Sequential hydrolysis of FAD by ecto-5′ nucleotidase CD73 and alkaline phosphatase is required for uptake of vitamin B₂ into cells

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Extracellular hydrolysis of flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to riboflavin is thought to be important for cellular uptake of vitamin B₂ because FAD and FMN are hydrophilic and do not pass the plasma membrane. However, it is not clear whether FAD and FMN are hydrolyzed by cell surface enzymes for vitamin B₂ uptake. Here, we show that in human cells, FAD, a major form of vitamin B₂ in plasma, is hydrolyzed by CD73 (also called ecto-5′ nucleotidase) to FMN. Then, FMN is hydrolyzed by alkaline phosphatase to riboflavin, which is efficiently imported into cells. We determined that this two-step hydrolysis process is impaired on the surface of glycosylphosphatidylinositol (GPI)-anchored protein (GPI-AP), dephosphorylates thiamine pyrophosphate and pyridoxal phosphate moiety, such as pyrophosphate, phosphoethanolamine, and PLP (8). There are four main isoforms of ALP in humans—tissue-nonspecific ALP (TNSALP), intestinal ALP, germ cell ALP, and placental ALP—all of which are GPI-APs (13). Among them, TNSALP is ubiquitously expressed and is the major isoform expressed in liver, bone, kidney, blood, and brain (14).

Although, Daniel et al. (11) reported that both FMN and FAD were hydrolyzed by ALP purified from the brush-border membrane of rat jejunum. ALP hydrolyzes compounds with a phosphate moiety, such as pyrophosphate, phosphoethanolamine, and PLP (8). There are four main isoforms of ALP in humans—tissue-nonspecific ALP (TNSALP), intestinal ALP, germ cell ALP, and placental ALP—all of which are GPI-APs (13). Among them, TNSALP is ubiquitously expressed and is the major isoform expressed in liver, bone, kidney, blood, and brain (14).

In food, vitamin B₂ is mainly in the form of FAD or FMN. Because they are hydrophilic, they are not imported directly into the intestinal epithelial cells; instead, they must be converted to riboflavin (RF, Fig. 1A) on cell surface for uptake (10, 11). RF uptake into cells is mediated by three kinds of RF transporters (SLC52A1, 2, 3) (9). FMN and FAD are then regenerated from RF in the cytoplasm by RF kinase and FAD synthetase (9, 12). These active forms of vitamin B₂ work as cofactors of various enzymes involved in redox reactions in many metabolic pathways, such as the tricarboxylic acid cycle, vitamin B₆ metabolism, and mitochondrial electron transport chain. Therefore, vitamin B₂ deficiency causes mitochondrial dysfunction as well as various metabolic disorders (9).

The water-soluble vitamins are essential to mammals and play roles in various metabolic reactions by working as coenzymes. The major forms of vitamins B₁, B₂, and B₆ in blood are phosphorylated or nucleotide forms (1–6) and they need to be hydrolyzed for uptake by cells. Alkaline phosphatase (ALP), a glycosylphosphatidylinositol (GPI)-anchored protein (GPI-AP), dephosphorylates thiamine pyrophosphate and pyridoxal 5′-phosphate (PLP) to thiamine and pyridoxal (PL), respectively (7, 8). However, it is uncertain whether the active forms of vitamin B₂, flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Fig. 1A), are hydrolyzed by the cell surface enzyme for their uptake. Moreover, enzymes which hydrolyze them are not known (9).

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hydrolyzes only FAD, and bile and pancreatic juice hydrolyze only FMN (16). Lee and Ford showed that an enzyme purified from placental trophoblastic microvilli possessed FAD pyrophosphatase activity and the enzyme did not hydrolyze FMN (17). These reports suggest a two-step hydrolysis of FAD (i.e., FAD → FMN, then FMN → RF).

Here, we show that, in human cells, FAD is hydrolyzed in two steps: FAD to FMN by CD73 and FMN to RF by ALP. CD73, also known as ecto-5′-nucleotidase, is a GPI-AP encoded by the NT5E gene and catalyzes conversion of adenosine 5′-monophosphate (AMP) to adenosine (13, 18).

