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In vivo incorporation of an azide-labeled sugar analog to detect mammalian glycosylphosphatidylinositol molecules isolated from the cell surface

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ABSTRACT

N-Acetylgalactosamine (GalNAc) linked to the first mannose of glycosylphosphatidylinositol (GPI) core has been previously reported to be heterogeneously present on some mammalian GPI-anchored proteins. Here we present a method for profiling GalNAc-containing GPI-anchored proteins in mammalian cells by metabolic labeling with tetraacetylated *N*-azidoacetylgalactosamine (GalNAz) followed by biotinylation of the incorporated sugar analog. We have labeled both endogenous and recombinant GPI-anchored proteins with GalNAz, and demonstrated that the azide-activated sugar gets incorporated into the GPI glycan, likely as an unsubstituted side branch of the core structure. GalNAz was detected only on GPI molecules attached to proteins, and not on GPI precursors, indicating that GalNAc modification takes place after the GPI anchor is transferred to protein. We have highlighted the utility of this cell labeling approach by demonstrating the ability to examine specific GalNAc-containing GPI-anchored proteins isolated non-destructively from separate membrane domains (apical and basolateral) in polarized epithelial cells. This study represents the first demonstration of site-specific in vivo labeling of a GPI moiety with a synthetic sugar analog.

1. Introduction

The glycosylphosphatidylinositol (GPI) anchor is an evolutionarily conserved glycolipid structure that is post-translationally attached to certain eukaryotic secretory proteins to anchor them to the cell surface. The precise structure of the GPI has been determined for numerous mammalian, protozoan, yeast, and plant GPI-anchored proteins (GPI-APs).[1,2] Comparison of these structures has revealed a conserved core composed of ethanolamine-*P*-Manα1–2Manα1–6Manα1–4GlcN-*myo*-inositol-lipid that is presumed to be common to all GPIs. This core structure is synthesized and transferred to protein in the endoplasmic reticulum, and many of the enzymes involved in this process have been identified.[1,3–6] Overall GPI structure differs considerably between species and even within an individual organism due to both a diverse and heterogeneous array of glycan moieties that can be appended as side-branches to the core glycan, and variations in the lipid composition of the phosphatidylinositol.[1–3] Furthermore, within any given organism, GPI structure is highly dynamic with each GPI molecule undergoing remodeling of both the glycan and lipid domains before and after its attachment to protein.[7–9]

Understanding GPI structure as it relates to potential functions of the GPI molecule represents a major challenge. To date, the only confirmed function of the GPI moiety is as a membrane anchor for certain proteins, however the GPI has been implicated in several important cellular processes such as signal transduction, apical targeting in polarized epithelial cells, and cell–cell communication (see Ref. 2 for a review). The dynamic structural complexity of GPIs suggests that elements of GPI structure could play a key role in these important processes, as well as in the biosynthesis and trafficking of GPI-APs. Indeed, roles of certain moieties found as side-branches to the conserved core of the mammalian GPI glycan are starting to be elucidated. The presence of phosphoethanolamine (EtNP) side-branched to the first GPI mannose (Man-1) is a prerequisite for the association of a GPI precursor with the human GPI transamidase complex, the enzyme that catalyzes the attachment of GPI to protein.[10] Phosphoethanolamine linked to the second GPI mannose (Man-2) residue is involved in regulation of mammalian GPI-AP transport from the ER to the Golgi.[9] More recently, it has been proposed that the synapse damage induced upon prion

Abbreviations: GPI, glycosylphosphatidylinositol; GPI-APs, GPI-anchored proteins; EtNP, phosphoethanolamine; Man-1, first GPI mannose; Man-2, second GPI mannose; Man-3, third GPI mannose; Man-4, fourth GPI mannose; Man_3-GPIs, trimannosyl GPIs; Man_4-GPIs, tetramannosyl GPIs; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GalNAz, *N*-azidoacetylgalactosamine; TEER, trans epithelial electrical resistance; Gluc, recombinant *Gaussia princeps* luciferase; Cluc, recombinant *Cypridina noctiluca* luciferase; PI-PLC, phosphatidylinositol-specific phospholipase C; UDP, uridine diphosphatidyl; PDM, protein deglycosylation mix; β-HexNAc-ase, jack bean β-*N*-acetylhexosaminidase; HF, aqueous hydrofluoric acid; PI, Phosphatidylinositol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IP, immunoprecipitated.

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protein clustering is mediated in part by the sialic acid-containing side branch of its GPI anchor.[11]

The significance of other moieties side-branched to the GPI glycan is unknown. For example, a fourth mannose (Man-4) may be added as a α1,2-linked side-branch of the third mannose (Man-3) on the GPI anchors of *Trypanosoma cruzi*, yeast, slime mold, and mammalian proteins. Addition of Man-4 is essential for GPI biosynthesis in yeast, whereas mammalian cells do not have this strict requirement and both Man_3- and Man_4-GPIs are transferred to proteins.[12] Interestingly, in mammalian cells the fourth GPI mannosyltransferase (PIG-Z) is expressed in a tissue-specific manner, suggesting that formation of tetramannosyl GPIs (Man_4-GPIs) is highly regulated on a tissue- or cell-specific level.[12] In mammalian cells, *N*-acetylgalactosamine (GalNAc) linked to Man-1 by a β1–4 linkage has been reported to be present on certain GPI-anchored proteins.[13–17] These studies involved the analysis of large amounts of purified protein through enzymatic and chemical digestion, thin-layer chromatography, gas- and mass spectrometry, and NMR.[13,15–18] Moreover, it has been demonstrated that the GalNAc addition to the GPI anchor is heterogeneous on an individual protein,[13,15–18] which makes their detection by conventional methods even more challenging. In some GPIs, GalNAc may be terminally capped with galactose and sialic acid.[17,19] The relative abundance of side-chain modifications on GPI anchors of mammalian proteins, the biological significance of the GalNAc modification, and the subcellular localization and mechanism of GalNAc addition are not known. The study of this modification is impeded in part by the lack of a simple and effective method to visualize GalNAc-containing GPIs without the requirement for purification of large amounts of protein.

