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Evaluation of Analogues of GalNAc as Substrates for Enzymes of the Mammalian GalNAc Salvage Pathway

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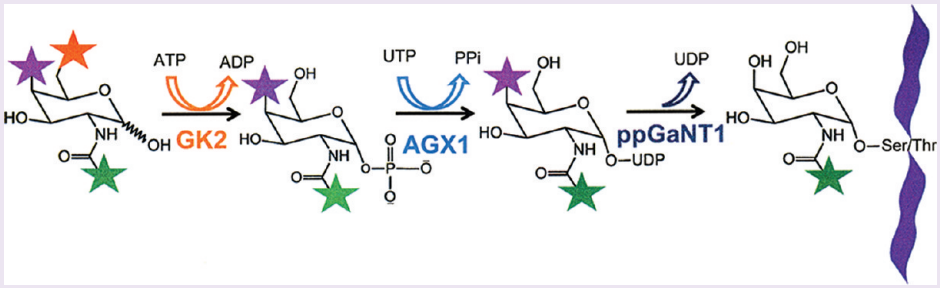
Supporting Information



ABSTRACT: Changes in glycosylation are correlated to disease and associated with differentiation processes. Experimental tools are needed to investigate the physiological implications of these changes either by labeling of the modified glycans or by blocking their biosynthesis. *N*-Acetylgalactosamine (GalNAc) is a monosaccharide widely encountered in glycolipids, proteoglycans, and glycoproteins; once taken up by cells it can be converted through a salvage pathway to UDP-GalNAc, which is further used by glycosyltransferases to build glycans. In order to find new reporter molecules able to integrate into cellular glycans, synthetic analogues of GalNAc were prepared and tested as substrates of both enzymes acting sequentially in the GalNAc salvage pathway, galactokinase 2 (GK2) and uridylpyrophosphorylase AGX1. Detailed *in vitro* assays identified the GalNAc analogues that can be transformed into sugar nucleotides and revealed several bottlenecks in the pathway: a modification on C6 is not tolerated by GK2; AGX1 can use all products of GK2 although with various efficiencies; and all analogues transformed into UDP-GalNAc analogues except those with alterations on C4 are substrates for the polypeptide GalNAc transferase T1. Besides, all analogues that could be incorporated *in vitro* into *O*-glycans were also integrated into cellular *O*-glycans as attested by their detection on the cell surface of CHO-ldlD cells. Altogether our results show that GalNAc analogues can help to better define structural requirements of the donor substrates for the enzymes involved in GalNAc metabolism, and those that are incorporated into cells will prove valuable for the development of novel diagnostic and therapeutic tools.

Carbohydrates contribute to life in many ways through their functions in energy metabolism and storage and as components of the genetic material as well as of structural elements. Carbohydrates attached to proteins or lipids (glycans) play important roles in cellular communication during cell differentiation and development. Glycans are assembled from monosaccharides, which can all be synthesized from glucose. In addition to a principal metabolic pathway, salvage pathways exist for most monosaccharides, which allow their incorporation directly from dietary sources into the biosynthetic routes of cellular glycan synthesis. Therefore, the addition to cells of labeled monosaccharides results in their incorporation into glycoproteins, glycolipids and proteoglycans. Using sialic acid analogues, it was discovered that sialyltransferases are rather permissive since they allow the integration of that monosaccharide modified with rather bulky groups into cellular glycoconjugates.[1] This observation was further extended to the sialic acid salvage pathway through integration of analogues of the precursor of sialic acid, *N*-acetylmannosamine (ManNAc).[2] Hexosamine analogues (*i.e.*, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), and ManNAc analogues) and sialic acid analogues were thus used to explore biological recognition events and downstream cellular behaviors[3,4] and have been introduced with great success into cells[5−7] as well as into living organisms[8,9] to probe either *O*- or *N*-glycans or glycolipids. For those purposes, azido analogues of hexosamines (GalNAz, GlcNAz, ManNAz) were incorporated through salvage pathways into glycans, expressed at the cell surface and then tagged by different techniques.[10,11] Other applications of hexosamine analogues have been described[12,13] showing that fluoro derivatives on the C4 position of GalNAc (4FGalNAc) or GlcNAc (4FGlcNAc) could be used to modify cellular recognition events since their incorporation into cells changed the glycan content of P-selectin glycoprotein ligand-1, reducing leukocyte adhesion via selectins. Recent developments in carbohydrate-based cancer vaccines have also led to the

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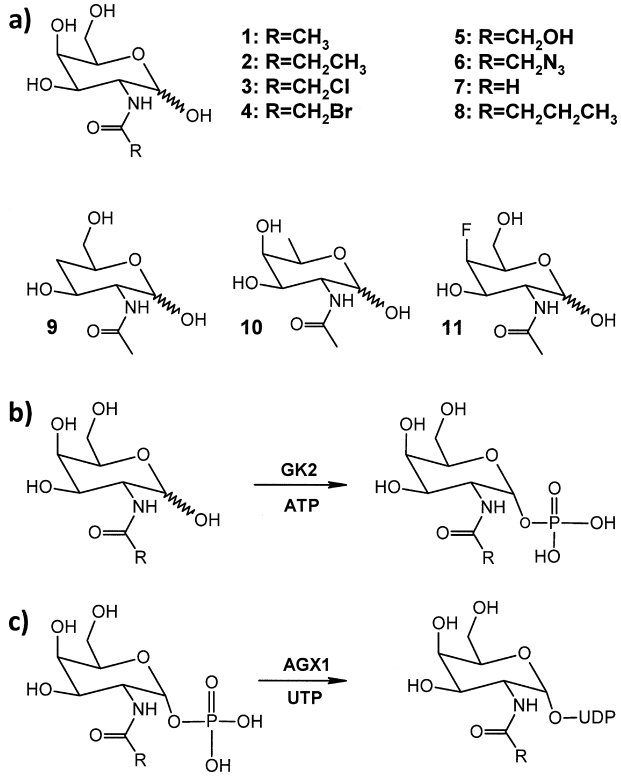
concept of introducing sugar analogues into tumor-associated carbohydrate antigen in order to improve the immunogenicity of those antigens.[14]