To determine the hydrolytic activity of human bone ALP and recombinant human CD73 toward vitamin B2 analogues, RF formation from FMN and FMN formation from FAD were measured. The contributions of these GPI-APs to vitamin B2 uptake, vitamin B2-dependent vitamin B6 metabolism, and mitochondrial function were determined using GPI-deficient cells. Because CD73 and ALP are both GPI-APs (13, 19), GPI-deficient cells contained a decreased amount of intracellular vitamin B2 when cultured with FAD as the vitamin B2 source, leading to changes in vitamin B6 metabolism and mitochondrial dysfunction. These lines of evidence suggest that the mitochondrial dysfunction seen in some severe cases of inherited GPI deficiency (IGD) might be caused by vitamin B2 deficiency, which would be prevented by the administration of RF.

Results

**RF formation from FMN by ALP and FMN formation from FAD by CD73**

To analyze the two-step hydrolysis of FAD, RF and FMN concentrations were measured in vitro by HPLC after incubation of 10 μM FMN and FAD, respectively, with human bone ALP and CD73 containing a C-terminal His tag in solution for 15 min. An increase in the RF concentration was detected after incubation of FMN with ALP but not with CD73. In contrast, the FMN concentration was increased after incubation of FAD with CD73 but not with ALP (Fig. 1B).

ALP-dependent RF formation from FMN is shown in Figure 2, A–C, including a time course and the dependence on the concentrations of ALP and FMN, respectively. RF production increased linearly for 10 min (Fig. 2A) in an ALP concentration-dependent manner (Fig. 2B). RF production from FMN by ALP was substrate saturable (Fig. 2C), the Km and Vmax values being 0.309 ± 0.051 μM and 7.47 ± 0.25 nmol/min/mg protein, respectively (Table 1). These results demonstrate that purified bone ALP, that is, TNSALP, hydrolyzes FMN, producing RF.

Recombinant CD73-dependent FMN formation from FAD is shown in Figure 2, D–F. Because FAD consists of RF, diphosphate, and adenosine moieties, AMP (adenosine + phosphate) or adenosine could be produced by FAD hydrolysis (Fig. 1A). To determine the products of FAD hydrolysis by CD73, we simultaneously determined FMN, AMP, and adenosine by HPLC after incubation of FAD with CD73. Increased concentrations of FMN and adenosine were detected, but no increase in AMP was observed. The concentrations of FMN and adenosine increased linearly for 30 min (Fig. 2, D and G) in a CD73 concentration-dependent manner (Fig. 2E and H). FMN and adenosine production by CD73 was dependent on the concentration of the substrate, FAD, and was saturable (Fig. 2, F and I). CD73 is known to hydrolyze AMP to adenosine; in addition, here, we show evidence that recombinant CD73 also hydrolyzes FAD to produce FMN and adenosine.

**Kinetic parameters of ALP and CD73 activities**

We determined kinetic parameters of the activities of ALP and CD73 (Table 1). Because AMP is a substrate for both CD73 and ALP (13, 18), the results for kinetic analysis of FMN and FAD hydrolysis are compared with those for AMP hydrolysis in Table 1. The Km value for FAD hydrolysis by CD73
was higher than that for AMP, and the $V_{\text{max}}$ value for AMP hydrolysis was higher than that for FAD hydrolysis, suggesting that CD73 binds AMP with higher affinity than FAD and more efficiently hydrolyzes AMP than FAD. In contrast, the $K_m$ values for FMN and AMP hydrolysis by ALP were comparable.

