Various *N*-glycoforms differentially upregulate E-NTPDase activity of the NTPDase3/CD39L3 ecto-enzymatic domain

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Abstract The GDA1/CD39 ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) superfamily is a group of eight heavily glycosylated ecto-enzymes that hydrolyze extracellular nucleosides di- and tri-phosphates in the presence of divalent cations, to generate the monophosphate derivatives. This catalytic process differentially regulates a complex array of purinergic signaling responses. NTPDase3/CD39L3 is dominantly expressed in pancreatic islet cells, where it may regulate insulin secretion, and has seven N-linked glycosylation sites with four close to five highly conserved domains called “apyrase conserved regions” (ACRs). In a manner similar to CD39, NTPDase3/CD39L3 uses ATP as its preferential substrate and also possesses significant activities toward other triphosphate and diphosphate nucleosides. To understand the mechanism of the ecto-NTPDase activity and substrate specificity, potentially impacted by *N*-glycans, we have generated soluble enzymatic domains of NTPDase3/CD39L3 in human embryonic kidney cells with four different glycan modifications. These include mannose_{5–9} glycans with kifunesine treatment, single GlcNAc-Asn by treatment with EndoH, de-glycosylated form by treatment with PNGaseF, and wild-type glycans. Our functional data indicate that the non-glycosylated NTPDase3/CD39L3 ecto-enzymatic domain retains activity, but that *N*-glycan attachments, such as the GlcNAc-Asn, substantially up-regulate specific NTPDase activity by 2–20 fold. Both the

V_{max} and the K_m on di- or tri-phosphate nucleosides are substantially and differentially altered by the glycan attachments. Structural modeling analysis based on putative structures derived from bacterial-originated CD39 domain proteins suggests that *N*-glycan modifications at Asn149 next to ACR2 and/or Asn454, N-terminal to ACR5 have critical roles in regulating the catalytic pocket of NTPDase3/CD39L3. Our data provide both new insights into the enzymatic mechanisms of NTPDase family members and further evidence that *N*-glycans directly modulate functional ectonucleotidase activities.

Keywords Glycosylation · CD39 · CD39L3 · ATP

Introduction

Most of the proteins of the human body are glycosylated, in that sugar structures are attached to parts of the protein through either the amino acids Asn (N-linked) or Thr/Ser (O-linked) [1, 2]. *N*-glycosylation occurs post-translationally requiring the sequon of Asn-X-Ser/Thr in the compartment of endoplasmic reticulum (ER) [3, 4] and is completed in the Golgi apparatus [5]. The pre-synthesized core units of 14 saccharide (Glc₃Man₉GlcNAc₂) structures are added en bloc to the Asn residues and are subsequently modified by a series of glycosidases and glucosidases in the ER. In the Golgi, these glycan structures are further processed by the actions of mannosidases and glycotransferases for trimming and branching. Then extensive sugar additions, with fucose, galactoses, GlcNAc, sialic acids, or sulfate, take place mostly in the *trans*-Golgi to add terminal structures to the ends of the *N*-glycans to form mature complex glycan structures.

The GDA1/CD39 protein family is comprised of highly glycosylated proteins [6, 7]. These are cell surface-localized nucleotide-metabolizing enzymes called ecto-nucleoside

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triphosphate diphosphohydrolases (E-NTPDases) [8]. These ecto-enzymes hydrolyze extracellular di- and triphosphates nucleotides in the presence of divalent cations and differentially regulate a complex array of purinergic signaling responses mediated via two purinergic receptor families [9–11]. Eight members of E-NTPDase families have been cloned and functionally characterized, including plasma membrane-bounded CD39 protein/NTPDase1, CD39L1/NTPDase2, CD39L3/NTPDase3, NTPDase8, and four intracellular organelle localized NTPDase 4, 5, 6, and 7.