In this study, we investigated the in vivo incorporation of a synthetic analog of *N*-acetylgalactosamine into the GPI glycan to visualize the GalNAc side-branch modification known to be present on some GPI-APs. Tetraacetylated *N*-azidoacetylgalactosamine (GalNAz) has been utilized in previous studies to label different classes of glycans in living cells, including N- and O-glycans.[20–24] This sugar analog is able to cross the cell membrane in living cells and is converted by the endogenous cellular machinery into its cognate nucleotide sugar (UDP-GalNAz) donor prior to incorporation into glycans.[23] It has also been reported that UDP-GalNAz can be epimerized into UDP-GlcNAz prior to integration into glycans in mammalian cells.[28] The work presented here demonstrates that GalNAz can be incorporated specifically into the GPI glycan. Using metabolic labeling of GPIs with GalNAz, we demonstrate the presence of the GalNAc side-chain modification on GPI-APs, but not GPI precursors. The utility of this method is highlighted by the demonstration of the ability to isolate GalNAc modified GPI anchors from different plasma membrane domains in intact polarized epithelial cells. Our findings provide the framework for the future study of the synthesis and function of GalNAc-containing side-branches of GPI glycans.

2. Results and discussion

2.1. A model system for sugar analog incorporation into GPIs

Metabolic incorporation of tetraacetylated *N*-azidoacetylgalactosamine (GalNAz), a synthetic azide-labeled GalNAc analog, has been used to label both N- and O-glycans in living cells.[20–24] GPI-anchored proteins (GPI-APs), but not GPI precursors, have been reported to heterogeneously contain a GalNAc side chain modification, suggesting that this modification occurs after GPI attachment to protein. Therefore, we examined whether metabolic GalNAz incorporation can also be used to label the GPI-glycan of GPI-APs in living cells. Two important considerations for this experimentation were the selection of a model GPI anchored protein and of a model cell line.

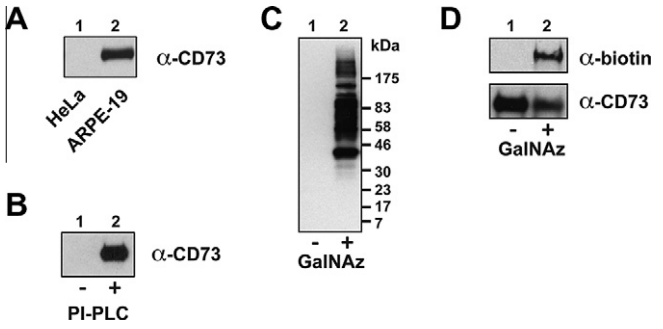

Figure 1. Expression of CD73 in different cell lines and metabolic labeling of ARPE-19 with GalNAz. (A) Expression of CD73 in HeLa (lane 1) and ARPE-19 (lane 2) cells. Whole cell lysates were analyzed by western blotting with anti-CD73 antibody. Note the strong expression of CD73 in ARPE-19 cells. (B) CD73 can be released from live ARPE-19 cells by treatment with PI-PLC. Cells were incubated in the absence (lane 1) or presence (lane 2) of PI-PLC, and then collected medium was analyzed by blotting with anti-CD73 antibody. (C) GalNAz is able to label total glycoproteins in ARPE-19 cells. Cells were incubated in the growth medium with (lane 2) or without (lane 1) 50 μM GalNAz. Cell lysates were prepared after 48 h, labeled with biotin–PEG_3–phosphine via the Staudinger ligation and analyzed by blotting with anti-biotin-HRP antibody. (D) CD73 can be specifically labeled with GalNAz. ARPE-19 cells were incubated in the presence (lane 2) or absence (lane 1) of 50 μM GalNAz. After 48 h, cells were lysed in RIPA buffer, CD73 was immunoprecipitated (IP) using the CD73 antibody, and then ligated to biotin–PEG_3–phosphine. Finally, the CD73 IP samples were analyzed by SDS–PAGE and immunoblotting with anti-biotin and anti-CD73 antibodies.

We sought a GPI-anchored protein that had been demonstrated to be robustly expressed in cells and to contain GalNAc on the GPI glycan. Furthermore, to minimize the challenges presented by the incorporation of the GalNAc analog into N- and O-linked glycan chains, we also sought a GPI-anchored model protein with a well-characterized glycosylation pattern and preferably lacking O-glycans. Based on these criteria, we chose CD73 (5′-nucleotidase) from a list of mammalian GPI-anchored proteins with characterized GPI structures. CD73 is N-glycosylated, but does not have O-linked glycans.[25,26] Additionally, the presence of HexNAc on the GPI anchor of the bovine CD73 has been reported previously.[18]

Expression of CD73 was examined in HeLa cells and the human retinal pigment epithelial cell line, ARPE-19, which can be grown either as an unpolarized monolayer, or cultured in a polarized fashion on membrane supports. These cell lines were selected based on observations that the majority of the GPI anchors from these cells detected by mass spectrometric analysis contain HexNAc attached to the first mannose residue of the core glycan (Nakayasu, E.; Vainauskas, S.; Taron, C.; Almeida, I. unpublished results). Consistent with previous reports,[27] we found that CD73 was highly expressed in ARPE-19 cells, but was virtually undetectable in HeLa crude cell lysates (Fig. 1A). Furthermore, CD73 could be efficiently released from the ARPE-19 cell surface by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) (Fig. 1B). Therefore, endogenously expressed CD73 from ARPE-19 cells was selected as a model system in which to test GalNAz incorporation into GPI anchors.

