Sialidases as regulators of bioengineered cellular surfaces

Cristina Y Zamora2,5, Matthew J Ryan2, Marc d’Alarcao3, and Krishna Kumar1,2,4

2Department of Chemistry, Tufts University, Medford, MA, USA, 3Department of Chemistry, San José State University, San José, CA, USA, and 4Cancer Center, Tufts Medical Center Boston, Boston, MA, USA

To whom correspondence should be addressed. Tel: +1-617-627-5651; Fax: +1-617-627-3443; e-mail: krishna.kumar@tufts.edu
5Present address: Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, MA, USA.

Received 9 November 2014; Revised 21 February 2015; Accepted 11 March 2015

Abstract

Human sialidases (NEUs) catalyze the removal of N-acetyl neuraminic acids from the glycome of the cell and regulate a diverse repertoire of nominal cellular functions, such as cell signaling and adhesion. A greater understanding of their substrate permissivity is of interest in order to discern their physiological functions in disease states and in the design of specific and effective small molecule inhibitors. Towards this, we have synthesized soluble fluorogenic reporters of mammalian sialidase activity bearing unnatural sialic acids commonly incorporated into the cellular glycocalyx via metabolic glycoengineering. We found cell-surface sialidases in Jurkat capable of cleaving unnatural sialic acids with differential activities toward a variety of R groups on neuraminic acid. In addition, we observed modulated structure–activity relationships when cell-surface sialidases were presented glycans with unnatural bulky, hydrophobic or fluorinated moieties incorporated directly via glycoengineering. Our results confirm the importance of cell-surface sialidases in glycoengineering incorporation data. We demonstrate the flexibility of human NEUs toward derivatized sugars and highlight the importance of native glycan presentation to sialidase binding and activity. These results stand to inform not only metabolic glycoengineering efforts but also inhibitor design.

Key words: 4-methylumbelliferyl, metabolic glycoengineering, neuraminidase, sialic acid, sialidase

Introduction

Metabolic glycoengineering utilizes carbohydrate derivatives and endogenous glycochemical synthetic pathways to incorporate unnatural functional groups into the glycomes of mammals and bacteria (Dube 2003). Glycosyltransferases and other cellular enzymes catalyzing the biosynthesis of N- and O-linked glycans tolerate a variety of chemical modifications in their substrates (Du et al. 2009; Dafik et al. 2010). In particular, the incorporation of “bioorthogonally reactive” functional groups (such as ketones, azides and alkynes) into the glycomes of mammalian cells continues to be a valuable tool nearly two decades after their introduction. Unnatural N-acyl mannosamine derivatives with these functional groups have been used as biosynthetic precursors to introduce N-acyl neuraminic acid (sialic acid) into the sialome of living cells. Through modified Staudinger ligation between azides, alkynes and their cognate reactants (Saxon and Bertozzi 2000; Hsu et al. 2007) and condensation of ketones with aminoxy or hydrazide groups (Yarema et al. 1998), a variety of functionalities may be “clicked” onto glycans (Campbell et al. 2007; Du and Yarema 2010). The glycocalyx of the cell is often the primary target of modification by glycoengineers as a means of studying critical cellular behaviors mediated by sialic acids, such as cell–cell communication, signaling and extracellular matrix binding (Chen and Varki 2010). Such efforts result in defined biological changes due to unnatural sialic acid presentation; however, any concomitant changes in the catabolism of altered sialoglycans have previously been overlooked in metabolic glycoengineering (MGE) studies, in part due to a paucity
of data on the behavior of cell-surface sialidases when presented with derivatized glycans. Sialidases [EC 3.2.1.18], also called neuraminidases (NEUs), are the family of enzymes responsible for removing sialic acid from endogenous glycoconjugates and thus regulate cell-surface sialic acid presentation. Human isoform NEU2 functions within the cytosol, while isoforms NEU1, NEU3 and NEU4 can be trafficked to the outer leaflet of the plasma membrane to various degrees to partake in cellular catabolism of sialylated glycans, in addition to their primary localization (Abdulkhalek et al. 2011; Sandbhhor et al. 2011; Shiozaki et al. 2011). The hydrolytic removal of neuraminic acids from glycans influences such diverse cellular behaviors as cell signaling (Tringali et al. 2007; Miyagi and Yamaguchi 2012), adhesion (Parker and Kohler 2010) and apoptosis (Kakugawa et al. 2002; Shkandina et al. 2012). Sialidase activity has also been implicated in various disease states such as chronic inflammation (Gadhoum and Sackstein 2008), lysosomal storage disorders (Pishehetsky et al. 1997; Pishehetsky and Hinek 2011) and cancer (Miyagi et al. 2004). Although well studied in viral systems, much less is understood about the function, structure and cognate ligands of human sialidase isoforms. We propose that decreases or increases in sialic acid hydrolysis by sialidases (NEUs) as a result of MGE not only affect the lifetime of these moieties within the glycome but may result in significant changes in sialidase-mediated cellular behaviors. Indeed, considering the ever-widening repertoire of functional and pathogenic roles played by NEU1–4 (Miyagi et al. 2004; Miyagi and Yamaguchi 2012), it is critical to characterize the structure–activity relationships (SARs) of these enzymes toward more effective inhibitor design and glycoengineering efforts.