While these proteins all share four [12] to five [13] highly conserved regions named apyrase conserved regions (ACRs), there are several important differences between them. Firstly, these ecto-nucleotidases are different with regard to their catalytic property. NTPDase1 and NTPDase 3 are considered to hydrolyze ATP and ADP well, whereas NTPDase3 (and 8) has ATP as a preferential substrate. NTPDase 2 hydrolyzes only ATP [14], and NTPDase 4, 5, and 6 have higher activity toward diphosphates [15–17]. Secondly, NTPDase 1, 2, 3, 4, 7, and 8 have two transmembrane domains anchoring the catalytic domain to the cell surface, while NTPDase 5 and 6 are secreted enzymes, allowing more freedom of motion for the catalytic domain. Transmembrane domains have long been proposed to regulate substrate specificities and hydrolysis mechanisms of NTPDases [18–20]. Thirdly, *N*-glycosylation site arrangements are very different [21]. Not only are there different numbers of sites but also the placement of the sites relative to the apyrase conserved regions is different as well. For example, four out of seven glycosylation sites in NTPDase 3 are near the ACR regions, whereas NTPDase 4 and 5 have no glycosylation sites approximate to ACR regions.

N-glycans have been confirmed to have many important functions, such as aiding in forming the tertiary structure of proteins, specifically in the folding part of the process, and playing a role in protein interactions. *N*-glycan modification has been shown to be critical for folding and surface expression of CD39 [21–23], albeit not for NTPDase 5 and 6 [16]. *N*-glycan modification is also required for NTPDase 2 for trafficking and folding [24]; mutating Asn443 in ACR5 retains the protein in ER. NTPDase3/CD39L3, the dominantly expressed NTPDase in pancreatic islet cells [25, 26], requires *N*-glycan for its folding and activity [27]. Mutating the highly conserved Asn81 N-linked site across all cell surface membrane NTPDases retains the cell surface expression, while these proteins exhibit significant loss of NTPDase activity [28]. Terminal glycosylation in Golgi is not essential for the ecto-enzymatic activity [29]. However, despite lacking *N*-glycans, bacteria-originated inclusion body refolded NTPDase 1–3 are also found to be active [30], implying that *N*-glycan might be only essential for correct protein folding in ER lumen. This notion is supported by the data that CD39 enzymatic domain retains full activity even when *N*-glycans are removed by glycopeptidase F (PNGaseF) from CHO-Lec1-

produced high mannose materials under native conditions [23]. The differences in the placement patterns of *N*-glycosylation sites adjacent to and related with ACRs in both NTPDase3/CD39L3 and NTPDase1/CD39 are established. These have prompted us to investigate whether or not NTPDase3/CD39L3 devoid of *N*-glycans under physiological conditions also retains NTPDase activity and whether or not the various glycoforms possess differences in terms of activity and substrate specificity.

In this study, we have generated a mannose_{5–9} glycan form of the NTPDase3/CD39L3 soluble enzymatic domains by adding glycosidase inhibitor kifunesine during HEK293 transfection [31]. Since the mannose_{5–9} glycan structure is known to be sensitive to the digestion of glycosidase enzymes under non-denaturing conditions [32], we have generated a glycoform of NTPDase3/CD39L3 in which all parts of the glycan structures were removed except for a single sugar GlcNAc after EndoH digestion. A fully non-glycosylated form of NTPDase3/CD39L3 was also produced by the treatment of PNGaseF, which completely removes all sugars. Along with the wild-type glycans without any treatment and serving as a control for the experiment, the four forms of NTPDase3/CD39L3 were studied to determine specific activities toward different concentrations of ATP, ADP, GTP, and GDP. The data indicate that non-glycosylated NTPDase3/CD39L3 retains activity, consistent with the result of *Escherichia coli* inclusion body refolded materials [30]. Importantly, the single-GlcNAc attachment could upregulate NTPDase activity by 2–20 folds. Both *V*_{max} and *K*_m on di- or tri-phosphate nucleotides were impacted by glycan attachment. Based on published structure from bacterial-produced CD39 domain protein [33], structural modeling analysis reveals that *N*-glycan modifications at Asn149 next to ACR2 and/or Asn454 N-terminal adjacent to ACR5 might impact the catalytic pocket of CD39L3. Our data provide not only new insights into enzymatic mechanisms of NTPDase family members impacted upon by *N*-glycans but also provide a feasible strategy for future studies of crystal structural analyses in NTPDase3/CD39L3.

