Engineering Sialic Acid Synthetic Ability into Insect Cells: Identifying Metabolic Bottlenecks and Devising Strategies To Overcome Them†

Karthik Viswanathan,‡ Shawn Lawrence,‡ Stephan Hinderlich,§ Kevin J. Yarema,|| Yuan C. Lee,⊥ and Michael J. Betenbaugh*,‡

Departments of Chemical and Biomolecular Engineering, Biomedical Engineering, and Biology, Johns Hopkins University, Baltimore, Maryland 21218, and Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Fachbereich Humanmedizin, Arnimallee 22, D-14195 Berlin-Dahlem, Germany

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ABSTRACT: Previous studies have indicated negligible levels of both sialylation and the precursor *N*-acetylneuraminic acid (Neu5Ac) in a number of insect cell lines grown in serum-free medium. The overexpression of the human sialic acid 9-phosphate synthase (SAS) in combination with *N*-acetylmannosamine (ManNAc) feeding has been shown to overcome this limitation. In this study we evaluated the potential bottlenecks in the sialic acid synthesis pathway in a *Spodoptera frugiperda* (Sf9) insect cell line and devised strategies to overcome them by overexpression of the enzymatic pathway enzymes combined with appropriate substrate feeding. Coexpression of SAS and UDP-GlcNAc 2-epimerase/ManNAc kinase, the bifunctional enzyme initiating sialic acid biosynthesis in mammals, resulted in Neu5Ac synthesis without use of any external media supplementation to demonstrate that Neu5Ac could be generated intracellularly in Sf9 cells using natural metabolic precursors. *N*-Acetylglucosamine (GlcNAc) feeding in combination with this coexpression resulted in much higher levels of Neu5Ac compared to levels obtained with ManNAc feeding with SAS expression alone. The lower Neu5Ac levels obtained with ManNAc feeding suggested limitations in the transport and phosphorylation of ManNAc. The bottleneck in phosphorylation was likely due to utilization of GlcNAc kinase for phosphorylation of ManNAc in insect cells and was overcome by expression of ManNAc kinase. The transport limitation was addressed by the addition of tetra-*O*-acetylated ManNAc, which is easily taken up by the cells. An alternative sialic acid, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (KDN), could also be generated in insect cells, suggesting the potential for controlling not only the production of sialic acids but also the type of sialic acid generated. The levels of KDN could be increased with virtually no Neu5Ac generation when Sf9 cells were fed excess GlcNAc. The results of these studies may be used to enhance the sialylation of target glycoproteins in insect and other eukaryotic expression systems.

Insect cells are widely used for the production of numerous recombinant proteins, typically through the baculovirus expression vector system (BEVS[1]). They often produce recombinant proteins in high yields and have the capacity to perform posttranslational modifications including glycosylation (*1*). The nature of the attached glycan can influence the structure, function, and stability of the protein (*2*, *3*). Unlike mammalian cells, insect cells are typically incapable of generating complex *N*-glycans with a terminal *N*-acetylneuraminic acid (Neu5Ac) (*4*, *5*). Insect cells often produce truncated or paucimannosidic *N*-glycans terminating in mannose (Man) or occasionally *N*-acetylglucosamine (GlcNAc) (*4*, *6*). As a result, glycoproteins derived from insect cells are likely to have shorter in vivo circulatory half-lives which lower the potential therapeutic value of these products (*7*). An insect cell line capable of producing sialylated proteins while retaining the other advantages of the BEVS such as high yields would represent a significant development to the biotechnology industry.

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* To whom correspondence should be addressed. Phone: (410) 516-5461. Fax: (410) 516-5510. E-mail: beten@jhu.edu.

‡ Department of Chemical and Biomolecular Engineering, Johns Hopkins University.

§ Freie Universität Berlin.

|| Department of Biomedical Engineering, Johns Hopkins University.

⊥ Department of Biology, Johns Hopkins University.

[1] Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; SAS, sialic acid 9-phosphate synthase; ManNAc, *N*-acetylmannosamine; Sf9, *Spodoptera frugiperda*; EpimKin, UDP-GlcNAc 2-epimerase/ManNAc kinase; GlcNAc, *N*-acetylglucosamine; Ac_4ManNAc, tetra-*O*-acetylated ManNAc; KDN, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; BEVS, baculovirus expression vector system; Man, mannose; Gal, galactose; CMP-Neu5Ac, cytidine monophosphate Neu5Ac; UDP-GlcNAc, uridine diphosphate GlcNAc; ManNAc-6-P, ManNAc 6-phosphate; Man-6-P, mannose 6-phosphate; CMP-SAS, cytidine monophosphate sialic acid 9-phosphate synthase; CHO, Chinese hamster ovary; CMP-KDN, cytidine monophosphate 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; AcSAS, *Autographa californica* baculovirus containing sialic acid 9-phosphate synthase gene; DMB, 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride; HPLC, high-performance liquid chromatography; PEP, phosphoenol pyruvate; Neu5Ac-9-P, *N*-acetylneuraminic acid 9-phosphate; AcEpimKin, *A. californica* baculovirus containing UDP-GlcNAc 2-epimerase/ManNAc kinase gene; AcKin, *A. californica* baculovirus containing mutated UDP-GlcNAc 2-epimerase/ManNAc kinase gene with only ManNAc kinase activity; AcEpim, *A. californica* baculovirus containing mutated UDP-GlcNAc 2-epimerase/ManNAc kinase gene with only 2-epimerase activity.

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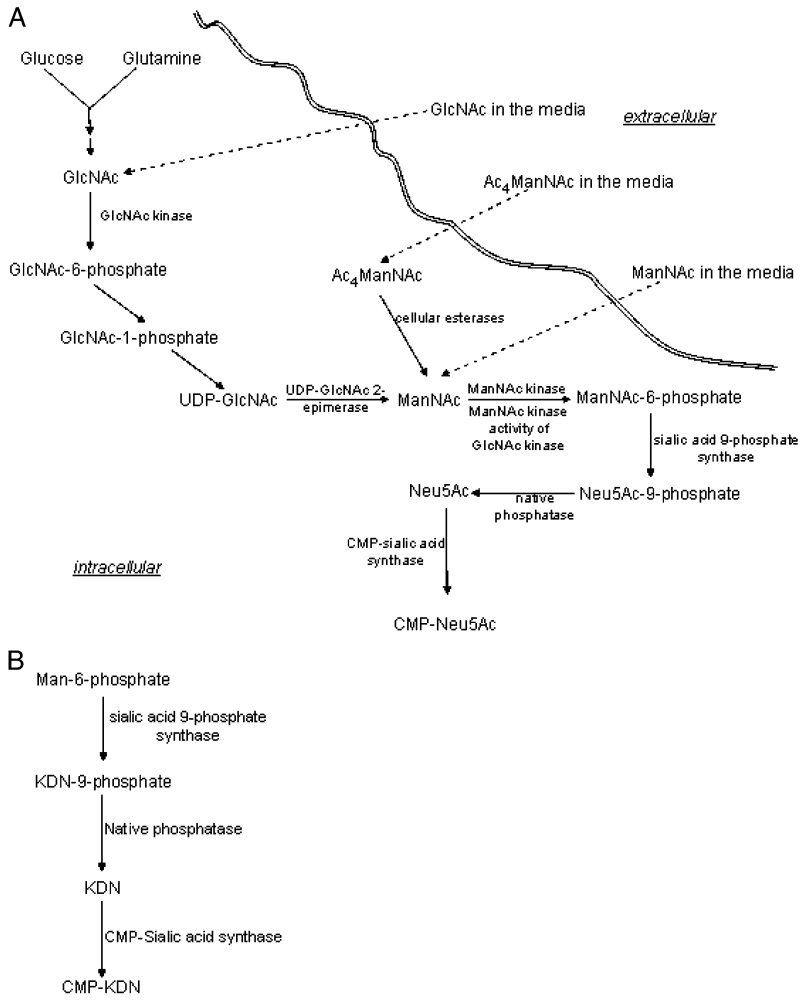

FIGURE 1: Schematic of the sialic acid biosynthetic pathways in mammalian cells. (A, top) Pathway for synthesis of Neu5Ac and CMP-Neu5Ac in mammalian cells. (B, bottom) Steps in the synthesis of KDN and CMP-KDN in mammalian cells.