If some hexosamine analogues have been already developed for metabolic labeling and chemical tagging, questions still arise with respect to competition with endogenous substrates and physiological consequences associated with the modifications introduced by the analogues. New hexosamines, and in particular GalNAc analogues, may present interesting properties for the possible inhibition of signaling pathways or the stimulation of cellular responses, particularly in the context of immunity and cancer. As incorporation of such analogues into cells is an important challenge for metabolic glycoengineering, we want to investigate which GalNAc analogues can be metabolized by cells. To be incorporated into cellular glycans, those analogues must first go through the biosynthetic steps of the GalNAc salvage pathway,[15] resulting in their conversion to the nucleotide sugar uridine diphosphatidyl GalNAc analogues (UDP-GalNAc analogues), which then serve as substrates for the GalNAc transferases acting downstream and allowing their incorporation into glycoproteins as well as proteoglycans and glycolipids. We thus analyzed *in vitro* which modifications of GalNAc could be accepted by the enzymes of the salvage pathway. This involves the phosphorylation on the C1 position of the analogues by galactokinase 2 and the use of the GalNAc analogues-1P as substrates of the uridylpyrophosphorylase AGX1, which produces the UDP-GalNAc analogues. Moreover, since for some purposes it is an advantage to bypass the GalNAc salvage pathway by using UDP-GalNAc analogues either for direct injection into cells to label mucin type *O*-glycans[9] or for synthesizing oligosaccharides or glycopeptides enzymatically,[16] we employed our previously described two-step enzymatic system[17] in order to obtain substantial amounts of UDP-GalNAc analogues. We also investigated whether those analogues could be attached *in vitro* to peptides or proteins by a polypeptide GalNAc transferase (ppGalNAc-T), which transfers GalNAc from UDP-GalNAc to Ser and Thr residues of proteins. Our *in vitro* analysis shows that several analogues are readily accepted by the enzymes of the salvage pathway and further incorporated into peptides. These results are confirmed by the incorporation of some of the analogues into the *O*-glycans of living cells.

RESULTS AND DISCUSSION

Synthesis of GalNAc Analogues. To determine the substrate tolerance of the GalNAc salvage pathway enzymes, we used a series of GalNAc analogues whose structures are given in Scheme 1a. Compounds **2**−**8** were prepared (see Supporting Information) from galactosamine hydrochloride. Analogues **2**, **3**, and **4** were obtained in good yield (67−74%) after treatment of galactosamine in a mixture of methanol/sodium methoxide with different acid anhydrides, as also described in ref 18, while compound **5** was obtained in lower amount (48% yield) after reaction of galactosamine with acetoxyacetylchloride. The azido derivative (**6**) was synthesized according to ref 5 and obtained in high yield (88%). On the other hand compound **7** was easily prepared, although with a 23% efficiency, through reaction between the amine and formic acid. Analogue **8** was recovered in a 21% yield after reaction of the amine with the NHS-butyrate ester. The two deoxy derivatives (**9**) and (**10**) were obtained after efficient deprotection of di-*O*-pivaloyl GalNAc derivatives prepared as previously described,[19] and 4-deoxy-4-fluoroGalNAc (4FGalNAc, **11**) was obtained from Dr Szarek.[20]

Scheme 1. Structures of GalNAc Analogues and Enzyme Reactions[a]



[a](a) Synthetic analogues of *N*-acetyl-galactosamine (GalNAc, **1**): GalNPr (**2**), GalNClAc (**3**), GalNBrAc (**4**), GalNGlycolyl (**5**), GalNAz (**6**), GalNFormyl (**7**), GalNBu (**8**), 4-deoxyGalNAc (**9**), 6-deoxyGalNAc (**10**) and 4FGalNAc (**11**). (b) Synthesis of [GalNAc or analogue]-1P. (c) Synthesis of UDP-[GalNAc or analogue].

Relative Specificity of Analogues toward GK2 and AGX1. Since we wanted to explore the ability of those GalNAc analogues to be metabolized inside the cell through the salvage pathway, we first tested them as potential substrates of the human GalNAc kinase 2 (GK2, Scheme 1b) and of the human UDP-GalNAc pyrophosphorylase (AGX1, Scheme 1c). For that purpose we used recombinant forms of the enzymes prepared as previously described[17] and ATP as phosphate donor for GK2 while UTP served as nucleotide donor for AGX1. Not all of the analogues tested were substrates of the enzymes, but for those that were substrates, Michaelis−Menten parameters were determined. The results are summarized in Tables 1 (GK2) and 2 (AGX1).

Most of the GalNAc analogues tested can be phosphorylated by the recombinant human GK2. The K_m value obtained for the natural compound, GalNAc (240 μM), is in very good agreement with those previously reported for the purified (140 μM[21]) as well as for the recombinant (220 μM[17]) GK2. The **6** and **3** derivatives show a similar K_m for the enzyme (values 1.3−2 times higher than for GalNAc) and in the case of **6**, in the same order of magnitude than that previously obtained (500 μM[17]). On the other hand, for **2**, **4**, **5**, and **8**, elongating the chain at the C2 position seems to highly reduce the affinity of the enzyme (K_m values from 15 to 30 times higher than for GalNAc), while for a shorter chain (**7**) the K_m value is intermediate between that of **3** and **6** and of **2**, **4**, **5**, and **8**. Considering the X-ray crystallography data for GK2,[22] the pocket formed by the aminoacids of the active site, in the proximity of