2.2. GalNAz can be incorporated into the GPI glycan of CD73

To determine if GalNAz can be metabolically incorporated into glycoproteins in APRE-19 cells, and specifically into the glycans of CD73, non-polarized ARPE-19 cells were grown in culture medium supplemented with GalNAz, after which GPI-anchored proteins were released from the cell surface with PI-PLC. Putative GalNAz-containing glycans were subsequently labeled with bio-

tin–PEG_3–phosphine via Staudinger ligation, and the biotinylated protein was subjected to western blot analysis using an anti-biotin antibody. Multiple proteins were labeled with GalNAz, suggesting that azide-containing sugar was efficiently incorporated *en masse* into multiple glycoproteins in ARPE-19 cells (Fig. 1C). For the analysis of CD73, PI-PLC released CD73 was affinity purified using an anti-CD73 antibody prior to biotin labeling and western blot analysis. Biotin was only found associated with CD73 isolated from cells that were exposed to GalNAz (Fig. 1D) confirming that GalNAz was efficiently incorporated into its glycans.

It has been reported that mammalian cells can epimerize the nucleotide sugar derived from GalNAz, uridine diphosphatidyl (UDP)-GalNAz, to UDP-GlcNAz.[28] In our study, both GalNAz and its epimerized form, UDP-GlcNAz, may be incorporated into the N-linked glycans of CD73. Because CD73 contains two different classes of appended carbohydrates (N-glycans and a GPI anchor), we sought specific evidence that GalNAz was being incorporated into the GPI moiety. We undertook two independent lines of experimentation on non-polarized cells to make this determination: (i) chemical and enzymatic treatment of glycans on CD73 and (ii) analysis of an engineered GPI-less version of CD73.

2.2.1. Chemical and enzymatic removal of glycans on CD73 demonstrates GalNAz labeling on the GPI glycan

To demonstrate that GalNAz is incorporated into the CD73 GPI glycan, we eliminated the biotin signal from azidosugars incorporated into non-GPI glycans by treating the PI-PLC-released and immunoprecipitated protein samples with protein deglycosylation mix (PDM; a commercial cocktail of enzymes including PNGase F, O-glycosidase, neuraminidase, β-galactosidase and β-N-acetylglucosaminidase) which removes both N- and O-linked glycans (Fig. 2A). PDM-treated CD73 decreased in molecular weight, consistent with the removal of N-glycans from the protein (Fig. 2B, lane 2). However, CD73 was still detected by western blotting with the anti-biotin antibody (Fig. 2B, lane 2), suggesting that biotinylated GalNAz remained associated with the protein, likely via the GPI anchor. Further treatment of PDM-deglycosylated CD73 with jack bean β-*N*-acetylhexosaminidase (β-HexNAc-ase) that liberates terminal β-linked HexNAc (Fig. 2A) completely eliminated the anti-biotin signal (Fig. 2B, lane 3). This indicates that the CD73-associated GalNAz is terminally exposed and is consistent with the notion that it is side-branched to the GPI glycan.

Lastly, we used aqueous hydrofluoric acid (HF) to cleave the phosphodiester bond and release the carbohydrate portion of the GPI from CD73 (Fig. 2A).[13] PDM-treated CD73 was immobilized by transfer onto PVDF membrane and treated with aqueous HF. Western analysis indicated that the anti-biotin signal was almost completely abolished following exposure of PDM-treated CD73 to HF (Fig. 2C, lane 2, top panel), whereas the control anti-CD73 signal showed little change following HF treatment (Fig. 2C, lane 2, lower panel). This data supports the conclusion that the GalNAz signal remaining after PDM treatment was associated with CD73 via a phosphodiester linkage, likely via the GPI anchor.

2.2.2. An engineered GPI-less CD73 molecule illustrates GalNAz labeling of the GPI anchor

Additional evidence that GalNAz was being incorporated into the GPI glycan was provided through expression of an engineered recombinant form of CD73 that lacked a GPI anchor. Two CD73 expression constructs were created that encoded: (i) a full length CD73 having its native GPI anchor (termed CD73–GPI) and (ii) a chimeric CD73 protein having its C-terminal GPI attachment signal peptide replaced with the transmembrane domain of membrane cofactor protein (MCP/CD46) (termed CD73–TMD).[29] The expressed CD73 proteins both correctly localized to the cell surface as visualized by immunostaining on transiently transfected, non-

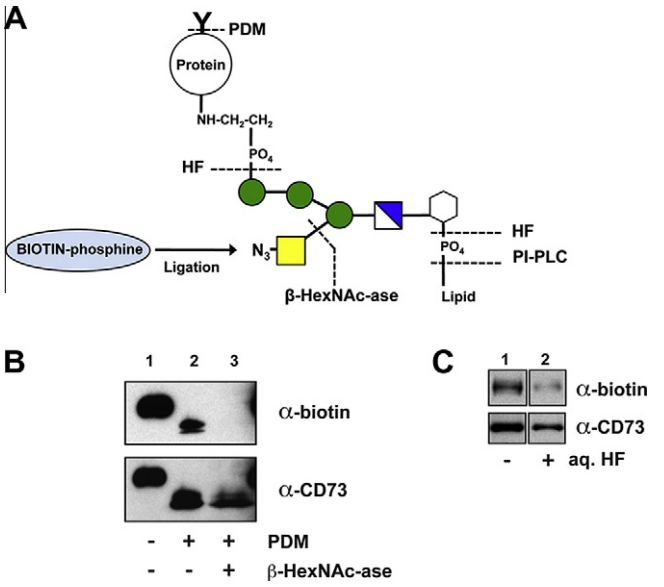

Figure 2. Demonstration of GalNAz labeling on the GPI glycan through chemical and enzymatic removal of glycans on CD73. (A) GPI anchor schematic, including sites of cleavage and the site of synthetic sugar analog incorporation and conjugation to biotin–PEG_3–phosphine. (B) Hexosaminidase-mediated removal of GalNAz to show incorporation into the GPI glycan. To verify GalNAz labeling within the GPI glycan, immunoprecipitated CD73 was either non-treated (lane 1), treated with PDM (lane 2), or treated with PDM followed by β-HexNAc-ase (lane 3) before labeling with biotin–PEG_3–phosphine. (C) Aqueous HF release of the GPI anchor results in the loss of the GalNAz signal. GalNAz-biotin-labeled CD73 was analyzed by SDS–PAGE and transferred onto PVDF membrane. Then the PVDF membrane with CD73 was incubated in the absence (lane 1) or presence (lane 2) of aqueous HF, followed by immunoblotting with anti-biotin and anti-CD73 antibodies. Glucosamine (blue/white half shaded square), mannose (green circle), and *N*-azidoacetylgalactosamine (yellow square-N3). PDM = protein deglycosylation mix), HF = aqueous hydrofluoric acid, PI-PLC = phosphatidylinositol-specific phospholipase C, β-HexNAc-ase = β-*N*-acetylhexosaminidase.