Metabolic engineering represents one approach for improving the glycosylation properties of insect cell proteins. Previous efforts in this area have focused primarily on the expression of transferases. *N*-Acetylglucosaminyltransferase-I (GlcNAcT-I) (*8*) and β-1,4-galactosyltransferase (GalT) (*9*, *10*) have been expressed in insect cells to improve the levels of GlcNAc and galactose (Gal) appearing on insect cell glycoproteins. Recently, the combined expression of *N*-acetylglucosaminyltransferase-II (GlcNAcT-II) and GalT in a *Trichoplusia ni* (TN-5B1-4) insect cell line resulted in glycoproteins containing more than 50% structures that were fully galactosylated on both branches (*11*). This cell line generated only paucimannosidic structures terminating in Man with a few structures containing GlcNAc on one of two Man branches in the absence of these heterologous transferases (*4*). Sialyltransferases have also been expressed in combination with galactosyltransferases in both mammalian and insect cells to improve sialylation of the proteins (*12*, *13*). However, the expression of these transferases will be most effective if there is an adequate supply of the substrates, the activated sugar nucleotides, which are utilized in the galactosylation and sialylation reactions.

The primary donor substrate for sialylation is CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac), and the metabolic pathway used by mammalian cells for generating CMP-Neu5Ac and its dedicated metabolic precursor, Neu5Ac, is shown in Figure 1A. UDP-*N*-acetylglucosamine (UDP-GlcNAc) generated from basic metabolites is first converted to *N*-acetylmannosamine (ManNAc) by the 2-epimerase activity of the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (EpimKin). ManNAc is then phosphorylated to ManNAc 6-phosphate (ManNAc-6-P) by the kinase activity present on the same enzyme (*14*). ManNAc-6-P is the substrate for sialic acid 9-phosphate synthase (SAS) to synthesize Neu5Ac 9-phosphate (*15*). The same enzymes can also yield an alternate sialic acid, KDN 9-phosphate (KDN = 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid), using Man 6-phosphate (Man-6-P) as substrate (Figure 1B). Unknown specific or nonspecific dephosphorylases act on these intermediates to yield Neu5Ac and KDN, which are in turn converted to CMP-Neu5Ac and CMP-KDN by CMP-sialic acid synthase (CMP-SAS) (*16*). These CMP-sialic acids are the activated molecules that transfer the sialic acid to the glycan on the proteins. As an alternative, ManNAc, the first dedicated precursor of Neu5Ac, has been added to CHO cell culture media to enhance sialylation of interferon-γ (*17*). This same pathway for sialic acid synthesis has been shown to utilize other unnatural ManNAc derivatives and synthesize unnatural analogues of sialic acid (*18*). The promiscuous nature of the pathway has been exploited to introduce reactive functional groups such as ketones (*19*) and azides (*20*) on the modified sialic acids at the cell surface. In addition, sialic acid when supplemented to certain eukaryotic cell line media has been shown to be incorporated into glycoproteins (*21*).

Sialylated structures have been detected in insect cells expressing recombinant galactosyltransferases and sialyltransferases (*22*, *23*). However, supplementation of the culture medium with serum, which is rich in sialic acids (*24*), was necessary to obtain these sialylated forms.

Applying metabolic engineering strategies to include the enzymes involved in sialic acid metabolism and transport represents another approach for altering the levels of necessary substrates for sialylation. Previous studies in insect cells have indicated negligible intracellular levels of endogenous sialic acids including Neu5Ac (*15*) and CMP-sialic acids, the active substrate for sialylation (*25*, *26*). The overexpression of the human SAS in combination with supplementation of ManNAc has been shown to overcome the absence of detectable Neu5Ac production in these insect cells (*15*). Interestingly, the insect cells apparently included endogenous specific and nonspecific kinase and phosphatase activities to phosphorylate and dephosphorylate ManNAc and Neu5Ac 9-phosphate, respectively (Figure 1A). Similarly, the expression of both human SAS and CMP-SAS in combination with ManNAc feeding is found to be sufficient to generate intracellular CMP-Neu5Ac in insect cells (*16*). In addition to Neu5Ac, the insect cells can generate the alternative sialic acid KDN (*15*). Following expression of SAS, KDN 9-phosphate is synthesized by conversion of endogenous Man-6-P. This KDN 9-phosphate is then converted to KDN by endogenous phosphatases present in insect cells. In cells infected with CMP-SAS, this KDN is converted to CMP-KDN (Figure 1B).

In the present study we further examine the metabolic pathways for sialic acid production in insect cells. Using the mammalian sialic acid synthesis pathway as a framework, we have identified the bottlenecks that exist in the sialic acid synthesis in insect cells. To elucidate bottlenecks, we incorporated the genes for selective enzymes of the pathway in combination with appropriate substrates. *Spodoptera frugiperda* (Sf9) cells were infected with baculoviruses containing the sialic acid 9-phosphate synthase gene (AcSAS) in combination with the AcEpim (baculovirus containing the functional UDP-GlcNAc 2-epimerase gene without ManNAc kinase activity), AcKin (baculovirus containing the functional ManNAc kinase gene without UDP-GlcNAc 2-epimerase activity), and AcEpimKin (baculovirus containing the UDP-GlcNAc 2-epimerase/ManNAc kinase bifunctional gene) in the presence or absence of ManNAc and GlcNAc in the media. By suitable infections and substrate feeding, we were able to overcome specific pathway limitations and enhance sialic acid production as well as control the ratio of the two different sialic acids (Neu5Ac and KDN) synthesized by the cells.

MATERIALS AND METHODS

Preparation of Sugar Solutions. Sugar solutions of ManNAc and GlcNAc (0.1 and 1 M each) (Sigma Chemicals, St. Louis, MO) were prepared by dissolving them in water followed by filter sterilization. Tetra-*O*-acetylated ManNAc (Ac_4ManNAc) was prepared by following a protocol adapted from a previously published procedure (*27*). Briefly, 7.0 mmol of ManNAc (Pfanstiel, Waukegan, IL) was dissolved in 30 mL of a 2:1 solution of pyridine and acetic anhydride. The mixture was stirred at room temperature for 12 h. Subsequently, the solvent was removed in vacuo, and the residue was dissolved in CH_2Cl_2 and washed successively with 30 mL of concentrated HCl (twice) and then 30 mL of saturated Na_2SO_4. Finally, the mixture was dried over Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography, eluting with a gradient of 2:1 to 1:2 hexanes/EtOAc. The resulting white amorphous foam was characterized by NMR as a mixture of anomers. The product was stored at −20 °C; periodically 100 mM stock solutions of tetra-*O*-acetylated ManNAc were prepared in ethanol and stored at 4 °C until use.

Cell Culture. Sf9 (ATCC, Manassas, VA) cells were grown in serum-free HyClone SFX media in shaker flasks. Approximately 0.8×10^6 Sf9 cells taken from cell cultures at densities between 1.5 and 2.5×10^6 cells/mL were plated on each well of a six-well plate. After attachment, the medium was removed and 2 mL of fresh medium was supplied to each well. The cells were infected with 20 μL of each virus or left uninfected. Baculovirus infections were performed as described by O'Reilly et al. (*1*).

The medium was supplemented by the appropriate sugar solution at the time of infection. For ManNAc supplementation, Sf9 cells were grown in media supplemented with 0.1, 2, and 5 mM ManNAc (from the 0.1 M ManNAc stock) and 10, 20, and 50 mM ManNAc (from the 1 M ManNAc stock). For GlcNAc supplementation, Sf9 cells were grown in media supplemented with 0.1, 2, and 5 mM GlcNAc (from the 0.1 M GlcNAc stock) and 10 and 20 mM GlcNAc (from the 1 M GlcNAc stock). For Ac_4ManNAc supplementation, the cells were grown in medium supplemented with 0.1, 0.2, 0.5, and 1 mM Ac_4ManNAc (from 100 mM Ac_4ManNAc).