Previously, we reported the use of derivatized sialic acids glycosidically linked to a fluorescent umbelliferyl aglycon as a platform for probing sialidase substrate specificity, as shown in Figure 1 (Zamora, d’Alarcao, et al. 2013). Hydrolytic activity of cell-surface sialidases was quantified in the human promyelocytic leukemia cell line HL-60 against a panel of neuraminic acids bearing varied N-acyl modifications and the enzymes were found to be amenable to R groups of varying hydrophobicity and bulk. These studies demonstrated that the lifetime of certain unnatural groups on the sialome, as appended by techniques such as MGE, may be modulated by cell-surface sialidases. Therefore, cell-surface sialidases may significantly alter the display of canonical MGE sidechains such as the ketone and “click” functional groups. However, the activities of sialidase isoforms against the N-levanoyl, N-pentynoyl and N-azidoacetyl moieties are unknown. Additionally, the substrate specificities of endogenous human sialidases against MGE-derivatized glycans displayed on cell surfaces have not been studied. We hypothesize that the incorporation efficiencies of unnatural sialic acids placed by MGE may be a function of both the promiscuity of the biosynthetic pathway and the sialidase activities resident on cellular surfaces.

Toward these needs, herein we report activities of human sialidase isoforms expressed on the Jurkat human T lymphocyte cell line, a commonly employed cell line in MGE. We utilized our fluorogenic sialic acid glycosides to quantify the activity of cell-surface sialidases against sialic acids bearing popular bioorthogonal MGE side chains, as well as hydrophobic and fluorinated functionalities known to exhibit noncovalent bioorthogonal behavior (Yoder et al. 2006; Dafik et al. 2008; Zamora, Dafik, et al.). To observe the activity of these enzymes against these unnatural sialic acids when natively displayed on the glycosylax, we employed metabolic glycoengineering to functiona- lize cells and quantified the hydrolysis of these derivatives by sialidase isoforms. These studies may inform drug design efforts and MGE alike, providing insight into the lifetimes and unnatural effects these functional groups confer to cellular sialomes, and lending further understanding to the role structure and presentation play in sialic acid catabolism.

**Results**

**Synthesis of sialic acid glycosides and mannosamine derivatives**

The synthesis of the derivatized sialic acid glycosides was carried out as previously reported (Zamora, d’Alarcao, et al. 2013) to afford compounds 1b–d shown in Figure 2. To complement the activity studies with our panel of glycosides 1a–i, we synthesized the corresponding panel of derivatized N-acyl mannosamines 2a–i (Figure 2) via reported routes (Dafik et al. 2008) for MGE studies with BJAB-K20 cells.