### Inhibition studies of ALP and CD73

Several types of inhibitors were used to characterize the hydrolytic properties of CD73 and ALP (Fig. 3). FMN hydrolysis and AMP hydrolysis by ALP showed similar patterns of inhibition (Fig. 3A). FAD hydrolysis and AMP hydrolysis by CD73 also showed similar patterns of inhibition (Fig. 3B). However, the inhibition properties of AMP hydrolysis mediated by ALP and CD73 were different. FMN and nicotinamide mononucleotide, which are phosphorylated vitamins, and

### Table 1

Kinetic parameters for hydrolysis reactions catalyzed by human bone ALP and recombinant human CD73

|       | $K_m$ (μM) | $V_{\text{max}}$ (nmol/min/mg protein) |
|-------|------------|----------------------------------------|
| a) ALP |            |                                        |
| FMN → RF | 0.309 ± 0.051 | 7.47 ± 0.25                           |
| AMP → adenosine | 0.538 ± 0.047 | 13.9 ± 0.4          |
| b) CD73 |            |                                        |
| FAD → FMN | 18.4 ± 0.3   | 12.5 ± 0.3                             |
| FAD → adenosine | 23.7 ± 1.5 | 13.2 ± 0.3                          |
| AMP → adenosine | 3.94 ± 0.51     | 188 ± 7                               |

The $V_{\text{max}}$ was expressed in product concentration per minute per mg or μg protein. Data represent means ± standard errors.

Abbreviations: ALP, alkaline phosphatase; AMP, adenosine 5'-monophosphate; FAD, flavin-adenine dinucleotide; FMN, flavin mononucleotide; RF, riboflavin.
FAD hydrolysis by GPI-anchored enzymes

**Figure 3. Inhibition of alkaline phosphatase (ALP) and CD73.** A, comparison of inhibitory effect of various compounds on hydrolysis of 1 mM flavin mononucleotide (FMN) and 1 μM adenosine 5’-monophosphate (AMP) by ALP. FMN hydrolysis activities were measured by riboflavin (RF) production from FMN; AMP hydrolysis activities were measured by adenosine production from AMP. The concentration of the inhibitors was 10 μM, except for αβ-methylene adenosine 5’-diphosphate (APCP; 2 μM) and levamisole (1 mM). Data represent means ± SD (n = 3). When the SD is smaller than the symbols, it is not shown. B, comparison of inhibitory effects of various compounds on hydrolysis of 10 μM flavin-adenine dinucleotide (FAD) and 4 μM AMP by CD73. FAD hydrolysis activities were measured by FMN production from FAD; AMP hydrolysis activities were measured by riboflavin production from AMP. The concentration of the inhibitors was 10 μM, except for APCP (2 μM) and levamisole (1 mM). Data represent means ± SD (n = 3). When the SD is smaller than the symbols, it is not shown. C, comparison of inhibitory effects of various compounds on hydrolysis of 1 and 4 μM AMP by ALP and CD73, respectively. AMP hydrolysis activities were measured by adenosine production from AMP. The concentration of the inhibitors was 10 μM, except for APCP (2 μM) and levamisole (1 mM). Data represent means ± SD (n = 3). When the SD is smaller than the symbols, it is not shown.

levamisole, which is an inhibitor of TNSALP (20), decreased the activity of ALP but not CD73. In contrast, αβ-methylene adenosine 5’-diphosphate (APCP), an inhibitor of CD73 (21), inhibited CD73 but not ALP. Guanosine 5’-monophosphate, a nucleotide, inhibited both CD73 and ALP, which is consistent with AMP being a common substrate of CD73 and ALP (Fig. 3C). Figures 2 and 3 demonstrate that TNSALP hydrolyzes FMN, producing RF, and CD73 hydrolyzes FAD, producing FMN.

**Extracellular hydrolysis and uptake of vitamin B2 and vitamin B2-dependent PLP and PL production in GPI-deficient cells**

Both CD73 and ALP are GPI-APs (13, 19) and phosphatidylinositol glycan anchor biosynthesis class T (PIGT) is required for GPI-AP generation, and therefore, PIGT-KO SH-SY5Y cells (PIGT− cells) were generated using the CRISPR/Cas9 system to obtain CD73- and ALP-defective cells. SH-SY5Y is the human neuroblastoma cell line. The activities of CD73 and ALP in PIGT− cells were significantly lower than in PIGT rescued cells (PIGT+ cells) (Fig. 4, A and B). Surface expression levels of CD73, TNSALP, and CD59 (another GPI-AP) on PIGT− and PIGT+ cells were compared by flow cytometric analysis (Fig. 4C). The surface expression of CD73, TNSALP, and CD59 was deficient in PIGT− cells.