Cells were harvested 72 h postinfection by removing the cell culture medium and washing the cells twice with phosphate-buffered saline (Life Technologies, Bethesda, MD). The cells were vortexed and then sonicated with a Tekmar sonic disruptor (Cincinnati, OH) for 30 s at 50% cycle at a power setting of 2.5. The samples were analyzed for total protein content with a Pierce BCA assay kit (Rockford, IL) and a 96-well plate reader (Molecular Devices, Sunnyvale, CA), and analyzed for sialic acids as described below.

Neu5Ac/KDN Detection. Previous studies have indicated the absence of sialic acid on cellular proteins for Sf9 cells lacking the expression of both heterologous galactosyltransferase and sialyltransferase genes (*13*, *23*). Consequently, any incorporation of Neu5Ac or KDN into cell proteins was ignored. The free sialic acid content was measured by the procedure as described by Hara et al. (*24*). A 95 μL sample of 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB; Sigma) reducing solution (7.0 mM DMB in 1.4 M acetic acid, 0.75 M β-mercaptoethanol, and 18 mM sodium hydrosulfite) was added to 5 μL of sample and incubated at 50 °C for 2.5 h, 3−10 μL of which was used for HPLC analysis on a Shimadzu (Columbia, MD) VP series HPLC instrument using a Waters (Milford, MA) Spherisorb 5-μm ODS2 column. A Shimadzu RF-10AXL fluorescence detector with 448-nm emission and 373-nm excitation wavelengths was used for detecting the peaks. An acetonitrile, methanol, and water mixture (9:7:84, v/v/v) with a flow rate of 0.7 mL/min was the mobile phase. Response factors of Neu5Ac and KDN were established with authentic standards on the basis of peak areas for quantifying sample sialic acid levels.

The sialic acid content was normalized on the basis of the total protein content measured with the Pierce BCA assay kit and a Molecular Devices (Sunnyvale, CA) microplate reader.

GlcNAc Kinase/ManNAc Kinase Activity Measurement. Enzyme activities were determined as described earlier (*28*). Briefly, assays contain 60 mM Tris/HCl, pH 7.5, 20 mM $MgCl_2$, 5 mM GlcNAc/ManNAc, 50 nCi [^{14}C]GlcNAc/[^{14}C]-ManNAc, and 20 mM ATP in a final volume of 200 μL. Assays were run for 30 min at 37 °C and stopped by addition of 300 μL of ethanol. Radiolabeled substrates were separated by descendent paper chromatography, and radioactivity was determined in a Tri-Carb 1900A liquid scintillation counter (Packard).

RESULTS

Effect of ManNAc Media Supplementation on the Sialic Acid Levels in AcSAS-Infected Cells. ManNAc is a metabolic precursor in the biosynthesis of Neu5Ac as shown in Figure 1A. This conversion involves the phosphorylation of ManNAc to ManNAc-6-P, which then fuses with phosphoenol pyruvate (PEP) to generate Neu5Ac-9-P. This intermediate is subsequently dephosphorylated to give Neu5Ac. In previous studies, Sf9 cells grown in serum-free media were observed to include negligible levels of Neu5Ac (*15*). To determine if insect cells could be engineered to produce Neu5Ac using ManNAc substrates, Sf9 cells were infected with the AcSAS baculovirus containing the sialic acid 9-phosphate synthase (sas) gene and supplemented with ManNAc at the time of infection. As shown in Figure 2A, these AcSAS-infected cells generated significant levels of Neu5Ac that varied with the amount of fed ManNAc. In the absence of ManNAc supplementation, however, the levels of Neu5Ac were very low (approximately 100 fmol/μg of total protein), suggesting that Sf9 cells have a limitation in the amount of ManNAc available for Neu5Ac synthesis. As ManNAc supplementation levels were increased, the levels of Neu5Ac synthesized increased over the entire range up to 27000 fmol/μg of total protein with 50 mM ManNAc supplementation as shown in Figure 2A. However, the increase in Neu5Ac obtained began to slow around 20 mM fed ManNAc, and there was only a relatively small increase in the Neu5Ac levels in changing from 20 to 50 mM ManNAc supplementation to the media.

A similar experiment was done with uninfected Sf9 cells and Sf9 cells infected with a control virus without any foreign gene (A35). The Neu5Ac levels in the uninfected cells as well as the cells infected with the A35 (negative control) virus also increased with ManNAc feeding (Figure 2B), with the same dependency on sugar concentration. However, these quantities were more than 2 orders of magnitude below those seen with the AcSAS infection. This difference suggests a native, albeit low, sialic acid 9-phosphate synthase activity in Sf9 cells. The lowest levels of Neu5Ac were observed following infection of the A35 virus and may reflect the negative effects of viral infection on host cell functions.

In addition to Neu5Ac, the levels of the alternate sialic acid KDN were also measured for these ManNAc feeding experiments. The sialic acid KDN is synthesized by SAS with Man-6-P and PEP as substrates. In A35-infected and uninfected (data not shown) cells, KDN levels were not detected. In Sf9 cells infected with AcSAS, the KDN levels were detectable at levels between 170 and 260 fmol/μg of total protein (Figure 2C). The levels of intracellular KDN decreased slightly as ManNAc was fed but still remained above 150 fmol/μg of protein. When the production of Neu5Ac relative to KDN is calculated, the Neu5Ac to KDN ratio rises rapidly with the initial addition of ManNAc due in large part to the rapid increase in Neu5Ac production accompanying ManNAc feeding (Figure 2C).

Effect of Coinfecting AcEpimKin and AcSAS. An alternative to ManNAc supplementation is to engineer the cells with the capacity to generate ManNAc, since Sf9 cells have negligible endogenous capacity to synthesize ManNAc (*15*). In mammalian cells ManNAc is synthesized in vivo by the epimerization of UDP-GlcNAc using UDP-GlcNAc 2-epimerase. The ManNAc is then converted to ManNAc-6-P by a kinase encoded on the same bifunctional complex (Figure 1A). The bifunctional rat enzyme (Figure 3) that generates ManNAc 6-phosphate from UDP-GlcNAc has been cloned into baculovirus (AcEpimKin), and infection of Sf9 cells with AcEpimKin results in the production of the active enzyme (*29*). To determine if Sf9 cells could be engineered to produce Neu5Ac without substrate supplementation, Sf9 cells were coinfected with the AcEpimKin and AcSAS baculoviruses simultaneously and then analyzed for intracellular sialic acid. Cells infected with AcEpimKin alone had negligible levels of Neu5Ac (Figure 4) as did cells infected with AcSAS alone (Figure 2A). However, coinfection with AcSAS and AcEpimKin enabled the cells to generate significant Neu5Ac (16 900 fmol/μg) intracellularly. This coinfection indicates that Sf9 cells generate the essential UDP-GlcNAc precursor for the synthesis of ManNAc and Neu5Ac endogenously. Thus, cells infected with AcEpimKin and AcSAS can synthesize Neu5Ac in the absence of any additional media supplementation. In fact, the Neu5Ac levels observed in the coinfected cells were comparable to the levels observed in the AcSAS-infected cells supplemented with 10 mM ManNAc (Figure 4).

Effects of GlcNAc Feeding on Neu5Ac Production. GlcNAc feeding represents another possible alternative for increasing the synthesis of sialic acid in insect cells. The substrate for AcEpimKin is UDP-GlcNAc, and a 10-fold increase in the UDP-GlcNAc levels was found with 10 mM GlcNAc supplementation to the media (data not shown). To see the effect of GlcNAc feeding on sialic acid production, cells were coinfected with AcEpimKin and AcSAS in the presence of different concentrations of GlcNAc (Figure 5). The amount of Neu5Ac generated increased with higher concentrations of GlcNAc, reaching a level of 115000 fmol/μg of protein at 10 mM fed GlcNAc. This Neu5Ac level was more than 6 times higher than the amount of Neu5Ac generated when the cells were infected with AcSAS and fed 10 mM ManNAc.