permeabilized HeLa cells (Fig. 3A), indicating that the GPI anchor of CD73 can be replaced with a transmembrane domain without detriment to the protein's folding or trafficking. Next, the CD73–GPI and CD73–TMD constructs were each individually expressed in ARPE-19 cells and subjected to GalNAz labeling. The cells were lysed with RIPA buffer, and the resulting immunoprecipitated and deglycosylated CD73 proteins were labeled with biotin–PEG_3-phosphine and analyzed by Western blot with anti-CD73 and anti-biotin antibodies. The GPI-anchored forms of endogenous and overexpressed CD73 (Fig. 3B) were detectable with both anti-CD73 and anti-biotin antibodies. Cells transfected with the CD73–TMD expression vector produced a slightly larger form of CD73 due to the presence of the appended transmembrane domain (Fig. 3, lane 3, lower panel). Importantly, this GPI-less form of CD73 did not contain biotinylated GalNAz (Fig. 3, lane 3, top panel) clearly demonstrating that GalNAz is incorporated into the GPI moiety.

While it is formally possible that GalNAz could also become incorporated into the GPI anchor as GlcNAz due to the reported epimerization of UDP-GalNAz to UDP-GlcNAz inside the cell,[28] the evidence strongly suggests that it is GalNAz. Only two aminosugars have been described to be associated with the mammalian GPI. The first is a GlcN residue that is an integral component of the core GPI glycan. GlcN is first added to the phosphatidylinositol (PI) as GlcNAc, after which its amino group is deacetylated prior to addition of the first GPI mannose. Thus, it is likely that GlcNAz incorporated at this position would either have its azide group similarly removed (also resulting in GlcN) thus becoming undetectable by Staudinger ligation with biotin–PEG_3–phosphine, or GlcNAz-PI would become a dead-end biosynthetic intermediate

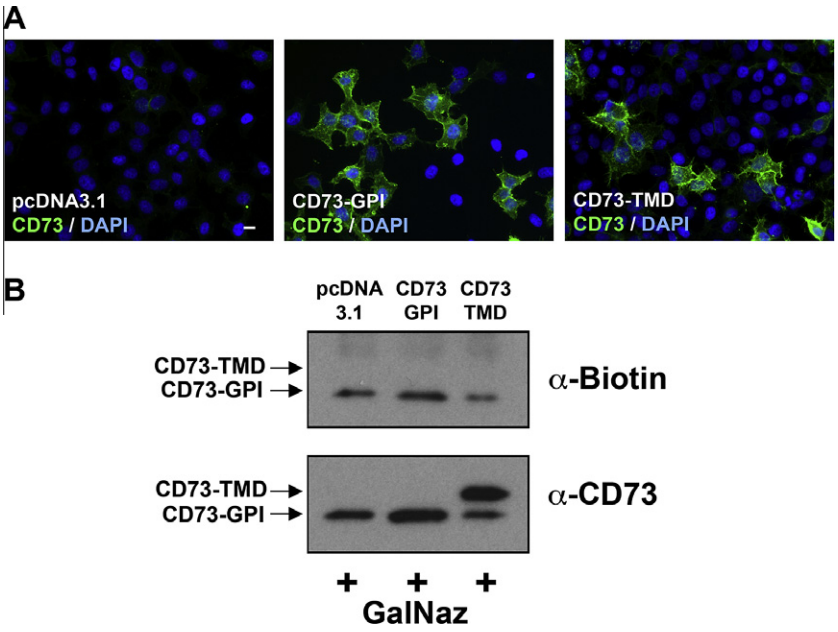

Figure 3. Biotinylated GalNAz is found on GPI-anchored CD73, but not transmembrane anchored CD73. (A) CD73–GPI and CD73–TMD correctly localized to the cell surface. HeLa cells were transfected with pcDNA3.1, CD73–GPI, and CD73–TMD plasmid constructs. Cells were immunostained under non-permeabilizing conditions with anti-CD73 and AlexaFluor488 anti-mouse IgG antibodies. (B) CD73–GPI contained biotinylated GalNAz, while CD73–TMD did not, demonstrating the specific incorporation of GalNAz into the GPI anchor. ARPE-19 cells were transfected with pcDNA3.1 (lane 1), CD73–GPI (lane 2), or CD73–TMD (lane 3) constructs and grown in the presence of GalNAz. Cells were lysed 48 h later and the immunoprecipitated CD73 proteins were deglycosylated and labeled with biotin–PEG_3–phosphine. The resulting CD73 IP samples were analyzed by SDS–PAGE and immunoblotting with anti-biotin and anti-CD73 antibodies. Scale bar = 10 μm.

that could not be further elongated or attached to proteins.[30] The second aminosugar known to be present on mammalian GPIs is a heterogeneously present GalNAc residue that is side-branched to Man-1 in a β1-4 linkage.[13,15,16] In our experiment, if this side-branching sugar was epimerized GlcNAz instead of GalNAz we would have observed a complete loss of biotin signal upon treatment with PDM mix alone, which contains a GlcNAc-specific β-*N*-acetylglucosaminidase that would remove a terminally exposed, side-branching GlcNAz. Instead, it was only upon treatment with both PMD and jack bean β-*N*-acetylhexosaminidase that we were able to achieve a nearly complete removal of the GalNAz–biotin signal, suggesting that it is terminal GalNAz, not GlcNAz that is incorporated into the GPI anchor.

