The absence of viral receptors is a major barrier to efficient gene transfer in many cells. To overcome this barrier, we developed an artificial receptor based on expression of a novel sugar. We fed cells an unnatural monosaccharide, a modified mannosamine that replaced the acetyl group with a levulinate group (Man-Lev). ManLev was metabolized and incorporated into cell-surface glycoconjugates. The synthetic sugar decorated the cell surface with a unique ketone group that served as a foundation on which we built an adenovirus receptor by covalently binding biotin hydrazide to the ketone. The artificial receptor enhanced adenoviral vector binding and gene transfer to cells that are relatively resistant to adenovirus infection. These data are the first to suggest the feasibility of a strategy that improves the efficiency of gene transfer by using the biosynthetic machinery of the cell to engineer novel sugars on the cell surface.

In many cells, lack of appropriate cell surface receptors is a major barrier to efficient viral-mediated gene transfer. One strategy to overcome this barrier is to chemically or genetically engineer new ligands onto the virus so that it will bind to existing cell receptors (1–3). A hypothetical second strategy would be to engineer the cell surface to display new receptors for existing gene transfer vectors. The goal of this work was to explore this second strategy.

N-Acetylneuraminic acid, a member of the sialic acid family, is the most abundant terminal sugar residue on mammalian cell glycoproteins and glycolipids (4). Biosynthesis of this sialic acid requires extensive enzymatic modification of N-acetylmannosamine before incorporation into the terminal position on oligosaccharides and expression on the cell surface (5, 6). The cellular enzymes responsible for synthesis of N-acetylneuraminic acid are known to tolerate substitutions at the N-acetyl position of N-acetylmannosamine. When various N-acetylmannosamines were present in the culture medium, modified sialic acids were incorporated into carbohydrates and expressed on the cell surface (7–9). Likewise, intraperitoneal injection of modified monosaccharides allowed expression of their metabolites on serum glycoproteins in rats (9). Bertozzi and co-workers (10) constructed a modified mannosamine that replaced the acetyl group with a levulinate group (named Man-Lev). They found that ManLev was metabolically incorporated into glycoconjugates and expressed on the surface of three cell lines. A unique feature of ManLev is that it places a ketone group on the cell surface. Because ketone groups are virtually absent from the surface of cells, metabolic incorporation of ManLev provides a novel functional group to which other molecules can be attached (10). In this study, we tested the hypothesis that ManLev could be used to create novel artificial viral receptors that would enhance adenovirus-mediated gene transfer in poorly infected cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Adenovirus Vector—**NIH-3T3 were cultured on 8-well plastic slides (Nalgé #177445, Naperville, IL) in Dulbecco’s minimal essential medium (high glucose) supplemented with 10% fetal calf serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. Primary cultures of human umbilical vein endothelial cells (HUVEC) were isolated as described previously (11) and plated on 8-well plastic slides in Media 199 supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin; the purity of HUVEC cells was >98%. A recombinant adenovirus vector expressing β-galactosidase (Ad2/LacZ) was prepared as described previously (12) by the University of Iowa Gene Transfer Vector Core at titers of approximately 2 × 10^10 IU/ml (particle/infectious unit ratio was approximately 50). The virus was applied to cells under indicated conditions at a multiplicity of infection of 100.

**Preparation of NeutrAvidin/Antibody Conjugate—**Two anti-adenovirus monoclonal antibodies were used, an antibody directed against fiber knob (1D6, a generous gift of Dr. David Curiel, University of Alabama at Birmingham) and an antibody directed against the fiber shaft (5C91, a generous gift of Dr. Beverly Davidson and Richard Anderson, University of Iowa). Antibody 1D6 inhibits infection by blocking fiber binding; antibody 5C91 does not inhibit infection. Both antibodies gave similar results in the binding and expression experiments. NeutrAvidin was coupled to the antibodies by sulhydryl linkage to the interchain sulf-hydryl groups using maleimide-activated NeutrAvidin as recommended by the manufacturer (Pierce). Antibody conjugates were separated from unreacted NeutrAvidin by purification through protein G columns. The final concentration of the purified NeutrAvidin/antibody conjugate was approximately 0.5 μg/ml. When the products of the reaction were examined by nonreducing SDS-polyacrylamide gel electrophoresis, the NeutrAvidin/antibody conjugate showed the expected electrophoretic shift (not shown). In addition, the NeutrAvidin/antibody conjugate was functional for adenovirus binding and biotin binding in an enzyme-
linked immunosorbent assay with adenovirus bound to a plate and detection with biotinylated horseradish peroxidase (not shown).