Effects of Infection with AcKin, a Virus with Only ManNAc Kinase Activity. Insect cells are capable of phosphorylating ManNAc to give ManNAc-6-P as evidenced by the generation of Neu5Ac in cells infected with AcSAS and supplemented with ManNAc. The SAS enzyme acts almost exclusively on ManNAc-6-P substrates (*15*). Previous studies showed that mammalian cells lacking the specific ManNAc kinase still display ManNAc kinase activity, which is derived from a secondary activity of GlcNAc kinase (*30*). To

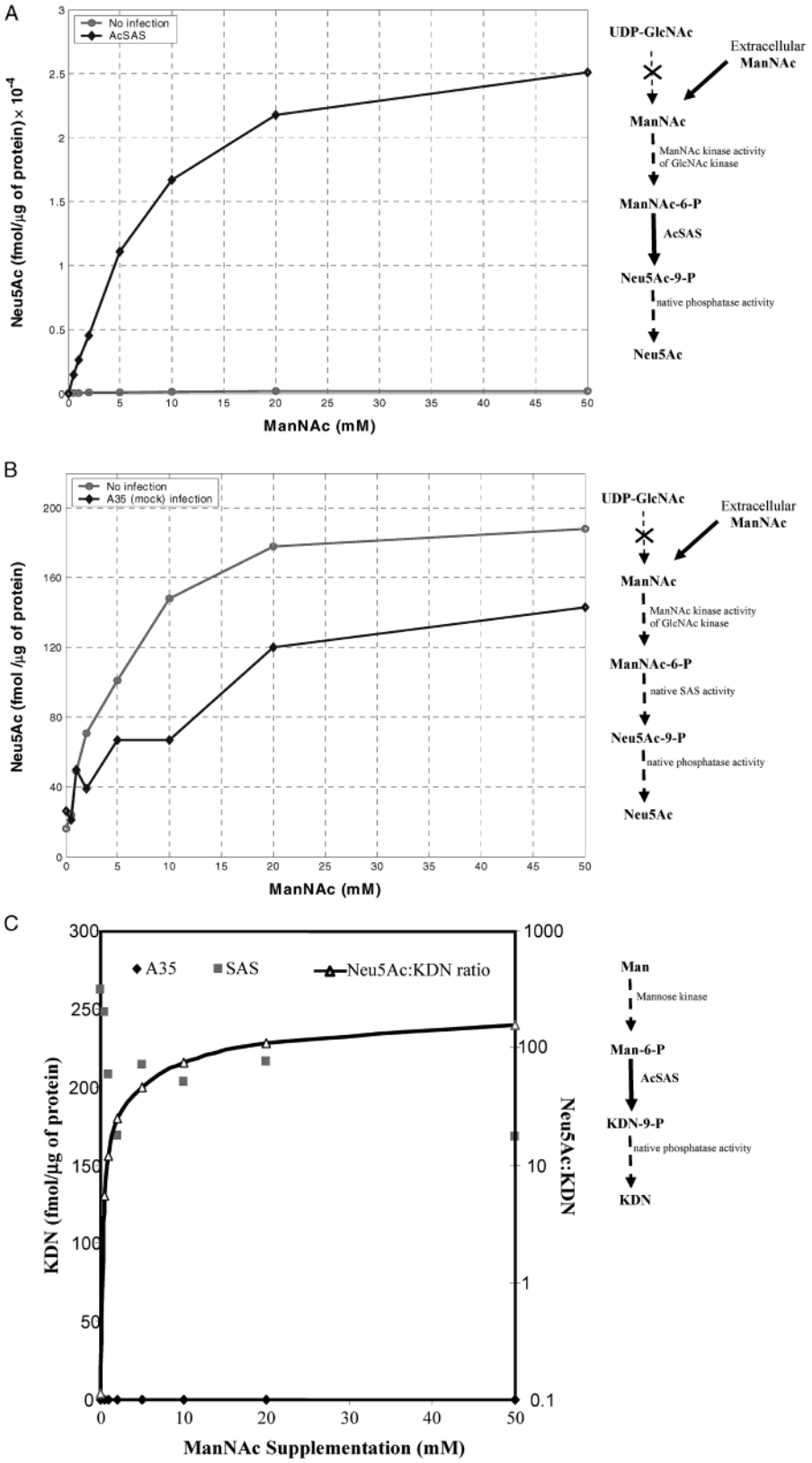

FIGURE 2: Effect of different ManNAc concentrations on Neu5Ac and KDN levels in Sf9 insect cells. (A, top) Intracellular Neu5Ac levels for cells infected with AcSAS virus or left uninfected in the presence of varying quantities of ManNAc. The cells were harvested 3 days postinfection, and Neu5Ac levels were measured by DMB labeling. The right panel includes the Neu5Ac synthesis pathway. The bold arrows represent recombinant activity, and the dashed arrows represent native activity. An "×" over the arrow represents a step in the pathway for which the enzyme activity is either negligible or not present at all. (B, middle) Intracellular Neu5Ac levels for cells infected with A35 (negative control virus) or left uninfected in media supplemented with varying quantities of ManNAc. The cells were harvested at 3 days postinfection, and the intracellular Neu5Ac levels were measured as before. (C, bottom) Absolute and relative KDN production levels for Sf9 cells infected with A35 (negative control virus) or AcSAS in medium supplemented with different ManNAc quantities. The left axis and closed symbols refer to the intracellular KDN content measured by DMB labeling. The right axis and open symbols refer to the ratio of Neu5Ac level to KDN level. All experiments were performed in duplicate with similar trends.

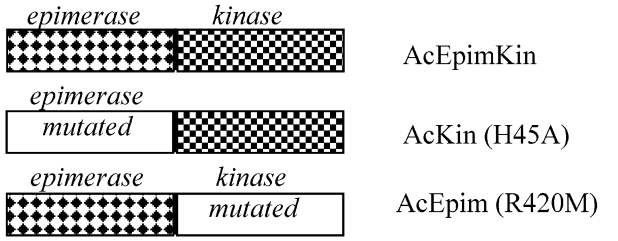

FIGURE 3: Schematic of the UDP-GlcNAc 2-epimerase/ManNAc kinase bifunctional gene along with mutants. The region of epimerase activity is located at the amino terminus of the protein, while the kinase active site is localized to the carboxy terminus of the protein. The AcEpimKin virus includes domains for fully functional epimerase and kinase activities. The AcKin virus contains an active kinase domain with a mutation in the epimerase domain (H45A) to eliminate epimerase activity. The AcEpim virus includes a functional epimerase gene with a mutation in the kinase domain (R420M).

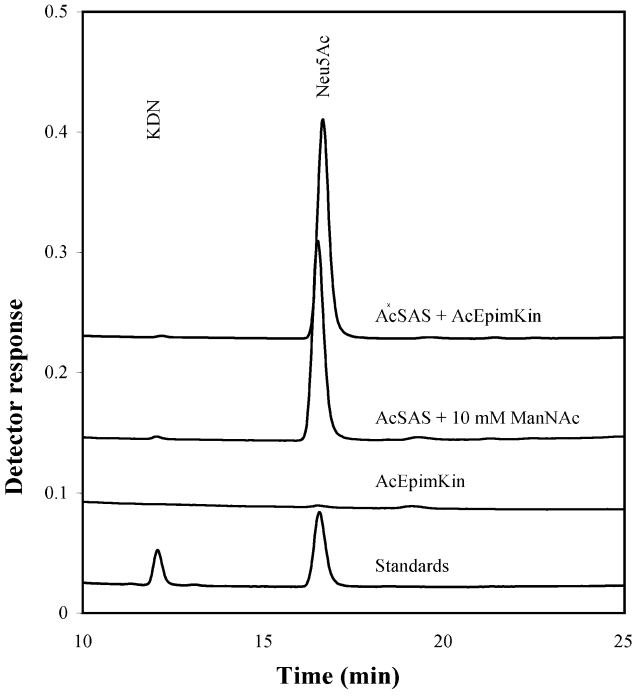

FIGURE 4: Sialic acid production as measured by an HPLC detector for Sf9 cells infected with AcSAS and AcEpimKin compared to production following infection with AcSAS alone in media supplemented with 10 mM ManNAc. Also included as a negative control is the activity following infection with AcEpimKin alone. Neu5Ac and KDN levels were measured following DMB derivatization using a fluorescence detector with a reversed-phase HPLC setup. Detector responses on the chromatograms have been normalized using the total protein concentration in lysates for each sample.

investigate if such an activity exists in insect cells as well, the ManNAc kinase and GlcNAc kinase activities in Sf9 cells were determined. The GlcNAc kinase activity was found to be 4.6 mU/mg and the ManNAc kinase activity 2.9 mU/mg. In the presence of equimolar ratios of GlcNAc and ManNAc, the ManNAc kinase activity dropped to less than 10% of its value measured in the absence of GlcNAc. This behavior is consistent with the activity observed for a mammalian GlcNAc kinase (*30*) and confirms that the ManNAc kinase activity in Sf9 cells is likely to be derived from the endogenous insect GlcNAc kinase.