PIGT− and PIGT+ cells were cultured in vitamin B2-depleted medium for 5 days, followed by culture for 24 h in a medium containing one of the vitamin B2 derivatives (FMN, FAD, or RF) or a medium without vitamin B2. Vitamin B2 concentrations in the cell and culture medium were measured by HPLC. FAD was the major form of vitamin B2 in the PIGT+ and PIGT− cells after cultured in medium containing RF, FMN, or FAD, indicating that imported RF was intracellularly converted to FAD (9). The intracellular total vitamin B2 concentrations were significantly lower in PIGT− cells than in PIGT+ cells after cultured in FMN- or FAD-containing medium, while they were similar after cultured in RF-containing or vitamin B2-depleted medium (Fig. 4D) (because RF can be transported into the cells by RF transporters).

In the media, total vitamin B2 and FAD were significantly higher (91.2 ± 2.1% versus 47.3 ± 0.3%, p < 0.01) and FMN was significantly lower (2.2 ± 0.4% versus 36.2 ± 2.0%, p < 0.01) for PIGT− cells cultured with medium containing FAD than for PIGT+ cells, suggesting that FAD was not efficiently hydrolyzed to FMN by PIGT− cells (Fig. 4E). FMN was significantly higher (80.1 ± 2.1% versus 75.0 ± 0.3%, p < 0.05) for PIGT− cells cultured with medium containing FMN than for PIGT+ cells, suggesting that FMN was not efficiently hydrolyzed to RF by PIGT− cells (Fig. 4E). However, when cultured with medium containing RF, there was no difference in vitamin B2 concentration in medium between PIGT− and PIGT+ cells (Fig. 4E).

These results indicate that the presence of FAD and FMN in the medium did not lead to efficient uptake of vitamin B2 into GPI-deficient cells; this is because FAD and FMN were inefficiently hydrolyzed to FMN and RF, respectively, because of
the defective expression of CD73 and ALP; this resulted in intracellular vitamin B₂ deficiency.

To analyze the effect of intracellular vitamin B₂ deficiency on the activity of vitamin B₂-dependent enzymes, concentrations of vitamin B₆ derivatives in the medium were measured. Pyridoxine (PN, a form of vitamin B₆) is imported into cells and phosphorylated to pyridoxine 5'-phosphate (PLP). PLP is then converted to PLP by the FMN-dependent enzyme PNP oxidase, and PLP is in turn dephosphorylated to PL; PL and PLP are efficiently exported to the medium (22–24). Extracellular PL and PLP should be a biomarker for intracellular vitamin B₂ status because sum of PLP and PL is a net produced metabolite by FMN-dependent PNP oxidase from PN which is contained in all precultured media as a vitamin B₆ source. After cultivation of PIGT+ and PIGT− cells in the presence of PN and FAD, the combined PL and PLP concentration in the medium was significantly lower for PIGT− cells than for PIGT+ cells (Fig. 4F). There was a significant difference (p < 0.01) in the sum of PLP and PL in the medium. ND: not detectable. E, intracellular vitamin B₂ amount after 24 h of cultivation of PIGT+ or PIGT− SH-SYSY cells in medium containing flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), riboflavin (RF), or no vitamin B₂. The residual amount of vitamin B₂ in medium is expressed as the percentage of the total vitamin B₂ concentration in cells. The data represent means ± SD (n = 4). **Indicates a significant difference (p < 0.01) compared with PIGT+ cells. F, CD73 activities were measured in lysates of PIGT+ and PIGT− cells. CD73 activities in PIGT+ or PIGT− SH-SYSY cells. CD73 activities were measured by ACP-C–fluorescent intensity (MFI) of PIGT+ versus PIGT−; CD73, 177 versus 82; ALP, 472 versus 102; CD59, 10,093 versus 77. The analysis was repeated at least three times. D, residual amount of vitamin B₂ in medium after 24 h of cultivation of PIGT+ or PIGT− SH-SYSY cells in medium containing flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), riboflavin (RF), or no vitamin B₂. The residual amount of vitamin B₂ in medium is expressed as the percentage of the total vitamin B₂ concentration (FAD + FMN + RF) in medium incubated without cells. The data represent means for PLP (gray), and RF (black). Error bars represent the SD for total vitamin B₂. †††Indicates a significant difference (p < 0.01) in the residual amount of total vitamin B₂ in the medium. ND: not detectable. F, ALP activity is detectable. G, correlation between intracellular FMN concentration and the sum of PLP and PL in the medium after 24 h of cultivation of PIGT+ (closed circles) or PIGT− (open circles) SH-SYSY cells in medium containing FMN, FAD, RF, or no vitamin B₂. The data represent means ± SD (n = 3). Statistical analysis: Student's t test for (A and B); ANOVA followed by the Tukey–Kramer test for (D–F); Pearson correlation for (G).
positive relationship between the sum of PL and PLP concentrations in the medium and the intracellular FMN concentration ($p < 0.001$, Fig. 4G). These results suggest that the amount of vitamin B$_2$ imported into the cells affected the vitamin B$_2$-dependent PLP and PL production.