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Table 1. Kinetic Parameters of GalNAc and Analogues toward Recombinant GK2[a]

sugar	$GalNH_2$ substitution	K_m (mM)	k_{cat} (s^{-1})	$k_{cat}K_m^{-1}$ (mM^{-1} s^{-1})	$k_{cat}K_m^{-1}$ (%)
1	*N*-acetyl	0.24 ± 0.03	2.7 ± 1.1	11.25	100
2	*N*-propionyl	6.47 ± 0.15	2.3 ± 0.17	0.35	3.1
3	*N*-chloroacetyl	0.49 ± 0.07	0.15 ± 0.04	0.31	2.8
4	*N*-bromoacetyl	3.75 ± 0.21	7.6 ± 0.7	2.03	18.0
5	*N*-glycolyl	3.54 ± 0.51	10.13 ± 3.7	2.86	25.4
6	*N*-azidoacetyl	0.32 ± 0.01	0.57 ± 0.04	1.78	13.0
7	*N*-formyl	1.08 ± 0.14	3.01 ± 0.5	2.79	24.8
8	*N*-butyryl	4.70 ± 0.5	0.26 ± 0.04	0.05	0.4
9	4-deoxy *N*-acetyl	1.88 ± 0.39	1.26 ± 0.05	0.67	5.9
10	6-deoxy *N*-acetyl	>10	<0.01		
11	4-deoxy-4-fluoro *N*-acetyl	1.10 ± 0.02	1.85 ± 0.8	1.68	14.9

[a] K_m and k_{cat} values were determined from Lineweaver–Burk plots obtained at different concentrations of GalNAc (**1**) and of each analogue (**2**–**11**) in the presence of a saturating amount of [^{32}P]ATP during 20–60 min of incubation.

the *N*-acetyl group of the co-crystallized GalNAc, is rather wide. It is thus possible that other substrates carrying longer or modified chains on the amino group of the sugar can be accommodated. On the other hand, modifications of the OH at position 4 of the sugar ring with smaller substituents seem to be well tolerated since both **9** and **11** are recognized by GK2 (K_m ~5 times higher than for GalNAc) even if in the crystal, there is one interaction of the OH on C4 with an Asp of the active site. Finally, only the 6-deoxy derivative (**10**) is not at all a substrate for GK2. This could be related to the implication of the 6-hydroxyl group of GalNAc in two interactions with His and Glu residues of the GK2 active site as seen in the crystal structure.[22] Moreover, it was already shown that an azido group on the C6 of GalNAc (6AzGalNAc) prevents the incorporation of that analogue into cell glycoconjugates,[5] suggesting that 6AzGalNAc is not an effective substrate of the GalNAc salvage pathway. Our results indicate that such inefficient incorporation could stem from GK2 essential recognition of the 6-hydroxyl group of GalNAc. Moreover, it should be noted that the specificity shown by GK2 is close to that observed for the bacterial *N*-acetylhexosamine 1-kinase (NahK), which presents similar K_m and k_{cat} constants for GalNAc.[23] Besides, NahK also accepts modifications of GalNAc on posistions C2 and C4 and displays a more limited tolerance for a C6 substitution[18] since 6-azido- and 6-deoxyGalNAc were transformed by NahK with significantly reduced yield.

If we now consider the catalytic efficiency $k_{cat}K_m^{-1}$, compounds **4**, **5**, **6**, **7**, and **11** are transformed by GK2 to analogues-1P with rather good efficiency since they all present values between 13% and 25% of that obtained for the natural substrate GalNAc. In fact it corresponds to what was observed in the next step, when sugars-1P were prepared from the different analogues (see Supplementary Table 1) in order to determine the kinetic parameters of AGX1. After purification, analogues-1P synthesized by GK2 were recovered in yields ranging from 57% for **1** to 10% for **8**. However, compound **10-P** was not synthesized by GK2, and compound **8-P** was obtained only in low amounts and could not be used in the determination of kinetic constants of AGX1.

Concerning the formation of UDP-analogues from the analogues-1P by AGX1, the K_m value obtained in this study for the natural substrate GalNAc-1P (1.17 mM) was similar to that previously reported (0.67 mM[17] and 0.5 mM[24]). Analogues **2-P**–**7-P**, **9-P**, and **11-P** were all recognized by the pyrophosphorylase, although to varying degrees. The **5-P** derivative showed a K_m value near that of GalNAc-1P, the **11-P**, and the **6-P** and the **7-P** derivatives presented a K_m about 2 times higher, while for **2-P**, **3-P**, **4-P**, and **9-P** the K_m values were 3–8 times higher than for GalNAc-1P. This shows that the recombinant form of AGX1 used in our study is able to interact with substrates modified on the *N*-acyl or on the C4 of the GalNAc moiety, in accordance with data obtained from the crystal structure of AGX1[24] where UDP-GalNAc was bound in the active site. The C4 hydroxyl group of the sugar moiety was found to interact with the enzyme in equatorial as well as in axial conformations, indicating the wideness of the binding pocket around this position. On the other hand, the *N*-acetyl arm of GalNAc was found in the crystal to establish extensive contacts with the enzyme suggesting a rather specific recognition for this part of the substrate. Our results indicate that the binding site of AGX1 can lodge shorter as well as longer chains on this arm. However, among the analogues tested only **2-P**, **3-P**, and **9-P** have a k_{cat} comparable to that of the natural substrate, all the others showing values 3–13 times lower than that of GalNAc-1P. As compared to the pyrophosphorylase GlmU, a GlcNAc-1-P uridyltransferase from *E. coli* previously employed[25,26] to prepare UDP-GalNAc analogues, AGX1 shows the same tolerance for the 4-deoxy and the 4-deoxy-4-fluoro analogues, but interestingly and contrary to GlmU, it can accept modifications on the *N*-acyl arm. Moreover, recent