**Sialidases on Jurkat cell surfaces bind and cleave unnatural sialic acid derivatives**

We began by measuring the activity of endogenous cell-surface siali-"
(4MU-Sia), which presents the natural ligand N-acetyl neuraminic acid. Intact Jurkat cells were suspended in acetate buffer and incubated with compound 1a at 37°C. Cell aliquots were carefully handled to ensure no cell lysis occurred. After 2 h, the reaction mixture was quenched by the addition of glycine buffer (pH 10.7) in order to effectively stop enzymatic activity and allow for optimum 4-methylumbelliferone fluorescence. Aliquots from each mixture were placed in a 96-well plate and fluorescence emanating from enzymatic cleavage and release of 4MU from 1a read immediately. Trypan Blue exclusion of cell samples confirmed lack of membrane rupture. We measured an average of 0.65 U of sialidase activity on Jurkat surfaces despite lower expression levels in similar cell lines (Pshezhetsky et al. 1997; Gadhoum and Sackstein 2008; Pshezhetsky and Hinek 2011) and suggests these enzymes may be readily available for unnatural sialic acid remodeling.

To understand how actively Jurkat sialidases might hydrolyze derivatized sialic acids, we treated Jurkat cells with the panel of compounds 1b–k and measured sialidase activity as above. Fluorescence intensities over background were compared with that of 1a to assess the effect unnatural functional groups have on sialidase function. To confirm the fluorescence observed originates from sialidase-mediated hydrolysis of our compounds, reactions were also carried out in the presence of NEU inhibitor 2-deoxy-2,3-dehydro neuraminic acid (DANA) which is known to inhibit all four human isoforms of the enzyme (Khedri et al. 2012). We hypothesized that cell-surface sialidase isoforms would hydrolyze glycosides 1a–i differentially and thus affect metabolic glycoengineering outcomes. In addition, we anticipated that sialidases might show similar activity against compounds 1b–d bearing the bioorthogonal ketone, alkyne and azide functionalities.

The results are shown in Figure 3. Sialidases on Jurkat plasma membranes showed a wide range of activities, with N-levanoyl compound 1b being cleaved to nearly 60% greater extent than the natural ligand 1a. In contrast, cells treated with compounds 1c and 1d, corresponding to the N-pentynoyl and N-azidoacetyl sialic acids, respectively, released no detectable fluorescence.

We also measured human NEU activity against sialic acid derivatives that varied in steric bulk and hydrophobicity. Fluorometric data from compounds 1e–i incubated with Jurkat cells exhibited similar trends to those we previously observed in HL60 (Zamora, d’Alarcão, et al. 2013), such as decreases in activity when N-acetyl chains are lengthened. As such, neuraminic acid 1i (bearing a trifluorobutyl side chain at C-5) showed 43% sialidase activity in Jurkat relative to control and similar compound 1h gave no detectable signal as seen in Figure 3. Isobutyryl derivative 1c, however, deviated from this trend, being hydrolyzed by cell-surface sialidases by 7% less compared with 1a.

Another trend previously observed was an increase in sialidase activity when sialic acids bore polar functionalities at C-5 (Zamora, d’Alarcão, et al. 2013). Although N-glycolyl neuraminic acid glycoside 1f was hydrolyzed slightly less than control in Jurkat (88% of compound 1a), we did observe an increase in cleavage by sialidases with the isosteric substitution of one hydrogen with fluorine in glycoside 1g, resulting in 114% activity. In all samples, treatment of cells incubated with compounds 1a–i with inhibitor DANA significantly inhibited action by sialidases (Figure 3), ruling out non-enzymatic hydrolysis mechanisms.

Metabolically incorporated sialic acid derivatives on cell-surface glycans are released by sialidases

Fluorogenic glycosides provide a sensitive and quantitative means of measuring the cooperative activities of all cell-surface sialidases and their SARs. However, soluble compounds 1a–i would have higher conformational and pose variability than derivatized sialic acids displayed on glycans as a result of metabolic glycoengineering. Membrane-bound glycans are conformationally and spatially restrained, and this may significantly alter the activities of cell-surface NEUs against them. We also expect freely diffusible compounds 1a–i to encounter NEUs with higher frequency and effective concentrations than when the same derivatives are incorporated into the sialome of the cell. Finally, the amount of neuraminic acids available within the sialome for cellular structure and function varies when MGE
techniques are applied as a result of varying tolerances for unnatural R groups (Whitman et al. 2011). This in turn may modulate the sialidase activity that occurs on cell-surfaces against native glycans with respect to what is observed in vitro.