**Synthesis and Purification of ManLev**—Solid N-hydroxysuccinimide (1.86 g, 16.11 mmol) was added to levulinic acid (1.70 g, 14.65 mmol) in CH₂Cl₂ (30 ml). The reaction mixture was cooled to 0 °C, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (3.09 g, 16.11 mmol) was added. After 10 min, the mixture was allowed to warm to room temperature and stirred overnight. The solution was diluted with CH₂Cl₂ and brine. After the organic phase was dried over MgSO₄ and filtered, the solvent was removed under reduced pressure to yield N-hydroxysuccinimide-Lev (2.65 g, 85%). § H NMR δ 2.89 (m, 4H), 2.83 (s, 4H), 2.21 (s, 3H), 1.48 (m, 4H), 0.63 (m, 2H), 2.49 (s, 3H). The hydroxide form of the DEAE-cellulose (Bio-Rad, 5 g) was allowed to stir in 1 M aqueous NaOH (100 ml) for 1.5 h. The resulting slurry was filtered and rinsed thoroughly with H₂O (4 × 100 ml). The resulting solid was air-dried, and the remaining traces of H₂O were removed under vacuum to give an off-white material with a Styrofoam-like appearance.

A solution of NaOCH₃ was prepared from Na+ metal (30 mg, 1.27 mmol) and CH₃OH (7 ml) at 0 °C. Once the reaction was complete, D-mannosamine hydrochloride (250 mg, 1.16 mmol) was added in one portion, and the resulting mixture was allowed to stir for an additional 45 min. N-Hydroxysuccinimide-Lev (372 mg, 1.27 mmol) in 2 ml CH₂Cl₂ was added via cannula, and the suspension was stirred overnight. The resulting slurry was filtered through Celite and rinsed with CH₃OH, and the filtrate was concentrated in vacuo to produce an off-white oily residue. This material was further dried under high vacuum to produce an off-white foam, which was purified by flash column chromatography (14 to 20% CH₃OH in CHCl₃) to provide Man-Lev (222 mg, 89%). § H NMR δ 5.12 (d, J = 1.5 Hz, 0.6H), 5.03 (d, J = 1.5 Hz, 0.4H), 4.46 (dd, J = 4.2, 1.5 Hz, 0.4H), 4.32 (dd, J = 4.8, 1.5 Hz, 0.6H), 4.06 (dd, J = 9.6, 4.8 Hz, 0.6H), 3.93–3.79 (m, 3H), 3.64 (dd, J = 9.9, 6.9 Hz, 0.6H), 3.53 (dd, J = 9.9, 9.6 Hz, 0.4H), 3.42 (ddd, J = 9.9, 4.8, 2.1 Hz, 0.4H), 2.92–2.85 (m, 2H), 2.63 (t, J = 6.9 Hz, 0.8H), 2.58 (t, J = 6.9 Hz, 1.2H), 2.25 (s, 1H), 2.24 (s, 1H), 13C (major epimer) δ 216.9, 178.6, 96.0, 74.8, 71.7, 69.6, 63.3, 56.0, 41.0, 32.1, 28.9.