Table 2. Kinetic Parameters of Each Analogue-1P toward Recombinant AGX1[a]

sugar-1P	$GalNH_2$ substitution	K_m (mM)	k_{cat} (s^{-1})	$k_{cat}K_m^{-1}$ (mM^{-1} s^{-1})	$k_{cat}K_m^{-1}$ (%)
1-P	*N*-acetyl	1.17 ± 0.12	1.81 ± 0.46	1.55	100
2-P	*N*-propionyl	9.25 ± 0.85	1.42 ± 0.5	0.15	9.7
3-P	*N*-chloroacetyl	5.08 ± 0.16	1.95 ± 0.05	0.38	24.5
4-P	*N*-bromoacetyl	7.26 ± 1.00	0.55 ± 0.23	0.08	5.2
5-P	*N*-glycolyl	0.57 ± 0.12	0.14 ± 0.09	0.25	16.1
6-P	*N*-azidoacetyl	2.57 ± 0.15	0.44 ± 0.02	0.17	11.0
7-P	*N*-formyl	2.32 ± 0.57	0.28 ± 0.06	0.12	7.74
8-P	*N*-butyryl	>50	<0.01		
9-P	4-deoxy *N*-acetyl	3.21 ± 0.12	1.12 ± 0.01	0.35	22.6
10-P	6-deoxy *N*-acetyl	n.d.[b]	n.d.		
11-P	4-deoxy-4-fluoro *N*-acetyl	1.99 ± 0.24	0.47 ± 0.09	0.24	15.5

[a] K_m and k_{cat} values were determined from Lineweaver–Burk plots obtained at different concentrations of GalNAc-1P (**1-P**) and analogues-1P (**2-P** to **11-P**) in the presence of a saturating amount of [^{3}H]UTP and during 20–60 min of incubation. [b] not determined.

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data[27] also show the broad specificity of AGX1 toward **2-P**, **6-P**, **8-P**, **9-P**, and **10-P**.

In terms of catalytic efficiency and according to the calculated value $k_{cat}K_m^{-1}$, besides the natural substrate **1-P**, compounds **3-P**, **5-P**, **6-P**, **9-P** ,and **11-P** are readily transformed by AGX1. This was effectively tested using GK2 in combination with AGX1 since *in vivo* both enzymes act in sequence, the product of the former being the substrate of the later. For that purpose, the different sugars were incubated overnight at 37 °C with GK2 and AGX1 in the presence of ATP and UTP. Products were prepared on the mg scale and submitted to extensive purification (ion exchange plus reverse phase chromatogrphy and gel filtration, see Supporting Information) before quantification (see Supplementary Table 2). As already seen from our previous analysis with each enzyme separately, neither **8** nor **10** could be used by GK2 and AGX1 together. On the other hand, if AGX1 presented a rather good catalytic efficiency for **1-P**, **3-P**, **5-P**, **9-P**, and **11-P**, when employed together with GK2, only **UDP-1** and **UDP-9** were produced in good amounts. As expected, the best efficiency was obtained for GalNAc (**1**, 43%) but compounds **2**, **3**, **4**, **5**, **6**, 7, **9**, and **11** could be also transformed into the corresponding UDP-analogues in milligram amounts and allowed us to test them as substrates for one of the next steps encountered *in vivo* for GalNAc: the transfer to an acceptor peptide.

Transfer of UDP-Analogues to Peptides by a Polypeptide αGalNAc Transferase. UDP-GalNAc formed in the cell is used by GalNAc transferases in order to build glycans. In a first approach we verified if our analogues could be substrates *in vitro* of the UDP-GalNAc:polypeptide αGalNAc transferase T1 (ppGalNAc-T1), an ubiquitous polypeptide αGalNAc transferase present in most tissues.[28] For that purpose we used the purified recombinant bovine enzyme[29] and UDP-analogues previously synthesized by the cooperative action of recombinant GK2 and AGX1. The acceptor substrates used were either a chemically synthesized peptide (STP_5) consisting of 5 repeated STP units or a recombinant protein ($MUC1_9$) produced and purified from *E. coli* and presenting 9 consecutive MUC1 tandem repeated motives. After incubation at 37 °C in the presence of the different donor substrates, we obtained highly glycosylated peptides and proteins that were characterized by mass spectrometry (Figures 1 and 2a).

UDP-4-deoxyGalNAc and UDP-4FGalNAc are not substrates of the ppGalNAc-T1 showing that the OH in position 4 of the sugar moiety is very important for this GalNAc transferase. Although it was already shown in a previous study,[30] it is however in contrast to the results reported in ref 12 where an incorporation of the 4FGalNAc was observed into cellular glycoproteins, including PSGL-1. This could be due to a transfer by another member of the ppGalNAc-T family showing a lower specificity for the donor sugar than the T1 enzyme, although no data are yet available on that point. On the other hand and as already demonstrated for GalNAz,[31] ppGalNAc-T1 tolerates modifications on the GalNAc amido group and gives good glycosylation ratios with all the corresponding UDP-analogues. According to the few available data on the UDP-GalNAc binding site in ppGalNAc-Ts, it was observed in the UDP-GalNAc:polypeptide αGalNAc transferase T10