Results

Producing three forms of the NTPDase3/CD39L3 extracellular ecto-enzymatic domain

Rat NTPDase3/CD39L3 extracellular ecto-enzymatic domain (ECD) refolded from *E. coli* inclusion body [30] lacks *N*-glycans. To generate soluble form of NTPDase3/CD39L3 with full *N*-glycan modification, plasmid DNA encoding extracellular domain (Q44-P485-GGHis6) of rat NTPDase3/CD39L3 fused to a signal sequence (Fig. 1a) was transfected into HEK293 cells.

Fig. 1 Producing three forms of NTPDase3/CD39L3 extracellular enzymatic domain. **a** Gp1b-rat-NTPDase3/CD39L3) extracellular enzymatic domain (Q44-P485-GGHis6). **b** Producing three forms of NTPDase3/CD39L3 extracellular domain. The open box represents ACR domains, and the lines represent cysteine residues

Secreted NTPDase3/CD39L3ECD could be purified by a Nickel-NTA column from conditioned medium. To generate NTPDase3/CD39L3ECD with mannose₅₋₉ glycan attachment (Fig. 1b), glycosidase inhibitor kifunesine was added the cell culture as described [31]. It is known that mannose₅₋₉ glycan is sensitive to glycosidase treatment under native condition [32]; therefore, purified mannose₅₋₉ NTPDase3/CD39L3ECD was treated either with EndoH to produce GlcNAc-Asn attached protein, or with PNGaseF to generate non-glycosylated protein (Fig. 1b). Indeed, as shown in Fig. 2, PNGaseF treatment resulted in a drastic molecule shift and produced NTPDase3/CD39L3ECD species with molecular weight (MW) without glycosylation (lane 3, ~ 51 kDa vs lane 1, ~ 65 kDa). This molecular weight is consistent with the expected 50.3 kDa MW of aglycosylated species. The PNGaseF-treated bands contain a doublet which could be due to additional post-translational modifications, such as O-linked glycan or N-terminal proteolytic cleavage. The EndoH treatment also produced similar species with expected molecular weight whereas about 55% of the total protein was not significantly affected by the treatment. Kifunesine was added 4 h post-transfection. This result indicates that about half of NTPDase3/CD39L3ECD with mannose₅₋₉ are sensitive to EndoH treatment for removing most of the sugars, other than GlcNAc. Longer incubation times or higher levels of EndoH enzymes did not increase the amount of expected protein derivative species. The high mannose glycans in undigested materials could contain some residual terminal sugars such as GlcNAc.

Neither PNGaseF treatment nor EndoH treatment changed the monomeric state of NTPDase3/CD39L3ECD

The EndoH and PNGaseF enzymes remove glycan structures from NTPDase3/CD39L3ECD, which could potentially

impact the monomeric state of the protein and cause protein aggregation. To test the possibility, protein samples treated or mock treated with EndoH or PNGaseF were analyzed by size exclusion column (SEC).

As shown in Fig. 3, EndoH treatment or PNGaseF treatment did not cause the peak of NTPDase3/CD39L3ECD to shift left in comparison with mock treated samples. Instead, the protein peak was shifted to right due to its loss of glycan structure.

Fig. 2 PNGaseF treatment completely removed all glycans and EndoH treatment resulted in nearly half of the single GlcNAc sugar substitution. Secreted NTPDase3/CD39L3ECD purified from conditioned medium cultured with kifunesine was digested with PNGaseF or EndoH, as described in “Materials and methods.” Two micrograms of protein samples were analyzed by SDS-PAGE and stained with Coomassie blue

Fig. 3 PNGaseF treatment does not change monomeric state of NTPDase3/CD39L3ECD and EndoH treatment did not change monomeric state of NTPDase3/CD39L3ECD. PNGaseF-treated or EndoH-treated NTPDase3/CD39L3ECD samples, along with PNGaseF

or EndoH, were analyzed by Superdex-200 size-exclusion column in PBS buffer. Gel filtration standard components (Bio-Rad) are vitamin B₁₂ (1.36 kDa), myoglobin (17 kDa), ovalbumin (44 kDa), γ -globulin (158 kDa), and thyroglobulin (670 kDa)

These results show that there is no protein aggregation in the NTPDase3/CD39L3ECD after the treatment of EndoH or PNGaseF and these proteins retain the monomeric state.