**Fluorescence-activated Cell Sorter Analysis and Direct Immunofluorescence**—Cells were cultured for 48 h with control media or media supplemented with 30 mM ManLev. Cells were rinsed once with 500 μl of Eagle’s minimal essential medium, and biotin-LC-hydrazone (Pierce) (10 mM in phosphate-buffered saline (PBS)) was applied to cells at room temperature for 1.5 h, which preliminary studies showed to be optimal. The cells were rinsed with cold media to remove excess biotin hydrazide. Cells were then briefly trypsinized and rinsed in cold media. Streptavidin-FITC (1 μg/ml, Vector Laboratories, Inc., Burlingame, CA) in PBS was applied for 10 min at 4 °C. Cells were then rinsed in 5 ml of cold media. Relative fluorescence was analyzed by fluorescence-activated cell sorter (Becton Dickinson, Mansfield, MA). For direct inspection of fluorescence after streptavidin-FITC treatment, cells were rinsed twice with 500 μl of cold media and then fixed with 4% paraformaldehyde/PBS.

**Binding of Virus and Beads**—To evaluate virus association with cells, adenovirus was labeled with the carboxy cyanine dye Cy3 (American Pharmacia Biotech) using methods described by Leopold et al. (13). Cy3 was covalently conjugated to capsid proteins of adenovirus by mixing 5 mM of Cy3 with 10⁻⁵ particles of virus in 1.5 ml of Na₂CO₃ at pH 9.0 for 2 h at 4 °C. The solution was subsequently transferred to a dialysis chamber (Slide-A-Lyzer, 10,000 molecular mass cutoff, Pierce) and dialyzed against two changes of PBS, 3% sucrose, pH 7.4 at 4 °C for 24 h. To attach labeled virus to cells with artificial viral receptors, NIH-3T3 cells were labeled with 10 μM biotin-LC-hydrazone (50 μg/ml) in PBS on ice for 30 min. Cells were rinsed with cold media, and 1 × 10¹⁰ adenovirus particles were applied for 30 min at 4 °C. Cells were rinsed twice with cold media and fixed with 4% paraformaldehyde/PBS. Fluorescence was assayed by confocal microscopy. To evaluate binding of particles, we applied streptavidin-coated yellow/green 40-nm Fluospheres (f-8780, Molecular Probes, Eugene, OR) to cells treated with biotin hydrazide.

**Evaluation of Transgene Expression**—β-Galactosidase activity was measured 24 h after application of vector as described previously (14). Individual experiments were performed using three sets of cells, and all experiments were repeated at least three times.
Expression of ManLev on the Cell Surface—Earlier work showed that when three immortalized cell lines, Jurkat, HL-60, and HeLa, were fed ManLev, the sugar was metabolized, and the downstream product, N-levulinoyl sialic acid, incorporated into cell surface oligosaccharides in place of the natural sialic acid (10). To test the potential utility of this system, we asked if ManLev could be incorporated into other cell lines and into primary cultures of human cells. We added ManLev (30 mM) to the culture medium and assayed expression of cell surface ketones by the covalent binding of biotin hydrazide to cells followed by detection with streptavidin-FITC. Fig. 1 shows that when primary cultures of HUVEC were fed ManLev, the coupling of biotin hydrazide increased, as evidenced by a 200-fold fluorescence shift on flow cytometry analysis and by direct inspection with fluorescence microscopy. There was little fluorescence in cells not fed ManLev. The increase in cell surface ketones was not restricted to HUVEC cells or to human cell lines; we saw a 25-fold increase in mouse NIH-3T3 fibroblasts, a 180-fold increase in human embryonic kidney cells (HEK293), a 20-fold increase in dog kidney epithelial cells (Madin-Darby canine kidney cells), a 35-fold increase in primary cultures of human airway epithelial cells, and a 200-fold increase in human cervical carcinoma-derived cells (HeLa) (not shown).