To address these considerations, we chose to measure the relative activities of sialidases against unnatural sialic acids once more, but this time when metabolically incorporated into cell-surface glycans as shown in Figure 4. We chose human Burkitt’s lymphoma subclone BJAB-K20 as the cell line for our MGE experiments that does not express UDP-GlcNAc 2-epimerase (Kepler et al. 1999) (Figure 4), resulting in a deficiency of N-acetyl-D-mannosamine, an essential and committed precursor to sialic acid synthesis (Tanaka and Kohler 2008). Cells cultured with N-acyl mannosamine derivatives (Figure 2, compounds 2a–i) regain sialylation due to tolerance of the metabolic pathway to chosen R groups (Tan et al. 2010). This process becomes the dominant source of sialic acids available for display on the glycocalyx, minimizing induced changes to the intracellular metabolic flux and competition between derivatives and endogenous N-acetyl neuraminic acid. Action by sialidases upon native glycans presenting sialic acid derivatives would result in their release from the glycocalyx into the growth medium, where they are isolated and quantified. Our approach allows for the first direct comparison with be drawn between the substrate specificities of cell-surface sialidases against membrane-associated and against free sialic acids.

BJAB-K20 cells cultured conventionally were conditioned to serum-free culture prior to metabolic incorporation. These conditions deplete cells of sialic acid precursors and produce a cell population with little to no sialic acid in its glycome. After conditioning, cells were incubated with mannosamine derivatives 2a–i for 72 h (a well-established MGE time frame; Yarema et al. 1998), before being gently pelleted and washed of all growth medium. Lymphoma subclone BJAB-K88 retains UDP-GlcNAc 2-epimerase activity and is thus able to synthesize sialic acids under serum-free conditions. This subclone was used as a positive control for the experimental conditions and detection of sialidase-liberated sialic acids in medium. Once collected, hydrolyzed sialic acids were extracted from growth medium samples and reacted with 4,5-methylenedioxy-1,2-phenylenediamine (DMB) for fluorescence quantification using reverse-phase high-performance liquid chromatography (HPLC; Hara et al. 1986). We expected to observe liberated sialic acids within the growth medium at the end of the 72 h incubation period typically employed in MGE. Any differences in the amounts of individual derivatized sialic acids hydrolyzed from cell surfaces is a function of the substrate specificities of the sialidases localized to plasma membranes alone and reflects their summative activity upon glycoengineered intact glycans.

DMB-HPLC analysis of growth medium samples from BJAB-K20 glycoengineering revealed the presence of derivatized neuraminic acids hydrolytically removed from cellular sialomes by NEUs (Figure 5). After co-culture of these cells with N-acetyl mannosamine (2a), N-acetyl neuraminic acid was detected in the growth medium indicating a restoration of sialylation due to MGE followed by hydrolysis by cell-surface sialidases. In comparison, medium samples from BJAB-K20 populations fed only vehicle contained on average 10% N-acetyl neuraminic acid, attributable to sialic acids present prior to serum-free conditioning. Analysis of medium samples from cells fed peracylated N-isobutyryl mannosamine (2e) found N-isobutyryl neuraminic acid in 200% relative concentration compared to populations fed 2a. This high amount of hydrolysis was also observed with compound 2g, which resulted in 102% relative concentration of N-fluoroacetyl neuraminic acid in medium. In both these cases, the comparative activity of cell-surface sialidases against these sialic acid derivatives on glycans matched that of solubilized glycodies 1e and g. However, the same substrate specificities were not observed for fluorogenic sialoses (Figure 3) and metabolically incorporated sialic acids. Medium samples from feeding of N-trifluoroacetyl compound 2b did not contain any of the corresponding sialic acid, as was also the case for N-trifluorobutryl compound 2i. Sialidase-mediated hydrolysis of N-glycolyl neuraminic acids (biosynthesized from cells 2f) from glycans was also depressed compared with hydrolysis of compound 1f (Figure 5) Trypan Blue exclusion confirmed membrane integrity was preserved throughout all experiments.