PNGaseF-treated NTPDase3/CD39L3ECD is significantly less active than those in different glycoforms when measured in a divalent cation-dependent NTPDase activity

To determine the activity of these NTPDase3/CD39L3ECD proteins in different glycoforms with presence of PNGaseF or EndoH, divalent cation-dependent ATPDase activity was measured. Calcium and magnesium ions were used to see whether the difference in cations impacted this. Each form of the NTPDase3/CD39L3ECD protein was added to the reaction mixtures containing one of the divalent cations and either ATP or ADP. Each reaction was incubated at 37 °C for 20 min, and solutions of malachite green and sodium citrate were added. Phosphate being released to the solution was measured by A660. Based on the phosphate concentration, the efficacy of the enzyme could be determined, which would form the trend for the effect of the glycan structures.

As shown in Fig. 4, all four forms of Rat NTPDase3/CD39L3 ECD had better activity in the presence of Ca²⁺ than Mg²⁺. PNGaseF-treated unglycosylated protein has a

2-fold higher ATPase activity than ADPase with magnesium ion which is similar to reported data from bacterial inclusion body refolded materials [30], but 5-fold higher ATPase activity than ADPase with calcium ion. For the three glycan-modified proteins, their calcium-dependent ATPase activities were roughly 2-fold higher than that of PNGaseF-treated samples whereas their magnesium-dependent ATPase activities were 12–20-fold higher. For the calcium-dependent ADPase activity, the increase approximated between 5 and 7 folds. For magnesium-dependent ADPase, the increase approximated 8-fold. The glycan-modified proteins exhibited similar activities for ATPase and ADPase, whereas ratios of ATPase and ADPase activity for PNGaseF-treated proteins approximated between two and five.

To further compare the activity toward other nucleotide substrates, GTP and GDP assays were performed. As shown in Fig. 5, GTPDase activity is higher in the presence of Ca²⁺ than Mg²⁺. Similarly, PNGaseF-treated unglycosylated NTPDase3/CD39L3ECD approximated 4-fold less active than those with different glycans for calcium-dependent GTPase activities: 10–16 folds less for magnesium-dependent GTPase activities, 4-fold less for calcium-dependent GDPase activities, and 7–14 folds less for magnesium-dependent GDPase activities. Tables 1 and 2 summarize the V_{max} and

Fig. 4 PNGaseF-treated NTPDase3/CD39L3 is significantly less active than those in different glycoforms, when measured in a divalent cation-dependent ATPDase activity. PNGaseF-treated, EndoH-treated, or mock-

treated NTPDase3/CD39L3ECD proteins were measured for ATPDase activity as described in “Materials and methods.” The error bars at each data point were calculated from two to five independent experiments

K_m of ATPDase and GTPDase activity in the presence of Ca^{2+} or Mg^{2+} for four glycoform materials of NTPDase3/CD39L3ECD. These data together have demonstrated that *N*-glycan modification could upregulate the NTPDase activity of NTPDase3/CD39L3ECD protein.

An inhibitor of E-NTPDase, POM6, differentially inhibits ATPDase activities of NTPDase3/CD39L3ECD between proteins with treated glycans and those with native, unmodified glycans

To further study the activity of NTPDase3/CD39L3ECD in different glycol-isoforms, polyoxometalates (POM6) [34], an inhibitor of E-NTPDase, was added to the ATPDase assay. As shown in Fig. 6a, the ATPase activity of NTPDase3/CD39L3ECD with wild-type glycans is less sensitive to the inhibition than EndoH-treated, PNGaseF-treated, and man5–9 proteins. Similarly for ATPase activity, NTPDase3/CD39L3ECD with wild-type glycans is less sensitive to POM6 inhibition than other forms.

ATPDase activities of EndoH-treated samples are significantly more sensitive to POM6 than that of the native protein ($p < 0.05$), whereas ADPDase activities of the PNGaseF-treated samples are more POM6-sensitive than that of the unmodified proteins is also statistically significant ($p < 0.05$).