The formation of a hydrazone bond between biotin hydrazide and the unnatural sialic acid will depend on the concentration of both substrates. Earlier work showed that a concentration of 20–40 mM ManLev in the culture medium yielded maximum expression of ketones on the surface of Jurkat cells (10). We obtained similar results with HUVEC, NIH-3T3, and 293 cells (not shown). To examine the effect of the amount of hydrazide, we varied the concentration of biotin hydrazide applied to primary cultures of human cells. We added ManLev (30 mM) to the culture medium and treated the indicated concentrations of biotin-LC-hydrazide. Cells were briefly trypsinized and rinsed before the addition of streptavidin-treated with the indicated concentrations of biotin-LC-hydrazide. Cells were then reacted with biotin hydrazide, labeled with streptavidin-FITC, and analyzed by fluorescence-activated cell sorter, exactly as described for Fig. 1. Data are mean fluorescence. Control, control cells. +AD, adipic dihydrazide (100 mM) was added to cells during biotin hydrazide reaction. +PDPH, 3-(2-pyridyldithio)propionyl hydrazide (100 mM) was added to cells as in B. +SA, unlabeled streptavidin (10 μg/ml) was added during streptavidin-FITC binding. No reaction, cells were not treated with biotin hydrazide or streptavidin-FITC.

![Fig. 2. Concentration dependence of biotin hydrazide binding to cells fed ManLev. HUVEC cells were grown in 30 mM ManLev and treated with the indicated concentrations of biotin-LC-hydrazide. Cells were briefly trypsinized and rinsed before the addition of streptavidin-FITC (1 μg/ml) for 10 min at 0 °C. Similar results were obtained in two other experiments.](image1)

**RESULTS**

**Expression of ManLev on the Cell Surface**—Earlier work showed that when three immortalized cell lines, Jurkat, HL-60, and HeLa, were fed ManLev, the sugar was metabolized, and the downstream product, N-levulinoyl sialic acid, incorporated into cell surface oligosaccharides in place of the natural sialic acid (10). To test the potential utility of this system, we asked if ManLev could be incorporated into other cell lines and into primary cultures of human cells. We added ManLev (30 mM) to the culture medium and assayed expression of cell surface ketones by the covalent binding of biotin hydrazide to cells followed by detection with streptavidin-FITC. Fig. 1 shows that when primary cultures of HUVEC were fed ManLev, the coupling of biotin hydrazide increased, as evidenced by a 200-fold fluorescence shift on flow cytometry analysis and by direct inspection with fluorescence microscopy. There was little fluorescence in cells not fed ManLev. The increase in cell surface ketones was not restricted to HUVEC cells or to human cell lines; we saw a 25-fold increase in mouse NIH-3T3 fibroblasts, a 180-fold increase in human embryonic kidney cells (HEK293), a 20-fold increase in dog kidney epithelial cells (Madin-Darby canine kidney cells), a 35-fold increase in primary cultures of human airway epithelial cells, and a 200-fold increase in human cervical carcinoma-derived cells (HeLa) (not shown).

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![Fig. 3. Competition for hydrazone formation and streptavidin binding. HUVEC cells were all grown in 30 mM ManLev. Cells were then reacted with biotin-LC-hydrazide, labeled with streptavidin-FITC, and analyzed by fluorescence-activated cell sorter, exactly as described for Fig. 1. Data are mean fluorescence. Control, control cells. +AD, adipic dihydrazide (100 mM) was added to cells during biotin hydrazide reaction. +PDPH, 3-(2-pyridyldithio)propionyl hydrazide (100 mM) was added to cells as in B. +SA, unlabeled streptavidin (10 μg/ml) was added during streptavidin-FITC binding. No reaction, cells were not treated with biotin hydrazide or streptavidin-FITC.](image2)
recombinant adenovirus. We chose not to directly chemically modify the viral capsid to display a hydrazide because our preliminary attempts to chemically link a hydrazide to adenovirus inactivated the virus (14). Therefore, to test the concept, we chose an alternate strategy (Fig. 5), using the uniquely reactive ketones as chemical handles for attaching artificial virus receptors; in our case we used biotin hydrazide and then a NeutrAvidin-conjugated anti-adenovirus antibody. The goal was to adorn the cell surface with these artificial receptors.