**Effect of intracellular vitamin B$_2$ deficiency on mitochondrial function**

FMN and FAD act as coenzymes in the mitochondrial electron transport chain, and a decreased intracellular FAD concentration might cause mitochondrial dysfunction (25). To compare the mitochondrial function between PIGT$^+$ and PIGT$^-$ SHSY5Y cells, O$_2$ consumption of cells was measured using a flux analyzer. After culture in vitamin B$_2$-depleted medium for 5 days, cells were cultured in medium containing a vitamin B$_2$ derivative (FAD, RF, or no vitamin B$_2$) for 24 h and their O$_2$ consumption was then measured (Fig. 5). PIGT$^-$ cells showed significantly lower O$_2$ consumption than PIGT$^+$ cells when they were cultured in FAD-containing medium, whereas those cultured in RF-containing medium showed a similar level of O$_2$ consumption to that in PIGT$^+$ cells. These results suggest that the GPI-deficient cells are susceptible to mitochondrial dysfunction. Additionally, non-mitochondrial O$_2$ consumption, which is the residual O$_2$ consumption after addition of rotenone/antimycin A, was also decreased in PIGT$^-$cells, suggesting the contribution of some flavo-enzyme oxidases.

**Discussion**

The present study showed that purified human TNSALP from bone hydrolyzed FMN and recombinant human CD73 hydrolyzed FAD. ALP catalyzes the hydrolysis of monoesters of phosphoric acid (14). Here, we showed that ALP hydrolyzes monophosphate vitamin B$_2$, FMN, but not dinucleotide-type vitamin B$_2$, FAD. Inhibition study showed that guanosine 5’-monophosphate and nicotinamide mononucleotide inhibited ALP activity, but NAD and FAD did not, suggesting that ALP has high affinity for compounds with a phosphomonoester moiety.

We also showed that adenosine and FMN were produced from FAD by CD73. Because AMP hydrolysis had a higher $V_{max}$ and lower $K_m$ than FAD hydrolysis by CD73, we speculate that adenosine was immediately produced from AMP after AMP and FMN production from FAD, with conversion of FAD to FMN being rate limiting. However, at the moment, we cannot completely eliminate the possibility of flavin-pyrophosphate as an intermediate.