Structural modeling analysis reveals that *N*-glycan modifications at Asn149 next to ACR2 and/or Asn454 N-terminal to ACR5 might impact folding of the catalytic pocket of NTPDase3/CD39L3

Crystallization and structure determination of rat NTPDase2/CD39L1ECD [35] and rat NTPDase1/CD39ECD [33] have been reported using protein refolded from bacterial inclusion bodies. No crystallization or structure determination has yet been obtained for NTPDase3/CD39L3ECD. To explore the potential molecular mechanisms of the *N*-glycan's upregulation on NTPDase3/CD39L3ECD activity, we did perform structural modeling analysis using the structure of NTPDase1/CD39ECD (PDB accession number 3ZX2).

As shown in Fig. 7, Asn residues of *N*-linked sites are in yellow and are all surface exposed, with Asn458 being part of the ACR5 helix. Mutating this residue in NTPDase2/CD39L1 causes protein retention in ER, indicating its critical role in protein folding. Interestingly, the corresponding position of the NTPDase3/CD39L3's Asn149 (pink) in the model is located in the loop connecting ACR2 (red) and a helix leading to ACR3 helix (red). *N*-glycan modification in this position could significantly affect the interdomain active site cleft clustered by ACRs.

Fig. 5 PNGaseF-treated NTPDase3/CD39L3 is significantly less active than those in glycoforms when measured in a divalent cation-dependent GTPDase activity. PNGaseF-treated, EndoH-treated, or mock-treated

NTPDase3/CD39L3 ECD proteins were measured for GTPDase activity as described in “Materials and methods.” The error bars at each data point were calculated from two to five independent experiments

The corresponding position of the NTPDase3/CD39L3’s Asn454 (pink), which is different from Asn458 of NTPDase1/CD39 and Asn443 of NTPDase2/CD39L1 on

ACR5, is at the N-terminal of ACR5. As seen in the model, *N*-glycan at this position could potentially impact entry of substrates into the catalytic cleft. These structural modeling

Table 1 Vmax and Km of NTPDase3/CD39L3ECD in different glycoforms for ATPDase activities

Substrates	CD39L3ECD	Vmax (μmol/min/mg)	Km (mM)
ATP (CaCl ₂)	Man5–9	137	0.319
ATP (CaCl ₂)	EndoH	178.8	0.473
ATP (CaCl ₂)	PNGaseF	86.2	0.954
ATP (CaCl ₂)	Wild type	162.3	0.521
ADP (CaCl ₂)	Man5–9	114.0	0.530
ADP (CaCl ₂)	EndoH	109.4	0.598
ADP (CaCl ₂)	PNGaseF	17.3	0.135
ADP (CaCl ₂)	Wild type	93.7	0.432
ATP (MgCl ₂)	Man5–9	168.3	0.826
ATP (MgCl ₂)	EndoH	206.9	1.307
ATP (MgCl ₂)	PNGaseF	11.0	0.419
ATP (MgCl ₂)	Wild type	136.5	0.745
ADP (MgCl ₂)	Man5–9	47.5	0.386
ADP (MgCl ₂)	EndoH	47.3	0.276
ADP (MgCl ₂)	PNGaseF	5.1	0.147
ADP (MgCl ₂)	Wild type	43.5	0.386

Table 2 Vmax and Km of NTPDase3/CD39L3ECD in different glycoforms for GTPDase activities

Substrates	CD39L3ECD	Vmax (μmol/min/mg)	Km (mM)
GTP (CaCl ₂)	Man5–9	128.2	0.346
GTP (CaCl ₂)	EndoH	131.7	0.323
GTP (CaCl ₂)	PNGaseF	30.3	0.095
GTP (CaCl ₂)	Wild type	135.0	0.358
GDP (CaCl ₂)	Man5–9	72.6	0.405
GDP (CaCl ₂)	EndoH	57.3	0.303
GDP (CaCl ₂)	PNGaseF	13.2	0.055
GDP (CaCl ₂)	Wild type	57.0	0.339
GTP (MgCl ₂)	Man5–9	64.8	0.264
GTP (MgCl ₂)	EndoH	50.7	0.147
GTP (MgCl ₂)	PNGaseF	3.8	0.046
GTP (MgCl ₂)	Wild type	60.2	0.239
GDP (MgCl ₂)	Man5–9	28.1	0.254
GDP (MgCl ₂)	EndoH	19.1	0.145
GDP (MgCl ₂)	PNGaseF	2.0	0.041
GDP (MgCl ₂)	Wild type	14.9	0.063

Fig. 6 An inhibitor of E-NTPDase, POM6, inhibits differently the ATPase activities of NTPDase3/CD39L3 in different glycoforms. PNGaseF-treated, EndoH-treated, or mock-treated NTPDase3/CD39L3 ECD proteins were measured for ATPase activity in the presence of 26- μ M POM6, as described in “Materials and methods.” The error bars at each data point were calculated from three independent experiments

data might provide molecular basis for the *N*-glycan upregulation on NTPDase3/CD39L3.