The high levels of Neu5Ac generated when ManNAc 6-phosphate is synthesized intracellularly using UDP-GlcNAc 2-epimerase/ManNAc kinase suggests a possible limitation in the production of ManNAc-6-P from the ManNAc added to the cell culture media. Even when ManNAc is fed up to 50 mM, the level of Neu5Ac saturates at 25000 fmol/μg of protein as compared to 115000 fmol/μg found for coinfection with AcSAS and AcEpimKin in the presence of 10 mM GlcNAc feeding. The limitation could occur in the intracellular enzymatic synthesis of ManNAc 6-phosphate from ManNAc, in the uptake of ManNAc by the cells, or at both steps. To examine for a limitation in the ManNAc kinase activity which is responsible for the synthesis of ManNAc-6-P from ManNAc, the cells were infected with an AcKin virus along with AcSAS in the presence of variable amounts of fed ManNAc. AcKin is a baculovirus that expresses a UDP-GlcNAc-2-epimerase/ManNAc kinase gene that contains unchanged ManNAc kinase activity but lacks any 2-epimerase activity due to the H45A point mutation in the epimerase domain of the bifunctional enzyme (Figure 3) (*29*). The Neu5Ac production levels for coinfections with AcSAS and AcKin were then compared with those for cells infected with AcSAS alone in the absence of any recombinant ManNAc kinase activity (Figure 6). At levels of ManNAc supplementation of 10 mM and below, the Neu5Ac yields were comparable in the presence or absence of the additional AcKin infection, suggesting that the ManNAc kinase activity of Sf9 GlcNAc kinase is sufficient for ManNAc phosphorylation. However, at levels of ManNAc of 20 mM and above, the Neu5Ac levels were significantly higher in the AcKin coinfected cells, reaching a level of Neu5Ac that was more than 2.5-fold higher at 50 mM ManNAc. This increase in Neu5Ac production in the presence of recombinant ManNAc kinase suggests a limitation in the endogenous ManNAc kinase activity of Sf9 cells at higher ManNAc feeding levels. However, even though the Neu5Ac levels increased with the AcKin infection and ManNAc supplementation, the total Neu5Ac yields were not as high as those obtained with AcEpimKin and AcSAS coinfection in the presence of fed GlcNAc, indicating other possible limitations in the conversion of fed ManNAc to Neu5Ac.

Effect of Feeding Ac_4ManNAc on Neu5Ac Production. To examine for a possible transport limitation in the uptake of ManNAc by insect cells, the ManNAc was tetra-*O*-acetylated prior to being provided as a substrate to the medium. *O*-Acetylation of ManNAc is known to facilitate its uptake in mammalian cells (*31*). *O*-Acetylation involves the chemical modifications of the hydroxyl groups to acetyl groups as shown in Figure 7A. The chemical modifications cause the ManNAc to be more lipophilic, which in turn allows the resulting Ac_4ManNAc to diffuse more easily through the cell membrane. Once inside the cell, the Ac_4ManNAc is deacetylated to free ManNAc by nonspecific esterases in the cytosol (*31*).

To investigate for a possible limitation in ManNAc transport, Sf9 cells cultured in serum-free media were supplemented with 0.2 mM Ac_4ManNAc and then coinfected with AcSAS and AcKin. The Neu5Ac level obtained using Ac_4ManNAc was comparable to the amount from cells coinfected with AcEpimKin and AcSAS in the presence of

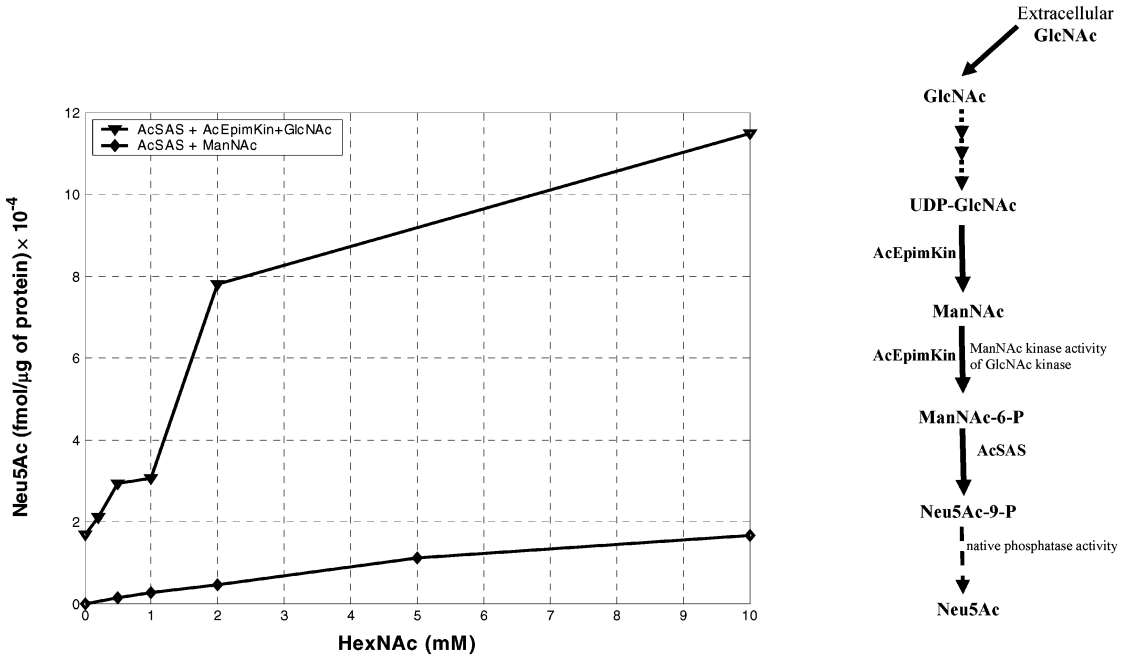

FIGURE 5: Effect of various HexNAc (GlcNAc or ManNAc) supplementation levels on Neu5Ac levels in Sf9 cells infected with AcSAS. Cells coinfected with AcEpimKin + AcSAS were supplemented with GlcNAc, while cells infected with AcSAS were supplemented with ManNAc. All experiments were performed in duplicate.