Adenovirus normally binds to cells via an interaction be-

Fig. 4. Binding of streptavidin-coated beads to the cell surface. HUVEC cells were cultured for 48 h (C and D) with HUVEC medium containing 30 mM ManLev or (A and B) HUVEC medium alone. Cells were treated with biotin-LC-hydrazide (10 mM in PBS) for 1.5 h at room temperature. Streptavidin-coated yellow/green 40-nm FluoSpheres (Molecular Probes, f-8780) were diluted 1:100 in PBS and applied to cells for 10 min. Cells were rinsed twice with cold HUVEC medium and fixed in 4% paraformaldehyde in PBS. Cells were examined by phase contrast (left) and confocal (right) microscopy. Similar results were obtained in two other experiments.

Fig. 5. Model of strategy to engineer novel viral receptor on cell surface. See text for details.
between its fiber protein and the cellular coxsackie-adenoviral receptor (15). Therefore, we chose NIH-3T3 cells for these studies because they lack this receptor, they do not readily bind adenovirus, and they are resistant to infection (16). To learn whether we could create a viral receptor that would enhance adenovirus binding to NIH-3T3 cells, we grew cells with and without ManLev and treated them with biotin hydrazide. We then exposed cells to the NeutrAvidin/antibody conjugate to generate a viral receptor. Finally, we applied Cy3-labeled adenovirus for 30 min and examined the cell surface using fluorescence confocal microscopy. Fig. 6 shows that virus bound only the ManLev-fed cells on which we had constructed the artificial receptor. In contrast, there was little or no bound virus in cells not fed ManLev or that lacked the NeutrAvidin/antibody conjugate.

**Construction of Artificial Adenovirus Receptors Enhanced Gene Transfer**—To test the hypothesis that increased binding could enhance gene transfer, we applied a recombinant adenovirus that expresses β-galactosidase to NIH-3T3 cells on which we had built an artificial adenovirus receptor as described above. Fig. 7A shows that the engineered receptors increased expression approximately 50-fold. Enhancement required that the cells were fed ManLev, and the cell surface was treated with biotin hydrazide. We also tested expression in primary cultures of HUVEC cells; the engineered receptor enhanced gene transfer approximately 9-fold (Fig. 7B). Enhanced expression required incorporation of ManLev and the NeutrAvidin/antibody conjugate. These results demonstrate that an engineered receptor can increase virus binding and enhance gene transfer. The smaller relative increase in HUVEC cells (9-fold) versus 3T3 cells (50-fold) might reflect a lower density of ketone-bearing sialic acids on the HUVEC cells. It is worthy of note that rapidly dividing cells have been shown to express unnatural sialic acids at a higher level than slowly dividing cells (17). The NIH-3T3 cells may engage in higher levels of sialic acid biosynthesis, resulting in higher levels of cell surface ketones and, correspondingly, higher densities of artificial adenovirus receptors. Nonspecific binding of the conjugate to the cell surface might account for the higher level of transgene expression under control conditions such as in the absence of biotin hydrazide (Fig. 7A). These results demonstrate that an engineered receptor can increase virus binding and enhance gene transfer.
Lack of cellular receptors to bind vectors currently limits gene transfer in a number of applications. We have taken a novel strategy to create a new cell surface receptor for an adenovirus vector. Fig. 5 schematically outlines the approach. ManLev included in the culture medium was biosynthetically converted to an unnatural sialic acid and incorporated into cell surface glycoconjugates. Thus, the cells were engineered to display a novel ketone on the cell surface. The ketone group provided a unique chemical handle to which we covalently attached biotin hydrazide. Biotin, in turn, was coupled to NeutrAvidin linked to an antibody specific to adenovirus. With this artificial receptor on the cell surface, binding and gene transfer with recombinant adenoviruses increased in cells that are normally resistant to adenovirus-mediated gene transfer.