Considered together, these data clearly indicate that GalNAz can be incorporated into the GPI glycan in vivo in mammalian cells, and that the sugar analog can be exploited to interrogate the presence or absence of GalNAc on individual GPI anchored proteins, such as CD73.

2.3. GalNAz is incorporated into the GPI after attachment to protein

Thus far, we have examined the ability of cells to incorporate GalNAz into the specific GPI-AP, CD73. However, while the GalNAc modification has not been previously observed on mammalian GPI precursors prior to their attachment to protein, the exact timing of GalNAc addition is unknown. Interestingly, biosynthesis of GPI intermediates containing GalNAc or Glc-GalNAc residues was previously reported in *Toxoplasma gondii*.[31] Therefore, we analyzed whether complete mammalian GPI precursors can be labeled with GalNAz prior to their attachment to protein.

HeLa cells were pulse-labeled with D-[2-^{3}H] mannose in the presence of GalNAz and then chased for 18 h with or without GalNAz. Cell lysates were subjected to Staudinger ligation with biotin–PEG_3–phosphine. Glycolipids were then extracted from the lysates and analyzed by thin layer chromatography (TLC). The mature GPI precursor H8 was the major labeled GPI accumulating in mammalian cells during metabolic radiolabeling, while H6 and H7 precursors were also detected as minor GPI species (Fig. 4, lanes 1, 3). All three detected precursors consist of a conserved core glycan (Manα1–2Manα1–6Manα1–4GlcN) linked to the 6-position of the D-*myo*-inositol ring of PI and differ only by a number of EtNP residues (1 (H6), 2 (H7), and 3 (H8)) appended to the core glycan. With increased incubation times, all of the H6 and H7 precursors were converted in the cell to H8 (Fig. 4, lanes 2, 4). If GalNAz could become incorporated into the GPI precursor, it would be expected that the mobility of the GalNAz-modified, radiolabeled GPIs would be altered relative to the non-GalNAz labeled GPIs due to the appended sugar and biotin tag. However, the mobility and relative abundance of the complete GPI precursor H8 extracted from cells grown in the presence or absence of GalNAz remained the same, indicating that GalNAz was not incorporated into GPI precursors (Fig. 4, lanes 2 and 4). Our results suggest that GalNAc modification occurs on protein-bound GPIs, but not on biosynthetic GPI intermediates or complete GPI precursors. This is in agreement with results from other studies where the GalNAc side-chain modification in higher eukaryotes was detected only on GPI-APs.[13,15–18] Thus, it is probable that side-branching GalNAc is added to the GPI post-attachment to protein, most likely in the Golgi. Our method and model system for observing GalNAz incorporation into CD73 thus provide a needed framework for ongoing investigation into the precise location and mechanism of GalNAc addition to the mammalian GPI glycan.

2.4. Detection of GalNAc-modified CD73 in polarized ARPE-19 cells

An association of GPI-APs with detergent-resistant microdomains in combination with their oligomerization[32–34] or N-glycosylation[35–37] has been implicated in the preferential localization of GPI-APs to specific plasma membrane domains in polarized cells. It is not known whether precise GPI structures, or, more specifically, the presence or absence of side-branching substituents on the GPI glycan, contribute to the polarized trafficking of GPI-APs. In

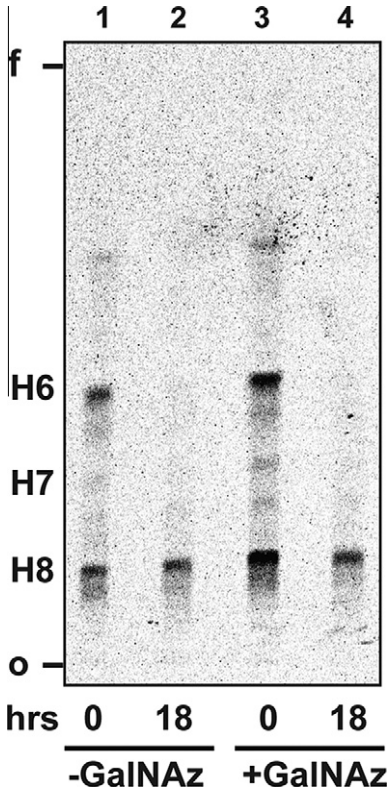

Figure 4. GalNAz does not get incorporated into GPIs prior to attachment to protein. HeLa cells were labeled with D-[2-^{3}H] mannose, then chased for 18 h in the presence (lane 4) or absence (lane 2) of 50 μM GalNAz. Cell lysates were prepared and followed by Staudinger ligation with biotin–PEG_3–phosphine. Non-protein associated GPI precursors were isolated by organic extraction and analyzed by TLC. O = origin, f = solvent front.

the present study, we utilized our GalNAz labeling method to conduct the first experiment aimed at comparing a structural feature of a GPI derived from the same protein isolated from two different membrane surfaces of polarized epithelial cells.

In several polarized epithelial cell lines, CD73 has been observed primarily on the apical membrane domain, but with lesser amounts also being present on the basolateral domain.[27,38] Using confocal microscopy, we confirmed that in polarized ARPE-19 cells, CD73 predominantly localized to the apical domain (Fig. 5A, asterisks). Some CD73 expression was also observed on the basolateral domain as indicated by partial co-localization with β-catenin, a marker of the basolateral compartment (Fig. 5A, arrows).[39]

To query the presence or absence of GalNAc on CD73 derived from both the apical and basolateral faces of polarized ARPE-19 cells, the cells were labeled with GalNAz for 48 h and CD73 was selectively released from each membrane by incubation with PI-PLC in the top (apical) and bottom (basolateral) chambers of the transwell filter. To ensure tight junction integrity was maintained during PI-PLC treatment, we developed a novel method using a highly sensitive luciferase-based assay as a means to monitor protein diffusion across the cell monolayer. Two luciferases of different sizes (20 kDa *Gaussia princeps* luciferase and 75 kDa *Cypridina noctiluca* luciferase) were added to the apical chamber concurrent with PI-PLC, and the diffusion of each enzyme to the basolateral chamber was measured over time (Fig. 4B). The experiments demonstrated no increase in enzyme diffusion during the PI-PLC incubation period, whereas a disruption of tight junctions (which are critical to maintaining barrier integrity) with EDTA and Triton X-100[40] led to a significant increase in epithelial permeability (Fig. 5B). This indicates that the tight junctions in the ARPE-19 polarized monolayer were maintained throughout PI-PLC treatment and ensures the integrity of our apical and basolateral PI-PLC fractions.