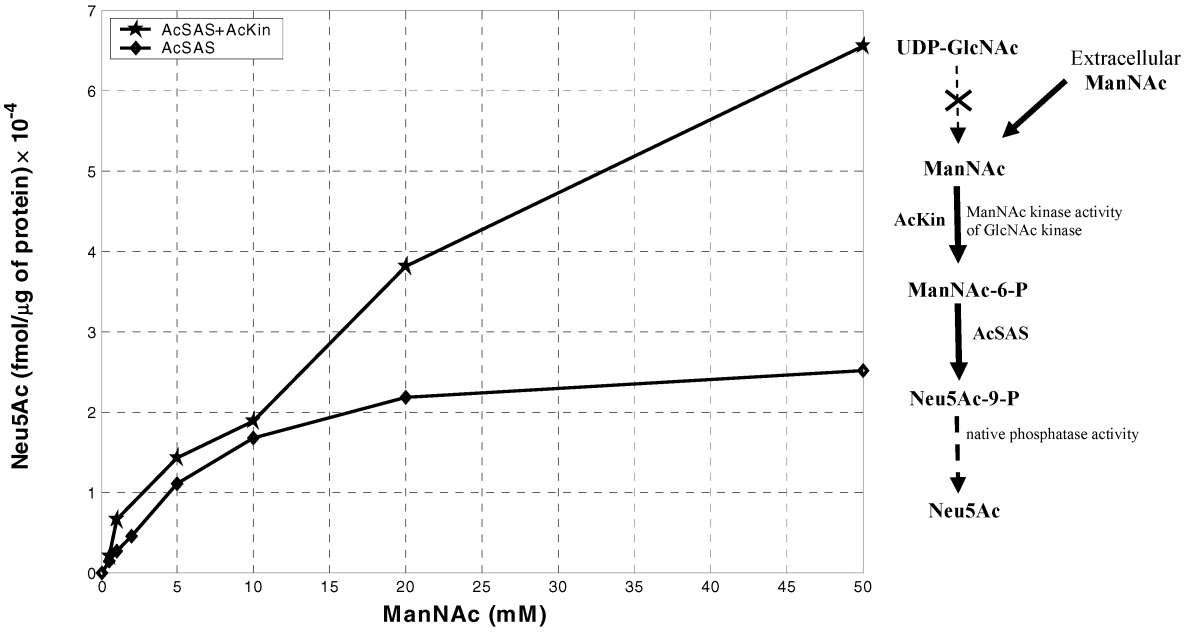

FIGURE 6: A comparison of Neu5Ac levels for Sf9 cells infected with AcSAS in the presence and absence of ManNAc supplementation. Experiments were performed in duplicate with similar trends.

an equivalent level (0.2 mM) of GlcNAc. Furthermore, this value was higher than the amount of Neu5Ac obtained with the same viruses (AcSAS and AcKin) supplemented with 10 mM ManNAc (Figure 7B). The production of slightly higher levels of Neu5Ac using much lower levels of fed Ac_4-ManNAc compared with free ManNAc suggests a limitation does exist in the ManNAc transport into insect cells, and this transport limitation inhibits the cells' ability to generate high levels of intracellular Neu5Ac. Unfortunately, Ac_4-ManNAc is also lethal to insect cells at elevated levels. For example, 1 mM Ac_4ManNAc supplementation resulted in the death of the majority of the cells after 2−3 days. Consequently, addition of Ac_4ManNAc was limited to experiments in which the Ac_4ManNAc concentration was held at or below 0.2 mM.

KDN Production in Insect Cells. Neu5Ac is the predominant form of sialic acid generated when the AcSAS infection is carried out in the presence of ManNAc or following coinfection with AcEpimKin. The alternate sialic acid KDN is also synthesized, albeit at much lower levels, by this same SAS enzyme using Man-6-P as the substrate (*15*) (Figure 1B). Man-6-P is generated by phosphorylation of Man in cells. To determine if the levels of KDN could be increased by substrate addition, additional Man was added to the medium. However, feeding Man did not alter the KDN levels significantly (data not shown). This absence of any effect

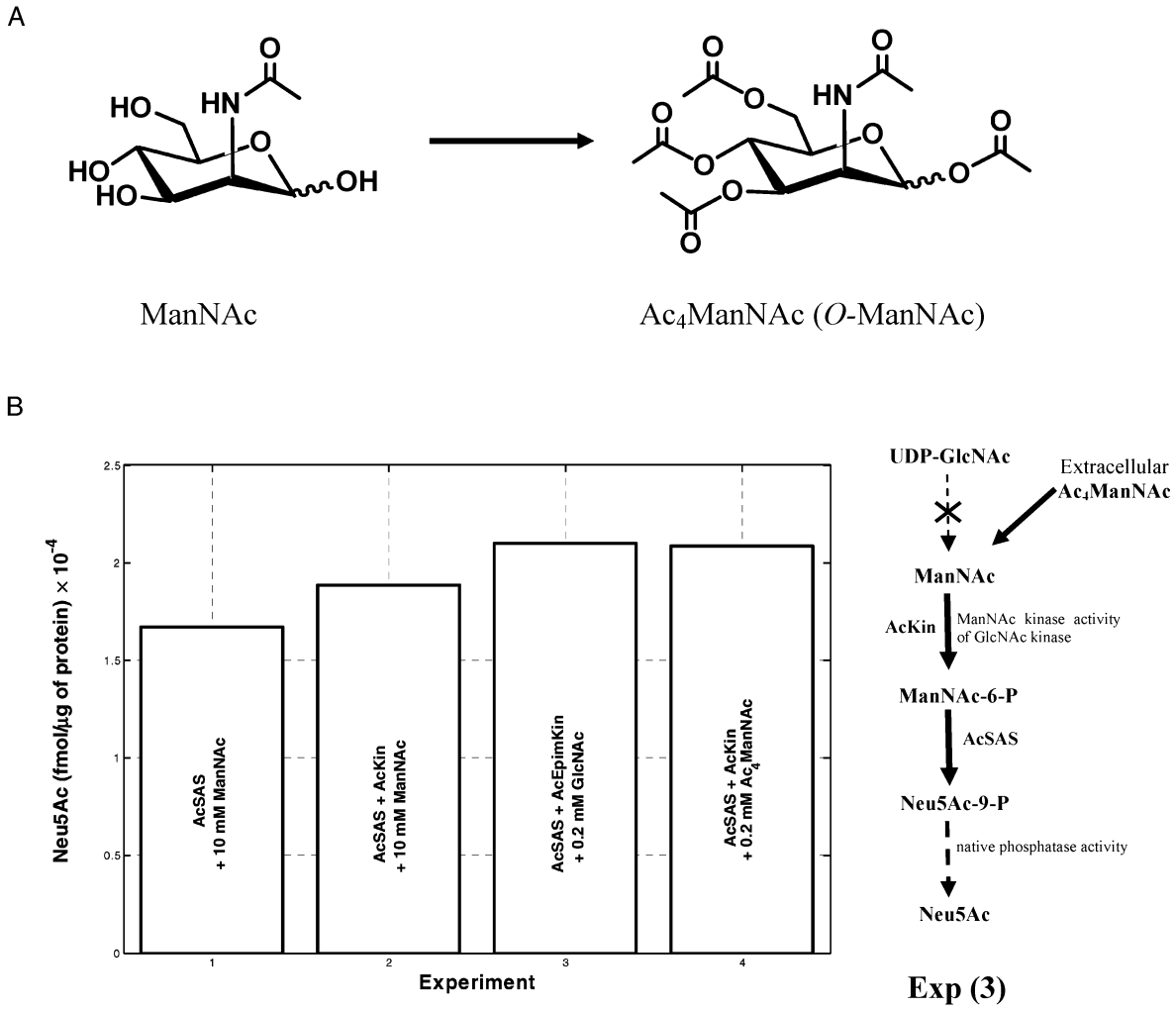

FIGURE 7: **(**A, top**)** The modification of the hydroxyl groups of ManNAc by *O*-acetyl groups leads to the generation of Ac_4ManNAc. (B, bottom) Levels of Neu5Ac in Sf9 cells coinfected with AcSAS and AcKin in the presence of Ac_4ManNAc compared to levels with GlcNAc feeding following AcEpimKin + AcSAS coinfection, and AcKin + AcSAS coinfection with ManNAc feeding. Infection with AcSAS supplemented with 10 mM ManNAc was also included as a comparison. Experiments in (B, bottom) were performed multiple times with similar results.