Fig. 4. Measurement of sialidase activity against sialylated glycans derivatized via metabolic glycoengineering. BJAB-K20 cells cultured in serum-free conditions lack UDP-GlcNAc epimerase activity and fail to synthesize ManNAc. Peracetylated mannosamine derivatives (1) bearing unnatural N-acetyl moieties at C-2 (ManNR) were fed to BJAB-K20 cells. After diffusion across the plasma membrane, mannosamines undergo biosynthetic transformation within the cell (2) before being incorporated within glycans as derivatized sialic acids (3). Hydrolytic removal of sialic acids due to endogenous NEU activity (4) results in desialylation and release of free sialic acids into the extracellular milieu. (5) Extracellular sialic acids are detected via DMB-HPLC analysis to measure neuraminidase activities against unnatural sialic acids. This figure is available in black and white in print and in color at Glycobiology online.
Sialic acid derivatives quantified via this method could only arise in BJAB-K20 cultures through hydrolytic action by sialidases off of cellular glycans after metabolic incorporation. We validated the incorporation of these derivatives into glycans by DMB-HPLC analysis of cell pellets from each MGE experiment (Dašik et al. 2008). Metabolic glycoengineering of BJAB-K20 utilizing peracetylated mannosamine derivatives has been successfully demonstrated for several side chains, among them N-glycolyl and N-azidoacetamido compounds 2d and f (Whitman et al. 2011). We found all corresponding sialic acid derivatives incorporated into the membrane fractions of our cell samples (Supplementary data, Figure S3), with varying efficacies of incorporation compared to the N-acetyl moiety 2a as expected. Compounds 2f and i were most effectively incorporated into the sialome after 72 h, with 57 and 59% incorporation, respectively, relative to 2a and are in good agreement with incorporations previously reported for BJAB-K20 (Whitman et al. 2011) and Jurkat (Dašik et al. 2008).

**Discussion**

Our probes of sialidase substrate specificities reveal a wide range of activities against the most popular R groups used in MGE (Figure 3). The results with compounds 1b–d differ from our prediction of decreased sialidase activity with lengthening acyl chain length. The van der Waals volumes of the N-acyl moieties of 1b–d, calculated according to the method of Bondi (1964), were found to be 65.4, 59.6 and 57.0 cm$^3$/mol, respectively. As all three side chains appear to be of comparable dimensions, this cannot be the only contributing factor to the observed substrate specificities. Indeed, the ketone in compound 1b provides two lone pairs capable of forming favorable hydrogen bond interactions with active site residues and water molecules. We previously observed that increases in hydrogen bonding capability of the ligand results in enhanced cleavage by human sialidases (Dube 2003; Almaraz et al. 2012; Zamora, d’Alarcao, et al. 2013). As such, we cannot discount the possibility of a favorable ligand conformation being induced upon sialidase binding.

The lack of glycosidic cleavage of “click” compounds 1c and d by Jurkat sialidases (Figure 3) may be attributable not only to steric bulk but also to a lack of moieties capable of hydrogen bonding. Alkynes and azides are largely inert toward biological molecules such as proteins and oligonucleotides in aqueous media (Campbell et al. 2007; Chang et al. 2009; Du et al. 2009; Dašik et al. 2010; Uhlig and Li 2011). In addition to their chemical stability, these functional groups may be unable to effectively form noncovalent protein–ligand interactions. A lack of stabilizing interactions within the active site would result in limited sialidase binding to these ligands. A search of the RCSB Protein Data Bank (PDB) revealed only four instances in which a crystal structure contains a ligand with a pent–4-yn–1-yl functionality (Wright et al. 2004; Patel et al. 2013). Of these records, none appear as N-pentynoyl and two are at an appropriate distance from phenylalanine, leucine and methionine residues to engage in hydrophobic contacts ($<4.5$ Å). Of 34 crystal structures deposited in the PDB containing a ligand bearing an azide, only one shows a N-azidoacetamido moiety within hydrogen bonding distance to a nearby histidine (Petsalakis et al. 2006). Our examination of the PDB revealed little evidence of strong interactions between either “click” moiety and protein surfaces, which may explain the lack of activity we observed. Nonetheless, cleavage of N-azidoacetamido neuraminic acids has been reported (Li et al. 2011). Li and coworkers assessed the substrate specificity of NEU2 with a panel of derivatized sialylgalactosides bearing a p-nitrophenol aglycon, reporting a 1.1-fold increase in conversion of the N-azidoacetamido functional group at C-5 of sialic acid over control. However, we note that these studies were performed on purified cytosolic isozyme NEU2 and isoforms NEU1–3 within cellular environments may certainly exhibit different substrate specificities despite highly conserved active site residues (Mageesh et al. 2006).