Following PI-PLC release, CD73 was collected by immunoprecipitation, PDM-treated to remove N-glycans, and incorporated GalNAz was labeled with biotin–PEG_3–phosphine. GalNAz was detected on both apically- and basolaterally-derived CD73 (Fig. 5C), though the signal for both CD73 and biotin was significantly lower on samples from the basolateral compartment, consistent with the predominant apical localization of CD73. This observation suggests that (i) GalNAc is added to the GPI glycan upstream of sorting of apically- or basolaterally-bound GPI-APs and (ii) that the presence or absence of side-branching GalNAc does not alone discriminate apical versus basolateral targeting of GPI-APs in human ARPE-19 cells. Furthermore, this experiment provides an example of the utility of sugar nucleotide analog incorporation into the GPI moiety as a means to probe for specific glycan modifications on two different surfaces of living cells.

3. Conclusions

In this study, we established a method to easily identify the presence of a GalNAc residue side-branched to the GPI glycan moiety of mammalian GPI anchored proteins. We utilized a metabolic labeling approach with tetraacetylated *N*-azidoacetylgalactosamine (GalNAz), an unnatural analog of GalNAc that crosses the cell membrane and is converted into UDP-GalNAz for incorporation into various cellular glycans. The incorporated glycan analog can be further modified by chemical coupling to a variety of phosphine-containing compounds (biotin, peptide epitopes, fluorophors) that permit its detection. We showed that mammalian cells are capable of metabolically incorporating a side-branching GalNAz in place of GalNAc in the GPI glycan moiety. In vivo labeling with GalNAz not only directly detects GPI-anchored proteins containing a GalNAc-modified glycolipid, but can also be used for specific enrichment of these GPI-APs through the immobilization of GalNAz containing GPI proteins via the azide group.

This approach will be useful for probing the structures of low abundance GPIs that are difficult to study using conventional mass spectrometry and NMR based techniques. Furthermore, this method may also aid in the identification of the glycosyltransferases responsible for the addition of the GalNAc side modification in a heterogeneous manner. We have also exemplified the utility of the method by showing that GalNAz is incorporated into the GPI moiety of CD73 separately isolated from different membrane domains of polarized epithelial cells. This highlights perhaps the most interesting application of this technique; the ability to examine the presence of specific GPI molecules and/or side branch modifications in living cells. This could be applied to such analyses as the investigation of GPI-APs on different membrane compartments of polarized cells (as demonstrated here) or the examination of GPI-APs on the cell surface during developmental or drug time course studies. The ability to specifically and non-destructively label and isolate the GalNAc-containing GPI anchors should therefore prove to be a useful tool for the analysis of GPI anchor structure, and even more importantly, may allow us to begin to elucidate the functional roles of GPI side branch modifications.

4. Experimental

4.1. ARPE-19 cell culture

ARPE-19 cells were obtained from American Type Culture Collection (ATCC). ARPE-19 non-polarized cultures were maintained