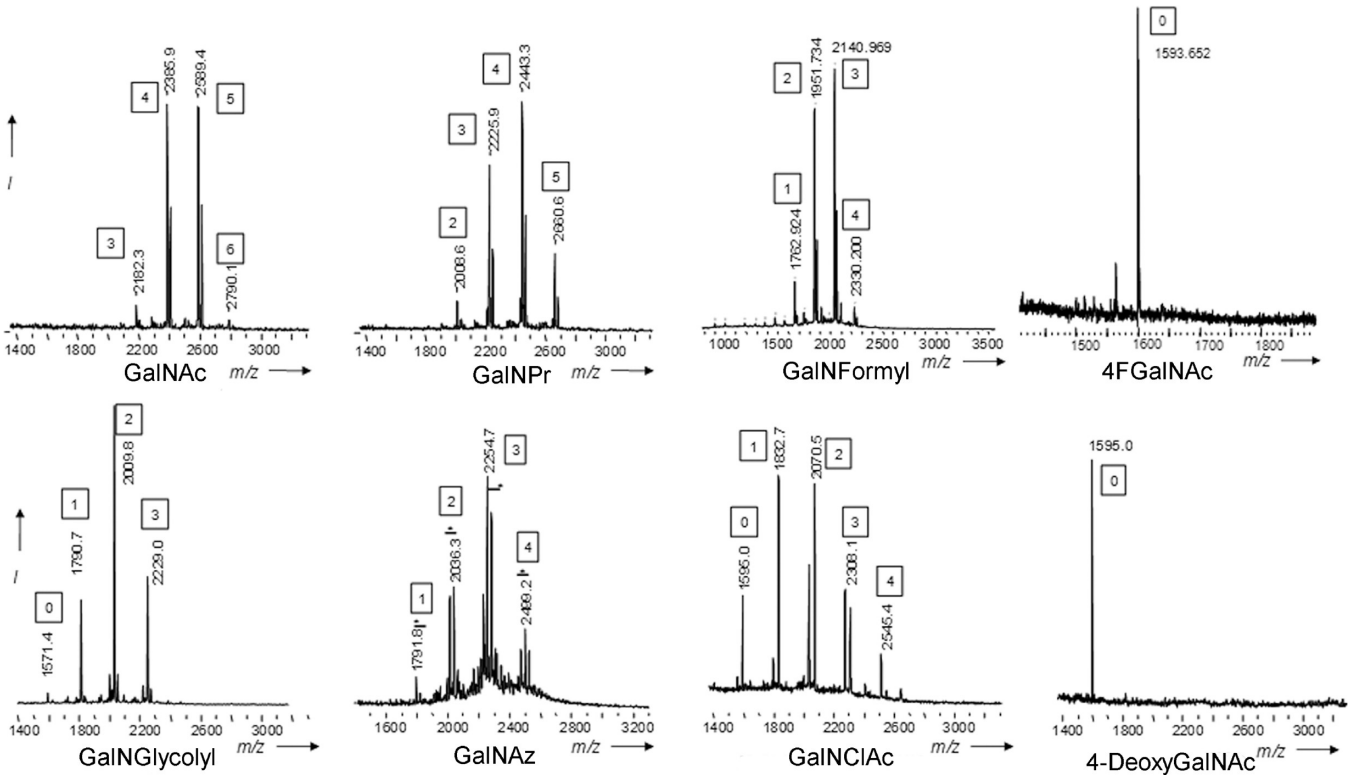

Figure 1. Mass spectra (MALDI-TOF) of STP_5 glycosylated peptides. STP_5 was glycosylated by the GalNAc transferase ppGalNAc-T1 starting from UDP-analogues. Above each peak is indicated, in a rectangle, the number of sugars incorporated onto the peptide. * A difference of −24 Da is observed between the calculated and observed masses of STP_5-GalNAz, which corresponds to the substitution of the N_3 group by a NH_2, due to a cleavage during mass spectrometry analysis. The mass difference of *ca.* +23 Da between nonglycosylated peptides seen for GalNGlycolyl (1571.4) and 4-FGalNAc (1593.7), 4-deoxyGalNAc (1595) and GalNClAc (1596) corresponds to Na^+ adducts. For the chloroacetyl derivative, the series of peaks with lower masses (Δ mass ∼36 Da) corresponds most probably to a loss of Cl occurring during mass analysis.

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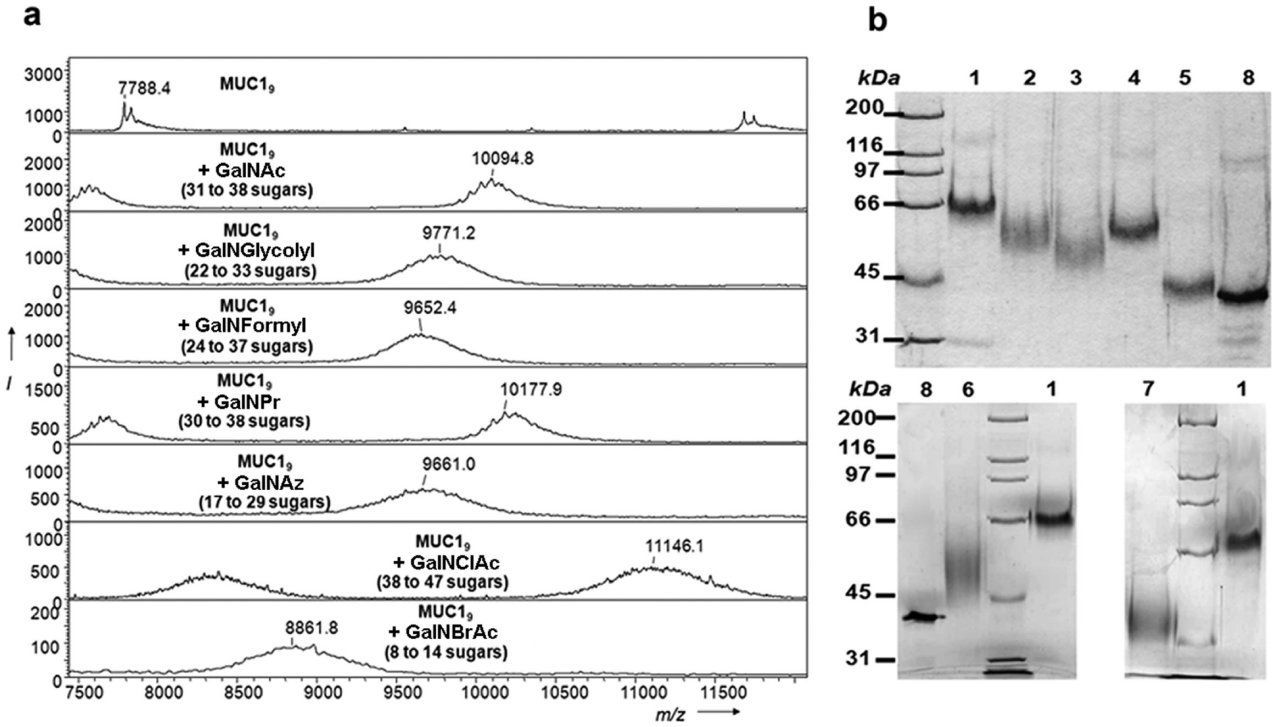