TNSALP from human bone was used in the present study. TNSALP hydrolyzes some phosphate compounds, such as inorganic pyrophosphate, PLP (vitamin B$_6$), and thiamine pyrophosphate (vitamin B$_1$). Hypophosphatasia (HPP) is caused by loss-of-function mutations in TNSALP. Decreased conversion of pyrophosphate to phosphate caused dysostogenesis (8). Because cell surface hydrolysis of PLP to PL is important for vitamin B$_6$ uptake into cells, decreased PLP hydrolysis activity causes vitamin B$_6$ deficiency, leading to dysfunction of various vitamin B$_2$-dependent enzymes such as glutamate decarboxylase which results in PN-dependent seizures (8, 26). In HPP, lowered levels of thiamine pyrophosphate in red blood cells were reported (7). Adenosine and γ-aminobutyric acid concentrations were lower in brain of Akp2 KO mice than in wild-type mice; this gene encodes TNSALP in mice (27). The present study showed that the phosphorylated form of vitamin B$_2$, FMN, is a substrate of human TNSALP. Because hydrolysis by ALP of vitamin B$_2$, B$_6$, and B$_8$ is required for their uptake, their uptake would be decreased in HPP, which will be a subject of future investigation.

Analysis using GPI-deficient cells, which are defective in both CD73 and ALP cell surface expression, showed that both FAD and FMN uptake activities were lower than in GPI-rescued cells, leading to intracellular deficiency of vitamin B$_2$. Thus, the GPI-deficient cells showed dysfunction of the vitamin B$_2$-dependent mitochondrial respiratory chain complex, as well as of PNP oxidase, and enzyme in vitamin B$_6$ metabolism. GPI-deficient cells showed significantly lower PLP and PL production and O$_2$ consumption when they were incubated with FAD than PIGT$^+$ cells, whereas those incubated with RF showed a similar level to that in PIGT$^+$ cells (Figs. 4 and 5A and 5, A and B). In addition, a significant positive relationship was found between the concentration of intracellular FMN and the sum of PLP and PL production (Fig. 4G). These results again suggest that cell surface hydrolysis of FAD to RF by the CD73 and ALP contributed to vitamin B$_2$-dependent functions (Fig. 5C). However, it might be still possible that some other GPI-APs contribute to these functions.

IGD is caused by mutations in genes involved in the biosynthesis or modification of GPI-APs. Major symptoms of patients with IGD are intellectual disability, developmental delay, and seizures. Because both ALP and CD73 are GPI-APs, expression of these proteins is decreased in some patients with IGD. Here, we demonstrated that GPI-deficient cells showed decreased intracellular vitamin B$_2$ levels when cultured with FAD, a major form of vitamin B$_2$ in blood, which led to mitochondrial dysfunction. This is consistent with reports that some severe IGD cases show mitochondrial dysfunction (28). In future, we are planning to analyze the metabolic conditions in vivo using IGD model mice (29). CD73 expression is decreased in phosphatidylinositol glycan anchor biosynthesis class G (PIGG) KO cells (30) and some cases with null mutation of PIGG also showed decreased expression of CD73 and mitochondrial dysfunction (31), suggesting that CD73 expression is important for uptake of vitamin B$_2$ in PIGG deficiency. Similar to HPP, some patients with IGD show decreased vitamin B$_6$ uptake and suffer from PN-dependent seizures (32). High-dose nonphosphorylated vitamin B$_6$ (i.e., PN) treatment was effective in treatment of seizures in more than half of patients with IGD (33). Two types of nonphosphorylated vitamin B$_6$ are imported into cells and converted to PLP in the cell. One of them, PL, is contained in food from animal sources. PN and its glycoside are contained in food from plant sources (34); PN is intracellularly converted to PNP, and PNP is converted to PLP by FMN-dependent PNP oxidase (22–24). In addition to decreased uptake of vitamin B$_6$, patients with IGD would show decreased intracellular conversion of PNP to PLP due to
dysfunction of this FMN-dependent enzyme caused by the decreased vitamin B2 uptake. Therefore, high-dose PN and RF treatment might be effective for patients with IGD. However, further study is required concerning vitamin B6 and B2 metabolism in patients with IGD.