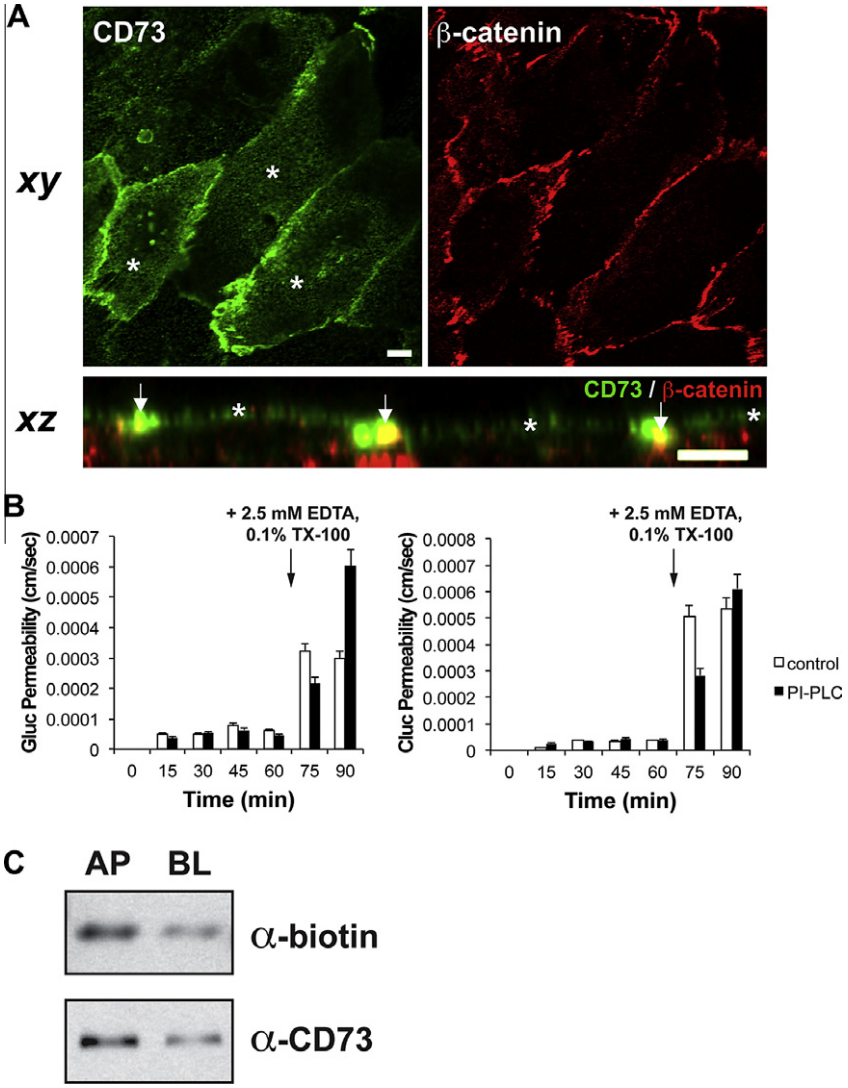

Figure 5. Examination of the GalNAc modification on the CD73 GPI anchor in live polarized cells. (A) CD73 preferentially localized to the apical membrane in polarized ARPE-19 cells. Cells were grown on filter supports and stained by indirect immunofluorescence followed by confocal microscopy. β-catenin staining was used as a marker for basolateral membrane expression. Note the expression of CD73 on the apical surface (asterisks) and the partial co-localization with β-catenin (arrows) on the basolateral surface. (B). The polarized cell monolayers were fully formed, as revealed by determining the permeability of the monolayer over time by measuring the diffusion of *Gaussia princeps* and *Cypridina noctiluca* luciferases (Gluc and Cluc, respectively) from the apical to the basolateral compartments (see Section 4). Only upon dissociation of the tight junctions by addition of EDTA and Triton X-100 was significant luciferase permeability observed. (C) CD73 containing a GalNAz-labeled GPI anchor was detected on both apical (AP) and basolateral (BL) membranes in polarized cells. ARPE-19 cells were grown on filter supports in the presence of GalNAz and then the live cells were treated on each membrane surface with PI-PLC. GalNAz-labeled CD73 released from apical and basal membranes was collected and immunoprecipitated separately. Deglycosylated CD73 was labeled with biotin–PEG_3–phosphine probe and analyzed by immunoblotting with anti-biotin and anti-CD73 antibodies. Scale bar = 5 μm.

in Dulbecco's modified Eagle's medium (DMEM): Ham's F12 medium with HEPES buffer containing 10% (v/v) fetal bovine serum (FBS; Thermo Scientific Hyclone), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were sub-cultured once a week. All cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C.

For polarized cell cultures, the cells were cultured on filters by seeding at a density of 1.6×10^5 cells/cm^2 on 75 mm diameter, 0.4 μm pore size, polycarbonate or 24 mm diameter, 0.4 μm pore size, polyester Transwell permeable supports (Costar). The cells were grown in DMEM/F12 medium (see above) with 10% FBS for 1–2 days, after which the medium was replaced with DMEM/F12 medium containing low serum (1%) and cells cultured for 3–4 weeks.

Trans epithelial electrical resistance (TEER) was used to monitor epithelial barrier formation and polarization using an EVOM with a STX2 electrode (World Precision Instruments). A plateau in TEER (35–45 Ω cm^2) was reached in 8–10 days and, thereafter, it remained essentially unchanged. Polarized cells were used for experiments after culturing for 3–4 weeks.

Diffusion of 0.2 ng of recombinant *Gaussia princeps* luciferase (Gluc, 20 kDa) and 0.1 ng of recombinant *Cypridina noctiluca* luciferase (Cluc, ~75 kDa) from the apical to basolateral chamber was used to measure the intactness of the cell monolayer during PI-PLC treatment. Every 15 min for 1.5 h, 30 μL aliquots were collected from the basolateral and apical chambers. Gluc and Cluc activities were measured in 5 μL of sample using BioLux *Gaussia* Luciferase and BioLux *Cypridina* Luciferase Assay Kits (New England Biolabs). Luminescence was read with a Berthold Centro LB 960 luminometer (Berthold Technologies). Diffusive permeability rates (P_o) in centimeters per second were calculated as previously described: [41,42]

$$P_o = [(\Delta L_B/\Delta t)V_B]/(L_A A)$$

where P_o is diffusive permeability (cm/s), ΔL_B is the change in basolateral luminescence, L_A is apical luminescence, Δt is the change in time, V_B is basolateral chamber volume (cm^3), and A is filter surface area (cm^2).

4.2. Plasmids

cDNA was generated via the SuperScriptIII reverse transcription kit (Invitrogen) from 2.5 μg total RNA isolated from ARPE-19 cells (RNeasy RNA extraction kit, Qiagen). To generate the CD73-GPI construct, PCR amplification of the full length gene was performed with Phusion DNA polymerase (New England Biolabs) using the following primers: CD73 Forward 5′-CATGAATTCCACAGCCATGTGT CCCCGAGCC-3′ and CD73 Reverse 5′-CATGTCTAGACTATTGGTATA AAACAAAGATCACTGCC-3′. The resulting PCR fragment was then cloned into the EcoRI-XbaI sites of pcDNA3.1(+) (Invitrogen). To generate the CD73–TMD construct, a knitting PCR approach was used. The CD73 gene upstream from the GPI attachment site was amplified using the following primers: CD73 Forward and CD73 Reverse-GPI 5′-CATGCTCGAGAAACTTGATCCGACCTTCAACTGCTG-3′. The C-terminal domain of MCP, including the transmembrane domain, was amplified from ARPE-19 cDNA with the following primers MCP Forward 5′-GTTGAAGGTCGGATCAAGTTTCTCGA-GAGGCCTACTTACAAGCCTCCAGTCTC-3’ and MCP Reverse 5’-CAT-GTCTAGAGTGGCATATTCAGCTCCACCATCTG-3′. The CD73–MCP fusion was then generated using the amplified CD73 and MCP fragments as templates and the CD73 Forward and MCP Reverse primers. The resulting PCR fragment was subsequently cloned into the EcoRI-XbaI sites of pcDNA3.1(+).