on KDN production with Man feeding could be due to high intracellular Man-6-P levels naturally or because high levels of Man are present in the serum-free media used. As an alternative, methods to increase KDN production relative to that of Neu5Ac were evaluated through the inhibition of the synthesis of ManNAc-6-P. As shown in Figure 1A GlcNAc is incorporated into the metabolic pathway as GlcNAc 6-phosphate. The phosphorylation of GlcNAc was carried out in Sf9 by GlcNAc kinase, which also includes a secondary ManNAc kinase activity. Previously, we observed that feeding an excess of GlcNAc could restrict the ManNAc kinase activity of GlcNAc kinase and limit the levels of ManNAc-6-P available to the cells. Therefore, GlcNAc feeding was considered as a possible method to limit Neu5Ac production in insect cells. Sf9 cells were coinfected with AcSAS and AcEpim (with and without AcKin) in the presence of fed GlcNAc, and the production of Neu5Ac and KDN was measured. AcEpim is a baculovirus that expresses a UDP-GlcNAc 2-epimerase/ManNAc kinase that contains unchanged epimerase activity but lacks any ManNAc kinase activity due to the R420M point mutation in the kinase domain of the gene for this bifunctional enzyme (*29*). Also this mutant enzyme is feedback inhibited completely by 0.1 M CMP-Neu5Ac (*29*). Cells infected with AcKin along with AcEpim and AcSAS synthesized high levels of Neu5Ac with GlcNAc feeding as expected (Figure 8). In contrast, in GlcNAc-fed cells infected with AcEpim and AcSAS in the absence of AcKin infection, the synthesis of Neu5Ac was limited to negligible levels and KDN became the predominant sialic acid synthesized (Figure 8).

DISCUSSION

Previous research in our laboratories has demonstrated that insect cells can be engineered to produce sialylation substrates (*15*, *16*). The present work focused on identifying particular bottlenecks that may exist in the sialic acid synthesis pathways of insect cells. In particular, we focused on the production of the sialic acids Neu5Ac and KDN in Sf9 cells. By varying the specific pathway genes as well as the substrates involved, we determined that particular processing steps can indeed limit the production of sialic acid. Furthermore, a suitable combination of substrate feeding alternatives and expression of various genes can be used to

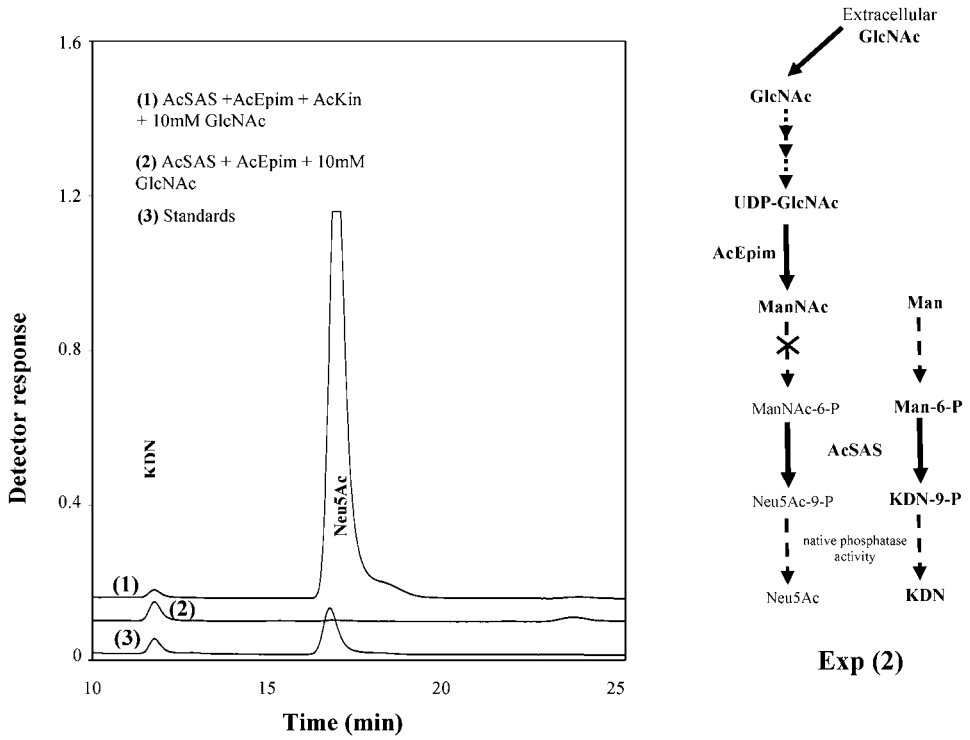

FIGURE 8: Inhibition of Neu5Ac synthesis in Sf9 cells caused by feeding GlcNAc. Neu5Ac and KDN levels were measured in Sf9 cells supplemented with 10 mM GlcNAc following AcSAS + AcEpim coinfection in the presence or absence of AcKin.

control the levels of sialic acid as well as the type of sialic acid formed.

As previously reported by Lawrence et al. (*15*), Sf9 cells synthesize Neu5Ac and KDN when infected with a baculovirus carrying the gene for sialic acid 9-phosphate synthase in the presence of exogenously fed ManNAc. In this study, the levels of Neu5Ac were observed to increase with ManNAc supplementation up to 20 mM fed ManNAc. This increase in Neu5Ac production clearly indicates a limitation in the available ManNAc for Neu5Ac synthesis in Sf9 cells. However, the addition of 50 mM ManNAc gave only a 12% increase in the synthesis of Neu5Ac over the level obtained with 20 mM ManNAc. Thus, a bottleneck in the sialic acid pathway is present in insect cells such that increasing the level of ManNAc present in the medium above 20 mM does not cause a significant enhancement in the amount of Neu5Ac generated. This bottleneck could exist either at the step involving ManNAc transport into the cells or in the metabolic conversion of ManNAc to substrates which can be utilized by the sialic acid synthesis enzyme. Nonetheless, the intracellular Neu5Ac content was still over 100 times higher in the AcSAS-infected lysates as compared to control culture lysates. However, the presence of detectable Neu5Ac in control cultures suggests that insect cells may contain very low endogenous levels of the enzymes for sialic acid synthesis. The gene for sialic acid synthesis indeed has been detected in *Drosophila melanogaster* (*32*) although the endogenous enzymatic activity was undetectable in Schneider S2 cell lines. KDN, an alternate sialic acid, was also generated in the current studies following AcSAS infection. The ratio of KDN to Neu5Ac decreased drastically following ManNAc feeding due to a rapid increase in the synthesis of Neu5Ac, indicating that ManNAc-6-P is the preferred substrate of SAS.

To determine if ManNAc feeding could be avoided and if insect cells could be engineered to generate Neu5Ac completely from intracellular metabolites, insect cells were coinfected with the AcEpimKin and AcSAS virus without any media supplementation. The coinfections of AcEpimKin and AcSAS were performed with the intent of avoiding potentially costly ManNAc feeding by introducing the enzymatic machinery for ManNAc production into the cells. The bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (*29*) present on AcEpimKin produces ManNAc and then ManNAc-6-P, the precursor for the SAS enzyme, from UDP-GlcNAc. Previous studies have indicated that Sf9 cells have significant levels of the UDP-GlcNAc present intracellularly (*26*). Indeed, the generation of Neu5Ac in the presence of AcSAS and AcEpimKin indicates that metabolic engineering can be used to complete the sialic acid synthesis pathway and that sialic acid can be synthesized in cells without any additional substrates. In fact, coinfection of AcSAS and AcEpimKin resulted in the production of Neu5Ac levels comparable to those seen with AcSAS infection and 10 mM ManNAc feeding. Furthermore, the coinfection of AcSAS and AcEpimKin in cells supplemented with 10 mM GlcNAc (a more cost-effective reagent than ManNAc) generated levels of Neu5Ac that were around 6 times higher than those from the coinfection of AcSAS and AcEpimKin without additional substrates and in fact saturated the experimental Neu5Ac detection system. These high levels of Neu5Ac observed with GlcNAc feeding also indicate that GlcNAc feeding is much more efficient for Neu5Ac generation as compared to ManNAc feeding.