Discussion

The NTPDase protein family represents an important class of ecto-enzymes with a wide range of pathophysiological functions and potential impacts on many disease conditions. For example, one of these NTPDase3/CD39L3's major biological functions appears to be in regulating insulin secretion from pancreatic β cells [25, 26]. A better understanding of the molecular mechanism governing NTPDase activity should facilitate drug-designing efforts on agonists and antagonists of NTPDase3/CD39L3 protein for potential clinical therapeutic application.

This study has uncovered the possibility that various *N*-glycan attachments can significantly increase activity of NTPDase3/CD39L3 enzymatic domain, when compared to non-glycosylated forms produced by its native host mammalian cells. Structural modeling analyses further reveal the potential involvements of Asn149 and N454 in influencing the catalytic cleft formed by ACR domains. These data should provide a new strategy to support *in silico* small molecule development and rational design of improved protein therapeutics.

Fig. 7 Structural modeling analysis reveals that *N*-glycan modifications at Asn149 next to ACR2 and/or Asn454 N-terminal to ACR5 might affect folding of the catalytic pocket of NTPDase3/CD39L3. The PDB accession number for rat NTPDase1 structure is 3ZX2. ACR domains are in red and numbered. N-linked residues are in yellow. **a**, **b** Two different views of the structure

This study has taken advantage of the glycosidase inhibitor kifunessine to generate mammalian cell-derived proteins with mannose₅₋₉ glycan modifications, which are sensitive to the treatments of EndoH and PNGaseF. The approach has allowed an efficient generation of presumably non-glycosylated and single-GlcNAc attached proteins as reported [23, 31, 32]. While four different glycosylated isoforms of NTPDase3/CD39L3ECD were produced by this method, we noted that *N*-glycan is not essential to the NTPDase activity for

NTPDase3/CD39L3ECD. Further, the attachment to protein, even only with a single-sugar, is sufficient to enhance NTPDase activity, when comparing to non-glycosylated proteins. Since no crystal structures have yet been obtained for NTPDase3/CD39L3, the findings from this study should provide a feasible way to provide relatively homogenous glycosylated protein materials for future structure study.

It has been well established for the E-NTPDase family that *N*-glycans are required for protein folding, intracellular trafficking, surface expression, and activity [13, 21–24, 27–29, 36]. Guidotti and coworkers have established critical roles of *N*-glycan of NTPDase1/CD39 in surface expression and activity [22, 23] and identified essential glycosylation sites [21]. For NTPDase3/CD39L3, an early work from Kirley and coworkers [27] has uncovered full activity loss with tunicamycin treatment and 50% loss on ATPDase activity with PNGaseF treatment on crude membrane from COS cells. These authors previously also identified that Asn81 near ACR1 is essential for full enzymatic activity [28] and have further shown that NTPDase3/CD39L3 with core glycan structure right after exiting ER during trafficking is active [29]. Our data with substantially pure materials support this conclusion. We further found that the activity difference between these materials was much bigger with magnesium than calcium, in the GTPDase assay than the ATPDase assay, and with diphosphate nucleotides than triphosphate nucleotides. Our results have also revealed that single GlcNAc attachment is sufficient for full activity. We have proposed two additional N-linked sites for potential glycan upregulation.