Figure 2. Analysis of glycosylated proteins. (a) Mass spectra (MALDI-TOF) of $MUC1_9$ glycosylated by the GalNAc transferase ppGalNAc-T1 starting from UDP-analogues, with a representation of the $[M + 3H]^{3+}$ ions; the extent of transferred sugars for each analogue is indicated on each spectrum. (b) Coomassie-stained gels after SDS-PAGE of glycosylated $MUC1_9$ for GalNAc (lane 1), GalNGlycolyl (lane 2), GalNFormyl (lane 3), GalNPr (lane 4), GalNAz (lane 5), GalNClAc (lane 6), GalNBrAc (lane 7), and non-glycosylated $MUC1_9$ (lane 8).

(ppGalNAc-T10) crystals that the residue Glu345 of the TrpGlyGlyGlu motif interacted with the oxygen on the C4 of the GalNAc moiety.[32] This TrpGlyGlyGlu motif is present in all ppGalNAc-Ts[33] and was found in the region of the active site in the crystal structure of ppGalNAc-T1.[34] In addition a mutation in the ppGalNAc-T1 of Glu316 to Gln, the equivalent of Glu345 in the ppGalNAc-T10, reduced dramatically the enzymatic activity.[35] The *N*-acetyl group of the GalNAc unit was found in the ppGalNAc-T10 crystallized enzyme in the proximity of a His residue (H370) and of a Gly (G320), which again are both situated in sequences conserved among all ppGalNAc-Ts. However, when His341 in ppGalNAc-T1, which is the equivalent of His370 in ppGalNAc-T10, was mutated to either Ala, Leu, Val, Lys, or Arg, it had little effect on enzymatic activity.[35] In agreement with these results the present data show that ppGalNAc-T1 can accommodate UDP-analogues carrying modified *N*-acyl groups on the amido function.

From the two acceptor substrates used in our study, slightly different results were obtained (compare the spectra in Figures 1 and 2a). STP_5 was predominantly substituted on 4 or 5 Ser/Thr when GalNAc was used and was mainly substituted on 4 residues for propionyl, 3 for the azido, 2 or 3 for the formyl and dropped to 1 or 2 for the glycolyl and chloroacetyl derivatives. In the case of $MUC1_9$, the chloroacetyl derivative gave the best yield of incorporation (42 units per peptide as a mean), GalNAc and propionyl gave the same ratio of substitution (around 35 units per peptide), while glycolyl and formyl were slightly less incorporated (about 28 analogues per peptide) followed by the azido derivative (23 units per peptide) and finally the bromo derivative with only 11 units transferred to each molecule of $MUC1_9$. SDS-PAGE (Figure 2b) carried out on $MUC1_9$ gives an illustration of the results obtained by MS. The naked peptide appears after Coomassie staining as a band with an apparent MW of 40 kDa. This protein migrates more slowly when glycosylated. However the distance of migration is not rigorously inversely proportional to the ratio of substitution by the different analogues. This may be due to a different adsorption of SDS depending of the quantity and type of analogue present on the peptide.

Our results show that the GalNAc analogues **2**, **3**, **4**, **5**, **6**, and **7** are substrates of the three enzymes GK2, AGX1, and ppGalNAc-T1 and thus could, as it was already demonstrated for **6**,[5] be incorporated *in vivo* in glycoproteins provided that they enter the cells. Besides, the chloroacetyl derivative is remarkably well incorporated in MUC_9 by the recombinant ppGalNAc-T1 used in our study, since it occupies between 38 to 47 over the 59 Ser and Thr present in the total peptide sequence, which represents a mean of 72% of the free acceptor sites. It is thus a very good donor substrate for that glycosyltransferase, indeed better than GalNAc, which under the same conditions is incorporated into 58% of the free Ser and Thr. Inversely, for the acceptor substrate STP5, GalNAc shows the best incorporation rate (45% of the free hydroxylated aminoacids), and the chloroacetyl derivative is significantly less transferred (15%). Although further investigations are needed in order to correctly interpret these discrepancies, it suggests that the incorporation of some sugar analogues may modify the structure of an acceptor protein.

Since five of the novel analogues presented here (**2**, **3**, **4**, **5**, and **7**) are substrates of the three enzymes previously tested, we checked whether these analogues could be taken up by cells and incorporated into glycoconjugates as has been demonstrated previously for **6**.[5]

Incorporation of the Analogues into CHO Cells. As a first approach for testing the incorporation of GalNAc analogues into cells, we choose the mutant CHO-ldlD cell