The AcEpimKin and AcSAS coinfection results present further evidence that a bottleneck to Neu5Ac synthesis exists following ManNAc feeding. The presence of efficient synthesis of high levels of Neu5Ac from intracellularly

generated ManNAc-6-P in these studies suggests that the bottleneck to Neu5Ac production for exogenously supplied ManNAc occurs in the ability of insect cells to either uptake or phosphorylate ManNAc. Although GlcNAc feeding led to higher levels of Neu5Ac than ManNAc feeding, understanding the bottleneck in the Neu5Ac synthesis from fed ManNAc is useful due to the feedback inhibition of the epimerization of UDP-GlcNAc by CMP-Neu5Ac (*33*). As a result, generation of high amounts of CMP-Neu5Ac may require the epimerization step to be bypassed and thus may ultimately depend on ManNAc feeding. To identify the bottleneck in the synthesis of Neu5Ac with fed ManNAc, both ManNAc transport from the media to the cells and the phosphorylation of ManNAc by ManNAc kinase were examined.

In the absence of any recombinant ManNAc kinase activity, Neu5Ac was still synthesized in cells infected with AcSAS and supplemented with ManNAc to indicate that the ManNAc was converted to ManNAc-6-P. As suggested previously in mammalian cells, the ManNAc kinase activity in insect cells was found to be derived from GlcNAc kinase (*30*). Indeed, when the ManNAc kinase activity was measured in the presence of equimolar ratios of GlcNAc and ManNAc, the ManNAc kinase activity fell to 10% of the activity found in the absence of GlcNAc. However, even though GlcNAc kinase can perform this enzymatic step, GlcNAc kinase is likely to be much less efficient than the activity obtained using a specific ManNAc kinase. We found that infection of the cells with AcKin (baculovirus containing the ManNAc kinase gene) in combination with AcSAS resulted in higher yields of Neu5Ac, especially at ManNAc levels greater than 20 mM. With 50 mM ManNAc feeding the coinfection of AcKin and AcSAS resulted in Neu5Ac levels more than 2.5 times higher than those found with AcSAS infection alone. This increase in Neu5Ac indicated that, in the absence of AcKin, a bottleneck exists in the phosphorylation of exogenously supplied ManNAc especially at higher concentrations of fed ManNAc.

Despite overcoming the limitation in ManNAc kinase activity using AcKin and AcSAS, the levels of Neu5Ac in insect cells were still found to be lower than those with infection with the AcEpimKin and AcSAS viruses complemented with GlcNAc feeding. To determine if this limitation was due to poor uptake of ManNAc by the cells, the supplemented ManNAc was modified by *O*-acetylation in an effort to enhance the efficiency of transport. In Jurkat cells *O*-acetylated compounds have been shown to be utilized as efficiently as the free sugars at concentrations 200-fold lower (*34*). The reason for this increase in efficiency is believed to be linked to the more efficient transport of highly lipophilic *O*-acetylated ManNAc through the cell membranes as compared to unmodified ManNAc. In our studies, Sf9 cells coinfected with AcKin and AcSAS with 0.2 mM Ac_4ManNAc were able to synthesize Neu5Ac at levels comparable to those of cells coinfected with AcEpimKin and AcSAS and supplemented with 0.2 mM GlcNAc or cells coinfected with AcKin and AcSAS with ManNAc feeding levels 50 times higher at 10 mM. Thus, Neu5Ac synthesis for AcSAS-infected Sf9 cells is limited at the point of ManNAc transport for almost all concentrations of fed ManNAc since the Neu5Ac levels obtained when using 0.2 mM Ac_4ManNAc exceeded those obtained for all ManNAc supplementation levels up to 10 mM. In contrast, the limitation in ManNAc kinase activity is only clearly apparent at levels of ManNAc exceeding 20 mM. *O*-Acetylation is a way of increasing efficiency of transport, but it cannot be used at higher concentrations because of its toxicity. A method for improving ManNAc transport efficiency without any toxic side effects may be beneficial for further enhancing the sialic acid metabolism from fed ManNAc.

Substrate feeding and metabolic engineering may also be used to control the type of sialic acid formed. The alternate sialic acid KDN is formed in addition to Neu5Ac when cells are infected with AcSAS (*15*). While Neu5Ac is derived from ManNAc-6-P, KDN is generated by the same enzyme from Man-6-P (Figure 1B). Furthermore, the levels of KDN synthesized increased when the cells lack the ManNAc-6-P precursor for Neu5Ac synthesis. One way to lower the ManNAc-6-P level is to eliminate ManNAc supplementation to the media, and another is to inhibit the intracellular formation of ManNAc-6-P. As Sf9 cells depend on GlcNAc kinase for phosphorylation of ManNAc, the synthesis of ManNAc-6-P can be restricted by feeding GlcNAc to the cells. Since the GlcNAc kinase preferentially acts on GlcNAc, this activity inhibits its ManNAc kinase function. Indeed, the addition of 10 mM GlcNAc was shown to favor KDN synthesis with little or no trace of Neu5Ac synthesis. In the absence of any available ManNAc-6-P in AcSAS-infected cells, the sialic acid 9-phosphate synthase enzyme acts on the available Man-6-P and converts it to KDN. However, the total levels of KDN obtained intracellularly were several orders of magnitude below those of Neu5Ac obtained when the AcSAS-infected cells were supplemented with 10 mM ManNAc. This low amount of cellular KDN synthesis may limit the feasibility of generating significant levels of glycoproteins terminating in KDN.

The current study has demonstrated that metabolic bottlenecks can exist in the production of sialic acids in engineered insect cells at the levels of expressed pathway genes and in the availability of substrates for the production of sialic acids Neu5Ac and KDN. These bottlenecks can be overcome by modifying the sialic acid pathway to include critical enzymes that are missing or at low levels and by altering the substrate or substrate transport in order to maximize sialic acid production. Previous studies with mammalian cells have revealed limitations in the sialylation pathway that can affect the level of sialylation observed on *N*-glycans (*35*). Insect cells represent an excellent model to evaluate sialylation bottlenecks since many of the genes are missing or expressed at very low levels in these cells.

The critical steps limiting complete sialylation of insect-cell-derived glycoproteins are the generation and transport of the nucleotide sugar CMP-Neu5Ac, the expression of transferases to yield acceptor substrates terminating in galactose, and the expression of sialyltransferase for transfer of Neu5Ac onto galactosylated acceptors. Previous research has shown that exogenous ManNAc feeding in combination with SAS and CMP-SAS expression will generate the essential nucleotide sugar for sialylation, CMP-Neu5Ac, as well as the alternative nucleotide sugar CMP-KDN (*16*). The expression of GalT in combination with GlcNAcT-II yields acceptor substrates terminating in galactose on both branches (*11*). Recently, the genes for these two bottlenecks were combined with sialyltransferase expression to generate fully

sialylated glycoproteins for insect cells grown in serum-free medium (*36*). Apparently, insect cells can achieve full sialylation without expression of the transporter that moves the CMP-Neu5Ac into the golgi apparatus. However, these studies were performed in medium supplemented with exogenous ManNAc. The current study shows that the requirement for any medium supplementation can be eliminated completely by expressing the UDP-GlcNAc 2-epimerase/ManNAc kinase gene in combination with other sialic acid pathway genes. Adding the UDP-GlcNAc 2-epimerase/ManNAc kinase gene to the others listed above will enable insect cells to become, to our knowledge, the first nonmammalian species to generate fully sialylated glycoproteins completely from intracellular metabolic components. Furthermore, the absence of these genes in insect cells has enabled the elucidation of specific bottlenecks in the sialic acid synthesis pathway. Critical bottlenecks including intracellular ManNAc transport as well as phosphorylation were identified to suggest ways in which production of CMP-Neu5Ac may be enhanced in the future. The elucidation of particular bottlenecks may be applicable to mammalian cell cultures as well since some of these pathway steps may also be limiting in these cells (*17*). Metabolic engineering efforts such as the ones described here will enable the optimization of intracellular pathways in insect cells, mammalian cells, and perhaps other species in order to achieve desirable levels of sialylation and other glycosylation modifications in the future.

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