On the whole, sialidase activity against glycosides 1e–i was greater than previously observed in HL60, a difference possibly attributable to contrasting global sialidase expression profiles or differing amounts of individual isoforms localized to plasma membranes between the two cell lines. However, cell-surface sialidase activities against individual compounds 1e–i incubated with Jurkat remained in relative agreement with that of cell-surface sialidases in HL60, validating SAR trends we anticipate will be of use in sialidase inhibitor design. Our work confirms that NEUs are a factor in the regulation of orthogonal substrates displayed on cell surfaces.

The activities of cell-surface sialidases against unnatural sialic acid derivatives engineered onto cell surfaces were often predicted by our soluble glycosides. We found that glycosyls that were poorly hydrolyzed by sialidases, such as 1a, 1d, and 1h, were also in low abundance or undetectable in the corresponding growth medium samples, indicating that these R groups when natively presented still present a challenge for enzyme binding and activity. In contrast, with the notable exception of 1b, R groups readily recognized and hydrolyzed by Jurkat sialidases as compounds 1e, 1f, and 1g, were also easily detectable in growth medium after 72 h, suggesting sialidases still cleave these derivatives as native glycans and at endogenous concentrations.

Our measurements in growth medium have shown cell-surface sialidases capable of cleaving sialic acid derivatives placed through MGE and likely do so throughout the 72-h time frame. In some instances, sialidase activity against derivatized sialylated glycans was greater than that of native ligand N-acetyl neuraminic acid, suggesting that these glycans may be able to adopt particularly favorable conformations for hydrolysis by NEUs. The anchoring of derivatives to cellular membranes as compared with our freely diffusible glycosides 1a–i
often did not significantly change the substrate specificities observed, indicating that our probes may continue to be an easy-to-execute alternative to more laborious methods of assessing SAR of sialidases. As a result of our findings, we conclude that differences in any MGE incorporation efficacies (such as those in Supplementary data, Figure S3) cannot be attributed to the permisiveness of biosynthetic pathways alone without also experimental inhibition of NEU isoforms.

As expected, however, native presentation of these derivatives on cell-surface glycans did modulate the sialidase activity observed in some cases. Although the amount of sialic acid derivatives presented to cell-surface sialidases may have differed, some well-incorporated compounds such as 2b resulted in no sialic acid release at all despite being highly cleaved by Jurkat sialidases (Figure 3). The importance of glycan presentation for binding under physiological conditions is well studied (Brewer et al. 2002; Grant et al. 2013; Tessier et al. 2013; Wang et al. 2014). Recognition of a low-energy conformation or induced pose of a particular glycan by glycan-binding proteins can vary significantly under physiological conditions such as presentation on cell membranes (DeMarco and Woods 2008). In addition to any conformational effects, we hypothesize that the cell-surface sialidase activity measured against a derivatized sialome may also be a function of sialyltransferase substrate preferences. Lectin affinity analysis of global sialylation in BJAB-K20 and Chinese hamster ovary Lec 3 cells after MGE with mannosamine derivatives has shown linkage-specific incorporation depending on the side chain. Kohler and coworkers found low levels of α2–3-linked N-glycolyl neuraminic acid on ganglioside GM3 compared with other incorporated functionalities (Whitman et al. 2011), the sialylation of which is solely regulated by sialyltransferase ST3GAL5 (Ishii et al. 1998). In contrast, this unnatural sialic acid was readily found linked α2–6 on cellular sialosides. Although still not fully characterized, human sialidase isoforms NEU1–4 have been found to exhibit marked linkage and ligand preferences in vitro and in other systems (Smutova et al. 2014). Differential sialylation of cellular glycans due to these preferences and/or limited substrate tolerances could account for the differences in sialidase activity we observe across either series and between fluorogenic sialosides and incorporated sialic acids. As the structures of NEU3–4 have not been fully characterized, we also cannot rule out allostery as a possible contributor to our fluorogenic assay data.