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line,[36] which is devoid of UDP-Gal/GalNAc 4-epimerase (GALE) activity. This enzyme being responsible for the interconversion of UDP-Glc/GlcNAc to UDP-Gal/GalNAc, CHO-ldlD cells cannot incorporate any GalNAc into their glycans unless this monosaccharide is added to the medium. Moreover, after penetration into cells, GalNAc has to follow the salvage pathway in order to be transformed into UDP-GalNAc. The ppGalNAc-transferases transfer the analogue from the nucleotide sugar to protein, and in CHO-ldlD cells, due to the absence of galactose, GalNAc or GalNAc analogues are the terminal sugars on cellular *O*-glycans which can be easily detected with GalNAc-specific lectins like *Vicia villosa* agglutinin (VVA).[37] To facilitate the passage through the cell membrane and thus the incorporation of the analogues, we peracetylated the monosaccharides with acetic anhydride in pyrdine. Peracetylated GalNAc was used as control. The peracetylated sugars were dissolved in DMSO and added to the cells at a final concentration of 12.5 μM. After 24 h the incorporation of the analogues was monitored by staining the cell surface with FITC-labeled VVA followed by immunofluorescence microscopy. Previous dot blot and Elisa tests had been carried out on the glycopeptides bearing GalNAc analogues prepared as described above, which revealed that VVA could bind all of the different GalNAc analogue α-Ser/Thr structures (Tn or Tn-like antigens) with a similar affinity (not shown). As illustrated in Figure 3, all analogues tested gave rise to an unambiguous fluorescent signal at the cell surface that was not present if no GalNAc or analogue had been incubated with the cells. The natural monosaccharide GalNAc gave the best signal with the fluorescent lectin, but **6**, **5**, and **3** showed also good staining, whereas **2**, **7**, and **4** resulted in slightly lower levels of lectin binding. 4FGalNAc could not be detected on cellular glycans by the fluorescent lectin since VVA, and all other GalNAc specific lectins tested did not recognize free 4FGalNAc in inhibition assays (not shown).

In summary we have shown that besides GalNAz, which is already successfully used in cells,[5] other analogues of GalNAc can be transformed into UDP-analogues through the GalNAc salvage pathway. The kinetic data presented in this study give some information on the substrate recognition of both enzymes GK2 and AGX1 that constitute the pathway. Moreover, with the exception of 4-deoxyGalNAc and 4FGalNAc, all analogues that can be readily transformed into UDP-GalNAc analogues first by GK2 and then by AGX1 could also be transferred to peptides *in vitro* by the ppGalNAc-T1. As for the two other enzymes, the analogues provide indications on the donor substrate specificity of this glycosyltransferase. In addition, the tools developed here are quite well adapted for the production

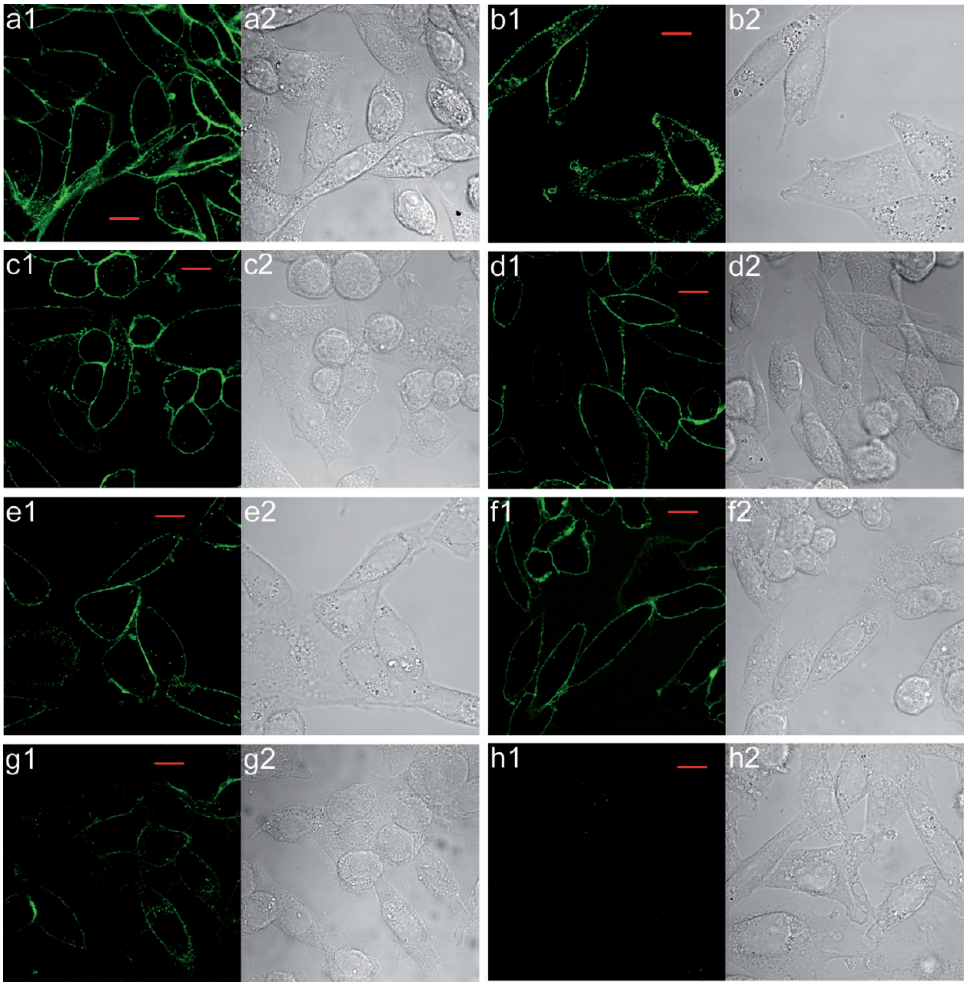

Figure 3. Incorporation of GalNAc analogues into cell glycans. Confocal fluorescence microscope images of CHO-ldlD cells incubated with peracetylated GalNAc analogues and fixed before staining with VVA-FITC. (a) GalNAc (**1**), (b) GalNGlycolyl (**5**), (c) GalNAz (**6**), (d) GalNPr (**2**), (e) GalNFormyl (**7**), (f) GalNClAc (**3**), (g) GalNBrAc (**4**), and (h) control, DMSO only. The numbers 1 and 2 indicate fluorescence and phase-contrast images of the same field, respectively. The scale bar (red) corresponds to 10 μm.