4.3. Incorporation of GalNAz into CD73

ARPE-19 cells (4–5 × 10^6 cells per 100 mm plate) were grown for 48 h in DMEM/F12 medium containing 10% (v/v) FBS with or without 50 μM tetraacetylated *N*-azidoacetylgalactosamine (GalNAz, Invitrogen). After two days, the medium was replaced with 5 mL fresh DMEM/F12 without serum and cells were treated with recombinant *Bacillus cereus* PI-PLC (3 U/mL final concentration) for 45 min at 37 °C. The recombinant *Bacillus cereus* PI-PLC was expressed in *Escherichia coli* and purified as described elsewhere.[43] Spent culture medium was harvested after PI-PLC treatment and centrifuged for 10 min at 3000*g* to remove any cell debris. The supernatant was incubated with 2 μg CD73 antibody (mouse monoclonal, Santa Cruz Biotechnology) and 50 μL Protein G magnetic beads (New England Biolabs) for 16 h at 4 °C. CD73 was eluted from the beads with 50 μL of 1% SDS/100 mM Tris·HCl, pH 7.5. A 20 μL aliquot of eluate (CD73 IP) was treated with protein deglycosylation mix (New England Biolabs) for 4 h as recommended by the manufacturer. Staudinger ligation was performed by reacting 25 μL of deglycosylated CD73 IP samples with 2 μL of 10 mM biotin–PEG_3–phosphine (Pierce Thermo Scientific) for 4 h at 37 °C. Then CD73 IP samples were run on a SDS–PAGE, transferred onto nitrocellulose membrane and blotted with anti-biotin-HRP (Cell Signaling Technologies), or anti-CD73 and anti-mouse IgG HRP-conjugated light chain specific (Jackson Immunoresearch) antibodies. The membrane was developed using Super-Signal West Pico or Dura Chemiluminescent Substrate (Pierce Thermo Scientific).

To study GalNAz incorporation into recombinant CD73 constructs, 1 μg of pcDNA3.1(+), CD73–GPI or CD73–TMD DNA was transfected into one well of a 6-well dish of ARPE-19 cells using Lipofectamine2000 (Invitrogen). Growth media was changed 6 h post-transfection, and GalNAz (Invitrogen) was added to the medium to a final concentration of 50 μM 24 h after transfection. Cells were harvested 72 h post-transfection by scraping in cold PBS and pelleting at 4 °C for 10 min at 1500 rpm. The cell pellet was resuspended in 100 μL PBS + 2 U PI-PLC and incubated for 45 min at 37 °C. Subsequently, 900 μL RIPA buffer containing protease inhibitors was added. Cells were vortexed and incubated on ice for 30 min, then spun down for 20 min at 4 °C at 14000 rpm. The supernatant was precleared with 25 μL protein G magnetic beads for 1 h at 4 °C, then was subjected to immunoprecipitation with CD73 antibody, followed by deglycosylation, labeling with biotin–PEG_3–phosphine via the Staudinger ligation, and detection of CD73 by western blotting as described above.

4.4. Treatment with β-*N*-acetylhexosaminidase and aqueous HF

CD73 IP samples were treated with 0.01 U of jack bean β-*N*-acetylhexosaminidase (Sigma–Aldrich) for 20 h at 25 °C in 50 mM sodium citrate, pH 4.5. CD73 samples transferred onto PVDF membrane were treated with 48% aqueous hydrofluoric acid for 60 h on ice.

4.5. Immunofluorescence

For immunohistochemistry, HeLa cells cultured in Nunc Lab Tek 8-well chamber slides (Thermo Scientific) were transfected with pcDNA3.1, CD73–GPI and CD73–TMD plasmid constructs using Lipofectamine2000. Cells were rinsed once with PBS and fixed for 15 min in 4% PFA at room temperature 48 h after transfection. For staining, all steps were performed at room temperature. Following fixation, cells were washed with PBS, then blocked in PBS containing 5% BSA (w/v) for 15 min, and then incubated with anti-CD73 antibody for 1 h diluted 1:100 in PBS containing 1% BSA (w/v). Cells were washed with PBS and incubated for 1 h AlexaFluor 488 anti-mouse IgG (Invitrogen) diluted 1:500 in PBS containing 1% BSA (w/v). Cells were washed with PBS, DAPI (Sigma–Aldrich) stained, and mounted with ProLong Gold Antifade mounting media (Invitrogen).

ARPE-19 cells were maintained in culture for 3–4 weeks on filters as mentioned above. Cells were washed once with DPBS containing calcium chloride and magnesium chloride (Invitrogen), and then fixed for 15 min in PBS containing 4% PFA (w/v). Cells were washed in PBS, quenched for 15 min in PBS with 75 mM ammonium chloride, 25 mM glycine, and the membrane filters were cut from the plastic inserts and placed into 24-well dishes for staining. Membranes were blocked in PBS containing 5% BSA (w/v) for 1 h at room temperature, and then incubated for 16 h at 4 °C in mouse anti-CD73 (Santa Cruz Biotechnology) primary antibody diluted in PBS containing 1% BSA (w/v). Filters were washed with PBS and then incubated with goat anti-mouse IgG AlexaFluor 488 in PBS containing 1% BSA (w/v) for 1 h. Filters were washed again with PBS and post-fixed with PBS containing 4% PFA (w/v) for 15 min at rt. Filters were washed with PBS, then blocked and permeabilized with PBS containing 5% BSA (w/v), 0.3% Triton X-100 (v/v) for 30 min. Cells were then incubated with rabbit anti-β-catenin (Cell Signaling Technologies) primary antibodies for 1 h at rt. Filters were washed in PBS, then incubated in anti-rabbit IgG 568 secondary antibodies (Invitrogen) in PBS containing 1% BSA (w/v) for 1 h. After washing in PBS, cells were DAPI stained and filters mounted onto glass slides with ProLong Gold Antifade mounting media. Images were acquired on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Inc.) with LSM software and analyzed using ImageJ and Adobe Photoshop CS4.

4.6. Labeling of HeLa cells with [2-^{3}H]mannose; extraction and analysis of radiolabeled GPIs

HeLa (~1 × 10^7) cells were labeled with D-[2-^{3}H]mannose and radiolabeled GPIs were extracted with chloroform–methanol–water (10:10:3, by volume), desalted by partitioning between

n-butanol and water, and analyzed by TLC as described previously.[44,45] The chromatograms were visualized and analyzed using a Typhoon PhosphorImager and ImageQuant image analysis software (GE Healthcare Biosciences).

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