fluorescence by glycosylation inhibitors. A, tunicamycin inhibited SiaLev expression on Jurkat cells (induced by exposure to 5.0 mM ManLev). B, α-BnGalNAc (4.0 mM) inhibited ketone expression in HL-60 cells and partially in HeLa cells.
There is increasing evidence suggesting that the context in which oligosaccharides are presented may have a profound influence on the outcome of biological investigations. This point has been illustrated in two examples from the recent literature. Sialyl Lewis x analogs that bound the leukocyte adhesion molecule L-selectin demonstrated a different rank order of affinities when immobilized on the surface of cells (22) than had been previously observed in solution binding assays (67). More recently, the relative binding affinities of disaccharides from a combinatorial library to a plant-derived lectin were found to depend on whether the carbohydrates were assayed as soluble analogs or immobilized on polystyrene beads (68). The information that dictates specificity and binding strength may be partially encoded in the sequence of individual oligosaccharide molecules, but the density and spacing of these epitopes on a cell surface may provide an additional level of information that governs the nature of the interaction (8).

The potential for context-dependent molecular recognition underscores the importance of methods that facilitate the investigation of carbohydrate epitopes within a more native context such as the surface of a cell. The cell surface glycoform engineering technique described herein is a straightforward method for accomplishing this goal. ManLev metabolism and concomitant ketone expression follow well defined and predictable kinetics. The density of cell surface ketones can be readily controlled by manipulating the concentration of ManLev in the media or the duration of the incubation, and the presence of millions of ketones on the cells has no observable adverse effect on cell viability. The simplicity of this technique and the precision with which cell surface reactivity can be controlled make it well suited for use in the study of carbohydrate-mediated cell surface interactions.

Because SiaLev residues reside in both N-linked and O-linked glycans, any sialylated human-derived cell is a potential candidate for glycoform remodeling. However, the permissive nature of sialoside biosynthesis, critical for the success of the method, does not necessarily extend to the homologous pathways in other species. While ManLev is readily converted to SiaLev in human cell lines, murine-derived cells metabolized ManLev at a rate that was significantly lower, resulting in ketone expression levels only 10% of those observed in similar human cells. This difference could not be accounted for by overall levels of normal sialic acid expression which were similar among the murine and human cells. Thus, it appears that the substrate specificity profile observed in human-derived cells lines is not identical to that in murine cells, and cells from other animals should be evaluated on a case-by-case basis prior to utilization of this technique.

In addition to native-like oligosaccharide epitopes, unnatural oligosaccharides or glycomimetics can be introduced onto cells through the simple coupling reaction with cell surface ketones. The method therefore establishes a foundation for studying structure-activity relationships among myriad cell surface epitopes while they reside within their native environment. It is notable that both the mechanism by which ketones are introduced onto cells and the chemical reaction with complementary nucleophiles are compatible with the physiological environment of a whole animal (26). This added dimension might allow for glycoform engineering in vivo, an interesting extension of the method.

There are a multitude of biological recognition events that may be studied using glycoform-engineered cells. For example, leukocyte-endothelial cell interactions central to the inflammatory response (69), and the attachment of certain bacteria and viruses to cells prior to infection (70), are processes that are known to involve carbohydrate-receptor interactions but remain poorly understood at the molecular level. Now that combinatorial libraries of carbohydrates are becoming available (71), new assay architectures for screening biologically relevant binding activities and downstream events such as microbial

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**FIG. 10.** Engineering increased ricin binding activity onto ManLev-treated cells with the synthetic epitopes Gal-ONH$_2$ and Lac-NHNH$_2$ (A). B, the synthetic carbohydrates compete with BH for reaction with cell surface ketones. Data points represent the average from duplicate experiments. C, ManLev-treated cells exposed to Gal-ONH$_2$ or Lac-NHNH$_2$ showed high ricin binding activity compared with control cells that were preincubated with ManNAc and therefore devoid of cell surface ketones. Similar results were obtained with the two cell lines Jurkat and HeLa. Data points represent the average from three experiments performed in duplicate and *error bars* represent the standard deviation.

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### DISCUSSION

Novel carbohydrate epitopes, and that the epitopes are accessible for recognition by protein receptors.

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invasion or cellular signaling will be increasingly in demand. It may be possible to screen libraries of oligosaccharide analogs in a rapid parallel fashion using glycofilm-engineered cells. We are presently exploring applications of metabolic cell surface engineering to these and related problems.

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