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of substantial amounts (in the milligram range) of glycopeptides bearing GalNAc analogues. To test the potential for metabolic engineering with the GalNAc analogues, we analyzed their incorporation into mammalian cells. Since the mutant cell line CHO-ldlD can incorporate into cell surface glycans only GalNAc or GalNAc analogues that have been added to the medium and gone through the entire GalNAc salvage pathway, the staining of the cell surface with a GalNAc-specific lectin strongly suggests that the analogues are substrates for the salvage pathway *in vivo* as well as *in vitro*. Therefore the results obtained with the GalNAc analogues *in vitro* are valuable indicators for their potential use in metabolic glycoengineering. The utility of this approach has recently come to light with the limited use of azidoGlcNAc for the labeling of *O*-GlcNAc structures[38] and with the continuing discussion about the incorporation of 4FGlcNAc into cellular glycans.[12,38,39] Work is in progress to determine whether these GalNAc analogues will be incoporated into more complex *O*-glycans. If they are recognized as acceptor substrates by downstream acting glycosyltransferases, they will be incorporated into tumor-specific glycans and could provide novel unnatural glycan structures on tumor cell surfaces useful as targets for immunotherapy.

METHODS

General. All chemicals used were reagent or analytical grade. Methods of synthesis, purification, and characterization of GalNAc analogues, GalNAc analogues-1P, and UDP-GalNAc analogues are given in the Supporting Information. Proteins were analyzed by MALDI-TOF mass spectrometry performed with an Autoflex I (Bruker Daltonics) fitted with a 337 nm nitrogen laser and operating in reflector positive mode for the peptide STP_5 and in linear positive mode for small proteins. Scintillation counting was performed in a Packard TriCarb liquid scintillation counter. Procedures for preparation of the recombinant enzymes, human GalNAc Kinase 2 (GK2), human UDP-GalNAc pyrophosphorylase (AGX1) and bovine UDP-GalNAc:polypeptide αGalNAc transferase 1 (ppGalNAc-T1) necessary for the following steps have been already described.[17] The peptide STP_5 (sequence: STPSTPSTPSTPSTPAG) was chemically synthesized by Philippe Marceau (CBM, Orléans), whereas the $MUC1_9$ (sequence: MH_6ARKKWKKAS(APDTRPAPGSTAPPAHGVTS)$_9$AL ALGSTAPPVHNVTSATSALGSTAPPVHNVTSATSKKWKKPSMRM) recombinant protein was produced in *E. coli*.[40]

Michaelis Constants Determination. Analogues or analogues-1P were incubated for 20−60 min at 37 °C in 25 μL Tris/HCl (75 mM, pH 8.5), $MgCl_2$ (5 mM), BSA (1 mg mL^{-1}), and either [^{32}P]ATP (Amersham, 0.5 mM, 10^4 cpm $nmol^{-1}$) with GK2 (2.1−4.2 μg mL^{-1}, 1.2−2.4 mU mL^{-1}) or [^{3}H]UTP (Perkin-Elmer, 4 mM, 600 cpm $nmol^{-1}$) with AGX1 (1.9−3.8 μg mL^{-1}, 7−14 mU mL^{-1}) and PPA (1.6 μg mL^{-1}, 3 U mL^{-1}). Incubations were stopped by dilution with water, applied to a column (0.7 cm × 3.0 cm) of Dowex 1 × 8 (Cl^-), the products were isolated by stepgradient elution, and the fractions were counted in a liquid scintillation counter. Controls without acceptor substrates were treated in the same way, and the column profiles were compared to detect the presence of overlapping radioactive peaks corresponding to degradation products. Whenever those peaks were present, they were subtracted from the assay chromatogram. Each experiment was repeated at least 3 times.

Synthesis of Glycopeptides Starting from the UDP-GalNAc Analogues. UDP-GalNAc analogues (2 mM) were incubated at 37 °C in 2-(*N*-morpholino)ethanesulfonic acid (50 mM, pH 6.5) containing dithiothreitol (2 mM), BSA (0.2 mg mL^{-1}), $MnCl_2$ (15 mM), recombinant bovine polypeptide αGalNAc transferase 1, (ppGalNAc-T1, 30 μg mL^{-1}, 40 mU mL^{-1}), and either STP_5 (0.4 mM) or $MUC1_9$ (0.06 mM). After 6 h calf intestinal phosphatase (CIP, New England Biolabs, 5.72 μg mL−1, 10^4 U mL^{-1}) was added, followed 1 h later by additional amounts of ppGalNAc-T1 (9.23 μg mL^{-1}) and UDP-GalNAc analogue (2 mM). Incubation was pursued for 24 h, and the reaction was stopped by the addition of MeOH (1 vol). The mixtures were kept on ice for 1 h in order to precipitate the proteins. After centrifugation, the supernatants that contain the glycopeptides were dialyzed against water and analyzed by mass spectrometry and also by SDS-PAGE (8% acrylamide) in the case of $MUC1_9$.

Incorporation of the Analogues in CHO-ldlD cells. The cells were maintained as described.[41] They were then incubated for 24 h with 12.5 μM peracetylated GalNAc analogues (from a 12.5 mM stock in DMSO), fixed with 3% (w/v) paraformaldehyde in PBS, and stained with VVA-FITC (5 μg mL^{-1} in PBS, 0.2% (w/v) BSA). Coverslips were mounted in 5 μL of mounting medium (5% (w/v) 1,4-diazabicyclo[2.2.2]octane in 50% (w/v) glycerol/50% (v/v) PBS) on glass slides and observed under a Zeiss Axiovert 200 M confocal laser microscope coupled with a Zeiss LSM 510 scanning device. Cells were observed with a Plan-Apochromat 63x objective and images were recorded with Carl Zeiss's LSM Image Browser software.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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