In summary, we have confirmed that the important role sialidases play in the regulation of unnatural sialic acid display within the glyco-calyx as a result of metabolic glycoengineering. Fluorogenic sialosides provide an expeditious means for assessing sialidase–sialoside interactions in the interest of informing glycobiological applications. Cell-surface sialidases are readily able to cleave functionalized sialic acids whilst free in solution, with these data often correlating with sialidase activity upon sialylated glycans endogenously displayed on cell surfaces. However, our data demonstrate the differences in hydrolytic activity against functionalized sugars that result as a function of native glycan presentation and underscore the importance for continued study into the conformational determinants of protein–glycan interactions and into the structures and function of this important family of catabolic enzymes.

Materials and methods
Reagents and enzymes
Methanol and dichloromethane were dried on an Innovative Technologies PureSolv 400 solvent purifier. NMR solvents were obtained from Cambridge Isotope Labs (Andover, MA, USA). Mannosamine hydrochloride was purchased from LC Scientific (Concord, ON, Canada). Boc anhydride and HBTU were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). N-Acetylgalactosaminic acid aldolase (EC 4.1.3.3) was purchased from Nacalai USA, Inc. (Tokyo, Japan). N-Acetylgalactosaminic acid and DANA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nutridoma SP was purchased from Roche Applied Science. DMB dihydrochloride was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). BJAB-K20 and BJAB-K88 cells were a much-appreciated gift from Dr. James C. Paulson at the Scripps Research Institute in La Jolla, CA, USA. All other chemicals and cell culture supplies were purchased from Sigma-Aldrich and Fisher Scientific.

Cell culture conditions
Jurkat cells (ATCC number: TIB-152) were cultured continuously as a suspension culture in RPMI 1640 culture medium supplemented with 20% fetal bovine serum. BJAB-K20 and K88 cells were cultured in RPMI 1640 containing 2 mM l-glutamine with 10% fetal bovine serum. Cell densities were typically maintained between 2.5 × 10^5 cells/mL and 2.0 × 10^5 cells/mL and passed every 48–72 h as needed. All cell lines were maintained under 5% CO2 and 37°C in a water-saturated cell culture incubator. Cell counts for each population of cells were determined by Trypan Blue exclusion with a hemocytometer. To achieve serum-free conditions, RPMI 1640 culture medium supplemented with 10% Nutridoma SP was used. BJAB-K20 and BJAB-K88 cells were gradually weaned of serum-containing media and cultured in Nutridoma-supplemented media over the course of three passages, maintaining cell densities between 2.5 × 10^5 and 2.0 × 10^5 cells/mL. After 8 days, cells were cultured in this medium exclusively and used in mannosamine feeding experiments.

Sialidase activity assay
Endogenous Jurkat sialidases were assayed as described elsewhere (Zamora, d’Alarcao, et al. 2013). Whole Jurkat cells were removed from suspension culture and seeded into 2-mL Eppendorf tubes at a density of 1–2 × 10^6 cells/mL in 200 µL of buffer (0.05 M NaOAc, pH 4.4). Samples were provided 125 µM 1a–II and allowed to incubate at 37°C for 2 h. Samples received 100 µM DANA in some cases as a negative control. After the reaction was complete, all samples quickly received 1000 µL of a quenching buffer (0.133 M glycine, 0.06 M NaCl, 0.083 M Na2CO3, pH 10.7) and an aliquot immediately taken for fluorometric determination of released 4MU at excitation wavelength 365 nm and emission wavelength 450 nm using a Tecan plate reader. The concentration of 4MU and units of sialidase activity were determined by subtracting the fluorescence reading of 1a alone in buffer from the cell replicate readings and comparing the result to a standard curve generated from solutions of free 4-methylumbelliferone. The relative fluorescence intensity was determined by subtracting the background fluorescence sample from the cell samples, and normalizing the sample to the fluorescence of 1a.