In terms of protein characteristics, NTPDase3/CD39L3 is a unique member of the E-NTPDase family. It has seven N-linked sites, four of which are next to the highly conserved ACR regions. NTPDase 5 and 6 have only two such sites, NTPDase 1 and 2 have three, whereas NTPDase 4 and 7 have none. Unglycosylated NTPDase 1 has a similar activity to the protein with wild-type glycans [23]. When compared to NTPDase1/CD39, NTPDase3/CD39L3 has the unique Asn149 near ACR2. NTPDase 2 also has a similar N-linked site near ACR2; it would therefore be of interest to see if there is any activity difference between its non-glycosylated and glycosylated form. Since the ACR regions are critical for NTPDase catalytic function, it is possible that *N*-glycans could change the confirmation of ACR structures and consequently upregulate enzymatic activity of NTPDase3/CD39L3. It is worth noting that non-glycosylated NTPDase3/CD39L3 is more sensitive to POM6 inhibition than those with glycans, suggesting that *N*-glycans might block the binding of the inhibitor.

In conclusion, this study has developed an efficient strategy in generating mammalian cell-derived rat NTPDase3/CD39L3 ECD with four different glycoforms. We have demonstrated that NTPDase3/CD39L3 ECD without glycan is significantly less active than those with different glycans, when measured in NTPDase activity in the presence of either

Ca²⁺ or Mg²⁺. Glycan structures seem to modulate the NTPDase3/CD39L3's NTPDase activities, suggesting that *N*-glycan modification has an additional role in impacting protein function besides protein folding and expression.

Materials and methods

Cell culture and protein purification Human embryonic kidney (HEK) 293 were grown and maintained in a humidified incubator with 5% CO₂ at 37 °C in a FreeStyle™ medium (Thermo Fisher, Waltham, MA). DNA transfection procedures in HEK293 were followed as described in the Thermo Fisher manufacturer's protocols. Briefly, DNA and a lipofectamine complex were inoculated with 1.0×10^6 /ml cells and expression enhancers were added 24 h post-transfection. To generate mannose5–9 glycan, 100 μM kifunesine was added to the cell culture 4 h post-transfection. Conditioned medium were harvested at day 6 and filtered with 0.2 μ filters. Two milliliters of Nickel-NTA column pre-equilibrated with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS) were loaded with conditioned medium and washed with 10 column volumes (CV) of TBS with 20 mM imidazole, before the protein was eluted using 100% step of 500 mM imidazole in TBS. The protein was dialyzed overnight against a 1000-fold TBS buffer overnight. Protein samples were pooled concentrated to 0.5 mg/ml using a 10-kDa MWCO centrifugal device.

Colorimetric NTPDase assay The colorimetric assay was performed as described [37]. Briefly, 0.045% malachite green hydrochloride (MG), 4.2% ammonium molybdate in 4 N HCl (AM), 4% solution of C12E10 (Polyoxyethylene 10 lauryl ether, Sigma-Aldrich) (CE), and 34% sodium citrate.2H₂O (*w/v*), were prepared. Prior to the assay, MG and AM were mixed in a 3:1 ratio, incubated for at least 20 min, and filtered through 0.2 μ membrane. 0.1 ml of the CE solution was added to every 5 ml of the MG/AG solution. The NTPDase buffer contained 20 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, and 0.5 mM EDTA. Five micromolars of CaCl₂ or MgCl₂ (50 ml) were added to reaction. During the NTPDase assay, NTPDase3/CD39L3 samples were added to initiate the reaction at 37 °C for 20 min. 0.8 ml MG/AG/CE solution and 100 μl citrate were added to stop the reactions. A660 reading was measured by pre-blanked with 1 ml MG/AG/CE buffer. A660 reading with CaCl₂ or MgCl₂ subtracted by A660 without divalent cation was converted to a phosphate standard curve.

EndoH treatment and PNGaseF treatment NTPDase3/CD39L3ECD-his6-Man5–9 (HEK293F treated with kifunesine) in TBS (5.6 μg) was mixed with 4 μl of EndoH in 40 μl at room temperature incubation overnight. For the PNGaseF treatment, NTPDase3/CD39L3ECD-his6-Man5–9

in TBS (5.6 µg) was mixed with 4 µl of PNGaseF in 40 µl at room temperature incubation overnight. A mock-treated control was incubated with TBS buffer at room temperature incubation overnight.

Compliance with ethical standards

Conflicts of interest Alexander H. Zhong declares that he has no conflict of interest.

Z. Gordon Jiang declares that he has no conflict of interest.

Richard D. Cummings declares that he has no conflict of interest.

Simon C. Robson declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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