This novel approach has several potential advantages. Because display of unique functional groups is accomplished through the biosynthetic processes of the cell, the method provides an opportunity to modify the cell surface simply by providing a readily incorporated substrate. Thus, it could potentially be applied to many different cell types, as evidenced by the successful incorporation of ketones onto primary human cell cultures and cell lines and cells from other species. Importantly, this strategy is targeted at the first, often rate-limiting step in gene transfer, vector binding to the cell surface. There are also opportunities for use of unnatural carbohydrate substrates other than ManLev; for example, the target monosaccharide or side chain could be changed and display of functional groups other than ketones may be feasible. Although we used an adenovirus vector to test the concept, the method is potentially applicable to creation of receptors or targets for other viral vectors as well as for nonviral vectors. Conversely, unique sugar groups might be incorporated into surface glycoproteins on viral vectors by feeding synthetic sugars to virus-producing cell lines.

The successful facilitation of gene transfer in vitro underscores the utility of this approach, but there remain limitations. One limitation is the complexity of the strategy depicted in Fig. 5. Three steps were used to enhance adenovirus binding and gene expression: incubation of cells with ManLev, application of biotin hydrazide, and addition of NeutrAvidin/antibody conjugates. Because each step will have some limitation in efficiency, this complexity will likely limit the number of functional receptors formed. Thus, an important future goal is to reduce the intermediate steps involved in receptor construction to achieve direct attachment of vectors to metabolically engineered cells. Another limitation is the high concentration of ManLev we used. This limitation might be circumvented in other cases as several ways, for example, by acetylation of the sugar to enhance entry into cells (18). Preliminary studies suggest that the same number of cell surface ketones generated by ManLev treatment can be obtained by treatment of cells with peracetylated ManLev at concentrations approximately 200-fold lower. Thus, future applications of the technique might be accomplished with either high concentrations of the free sugar, which is more conveniently prepared, or lower concentrations of an acetylated or otherwise modified sugar if concentration is an issue.

The ability to use an unnatural sugar introduced into many different cellular glycoproteins and glycolipids suggests that adenovirus can use artificial receptors for infection; the endogenous adenovirus receptor CAR (coxsackie-adenoviral receptor) (15) is not essential. The conclusion that specificity in binding is not required for adenovirus infection is supported by earlier work in which an increase in nonspecific binding improved gene transfer to adenovirus-resistant cells (14, 19, 20). The fact that enhancement of binding, even that which is not specific, can facilitate gene transfer suggests the feasibility of future approaches that alter either the viral capsid or the cell surface to introduce new ligands and/or receptors.

Our data suggest the feasibility of a gene transfer strategy in which the biosynthetic machinery of the cell is used to engineer novel receptors on the cell surface. Such a strategy might provide significant advantages for ex vivo gene transfer applications in which the target cell is relatively resistant to a vector that otherwise might have significant advantages. Could a similar strategy be developed for in vivo gene transfer? There are several additional considerations. First, any cell decorated with sialic acid could potentially be modified with ketone groups; cells with the highest flux in the sialic acid pathway would likely display the greatest number of ketones on their surface. Thus, one would not expect to achieve significant selectivity among cell types. In some cases this could be an advantage. However, in other cases targeting might require selective delivery, for example into the airway lumen, into the brain, etc. Thus, the development of mechanisms for delivering ManLev or other carbohydrates to specific tissues is a subject of current interest. Second, as described above, the current system is too complex for in vivo use, and some of the reagents (e.g. NeutrAvidin) would be immunogenic. Future modifications and developments would be required for in vivo use, with a focus on eliminating the need for intermediate protein conjugates between virus particles and unnatural cell surface epitopes. Third, considerations of efficiency are similar to those with other vectors; studies in animals will be required to learn where the general strategy we describe can also increase gene transfer to resistant cells in vivo. Finally, safety considerations would be critical. In earlier studies, Keppler et al. (7) administered N-propanoyl-D-mannosamine and N-propanoyl-d-glucosamine intraperitoneally to rats; there were no adverse effects, and substitution of native N-acetyleneuramic acid residues with nonnative analogs occurred in all organs. However, additional studies of safety would be required. Still it is possible that short-term administration of future generations of unnatural sugars, receptors, and vectors could prove to be an effective means of facilitating gene transfer.

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