Metabolic glycoengineering of BJAB cells
Peracetylated mannosamine derivatives were dissolved in ethanol to give 10 mM stock solutions. Aliquots of the appropriate derivative were dispensed onto the bottom of empty sterile tissue culture plates and the ethanol allowed to fully evaporate. BJAB-K20 cells cultured under serum-free conditions were seeded in these plates to a final density of 2.5 × 10^5 cells/mL in 12 mL and a final mannosamine derivative concentration of 200 µM. Plates were incubated as described.
Isolation of membrane sialic acids from cell pellets

Cell pellets from mannosamine feeding experiments were washed thrice with PBS, suspended in DI water, and lysed mechanically via freeze–thaw cycles. Samples were centrifuged at 10,000 × g for 15 min and the supernatant decanted. Pellets were each extracted once with 200 µL of 2 : 1, 1 : 1, and 1 : 2 chloroform–methanol solutions. The methanolic phase of each extraction is retained and the organic phase discarded. Samples were centrifuged and concentrated to dryness. Once concentrated, 200 µL of 2.0 M acetic acid was added and samples incubated for 3 h at 80°C to hydrolyze glycans, passed through a 3000 MWCO Millipore YM-3 filter assembly and the filtrate concentrated under vacuum to dryness.

Isolation of hydrolyzed sialic acids from growth medium samples

Growth medium was collected from 12-ml BJAB-K20 cultures after being grown in the presence of mannosamine derivatives for 72 h. Samples were lyophilized to dryness, dissolved in 2 mL of water and loaded onto a 10-mL column packed with Bio-Rad AG 1-X8 formate form resin in water. The column was washed with three column volumes of water to elute impurities and excess mannosamine derivatives, followed by elution of sialic acids with four column volumes of 1 M formic acid. Eluent was concentrated under vacuum to dryness for DMB-HPLC analysis.

DMB-HPLC analysis of samples

Supernatant and cell pellet samples from mannosamine feeding experiments were derivatized with DMB for reverse-phase HPLC resolution and sialic acid quantification. To a 30 µL aliquot of sample was added under darkness 30 µL of a solution containing 7.0 mM DMB, 20.0 mM Na2S2O4 and 681 mM β-mercaptoethanol in 1.4 M AcOH. Samples were incubated for 2.5 h at 50°C to achieve DMB labeling. Once complete, samples were diluted by 1 : 10 with HPLC solvent A and injected onto a Tosoh C18 reverse-phase column (TSK Gel ODS-120T, 4.6 × 250 mm; 5 µm) at a flow rate of 1.0 mL/min. HPLC solvent system was as follows: solvent A: 98% water, 2% acetonitrile; solvent B: 99% acetonitrile, 1% water. Fluorescence was read using a Hitachi L-7480 detector with excitation at 372 nm and emission set at 448 nm.

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

Funding

This work was supported in part by the National Institutes of Health (1 RO1 CA125033 to K.K. and M.D.). C.Z. was supported in part by a Department of Education GAANN fellowship. The ESI–MS and NMR facilities at Tufts are supported by the National Science Foundation (0320783, 821508).

Acknowledgements

We thank Prof. Michael Pawlita (Deutsches Krebsforschungszentrum, Heidelberg, Germany) for permission to use BJAB-K20 cells and are very thankful to Prof. James C. Paulson (The Scripps Research Institute) for sharing the BJAB-K20 cells, K88 cells and the group’s expertise. We also sincerely thank D. Walt (Tufts University) for the use of his tissue culture facilities; Dr D. Wilbur (Tufts University) for his assistance with the HPLC instrument; and J. Kritzer and C. Mace (Tufts University) for helpful discussions. This article is dedicated to Prof. Iwao Ojima (Stony Brook University) on the occasion of his 70th birthday.

Conflict of interest

None declared.

Abbreviations

4MU, 4-methylumbelliferone; DANA, 2-deoxy-2,3-dehydroxy neuraminic acid; DMB, 4,5-methylenedioxy-1,2-phenylenediamine; HPLC, high-performance liquid chromatography; MGE, metabolic glycoengineering; NEU, neuraminidase; PDR, Protein Data Bank; SAR, structure–activity relationship.

References


Sialidases as regulators of bioengineered cellular surfaces