Adenosine, produced by CD73 from AMP, is an immune inhibitory molecule through its receptor expressed on immune cells (35, 36). Some tumors show upregulation of CD73, and adenosine promotes both migration and proliferation (37). Therefore, CD73 is a target for immunotherapy for cancer. Antibody against CD73 has been used in a phase 1 clinical study (36). Here, we show the importance of CD73 for vitamin B2 uptake. In cancer therapy, the effect of antibody against CD73 on vitamin B2 metabolism should be studied in future study.

**Experimental procedures**

**Materials**

Human bone ALP was purchased from Calzyme. Human CD73 His-tag was purchased from BPS Bioscience. All other reagents were of analytical grade.

**Measurement of hydrolysis of FAD**

For measurement of hydrolysis of FAD, FAD and human bone ALP or human CD73 were incubated in reaction buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, and 0.002% bovine serum albumin, after separately preincubation of FAD and the enzyme at 37°C for 3 min. At an appropriate time, the reaction was stopped by addition of ice-cold perchloric acid and the mixture was centrifuged (9000 g for 5 min). The supernatant was transferred to a fresh tube, neutralized by KOH, and recentrifuged. The supernatant was filtered through a 0.45-μm membrane and simultaneously analyzed for FMN, RF, AMP, and adenosine concentrations by HPLC. The HPLC conditions were optimized from a previously reported method (38). The HPLC apparatus consisted of a Shimadzu LC-10ADvp system equipped with an RF-10Axl spectrophotometer and SPD-10Avp UV-VIS detector (Shimadzu). Chromatographic separation was performed on an InertSustain AQ-C18 column (150 x 4.6 mm, i.d. 5 μm; GL Sciences) using a gradient elution mode at a flow rate of 1.0 ml/min. The column temperature was 25°C. Mobile phase A was 10 mM potassium phosphate containing 5 mM EDTA-
disodium salt, adjusted to pH 6.0; mobile phase B was methanol. We used a linear gradient from 8% to 25% mobile phase B from 3 min to 6 min, followed by holding at 25% B until 25 min after injection. Fluorescence measurements were made with excitation at 440 nm and emission at 560 nm to determine FMN and RF concentrations. Absorbance measurements were made at 260 nm for the determination of AMP and adenosine.

Hydrolysis activity was calculated as observed production minus nonenzymatic production, which was determined in negative controls from which enzyme was absent otherwise using the same procedure as described previously.

**Measurement of hydrolysis of FMN**

For measurement of hydrolysis of FMN, FMN and human bone ALP or human CD73 were incubated in reaction buffer containing 50 mM Tris-HCl (pH 7.4) and 5 mM MgCl₂ at 37 °C for 3 min (39). The reaction procedures were the same as for analysis of FAD hydrolysis. The produced RF concentration was determined using an isocratic HPLC method with 75% buffer A and 25% buffer B (other conditions the same as for analysis of FAD hydrolysis).

**Measurement of hydrolysis of AMP**

For measurement of hydrolysis of AMP, AMP and human bone ALP or human CD73 were incubated in reaction buffer containing 50 mM Tris-HCl (pH 7.4) and 5 mM MgCl₂ at 37 °C for 3 min (39). The reaction procedures and HPLC conditions were the same as those for analysis of FAD hydrolysis.

**Generation of GPI-deficient cells**

PIGT−KO cells were generated from SHSY5Y cells (a human neuroblastoma-derived cell line) using the CRIPR/Cas9 system. Plasmid pX330 for expression of human-codon-optimized *Streptococcus pyogenes* (Sp) Cas9 and chimeric guide RNA were obtained from Addgene. The seed sequence for the SpCas9 target site in the target gene was tcggtgca- (underline; PAM sequence). SHSY5Y cells were transfected with pX330 containing the gRNA of the target site using Lipofectamine 2000 (Invitrogen). KO clones were obtained by limiting dilution, and KO was confirmed by sequencing the target sites in the genomic DNA and by flow cytometric analysis of CD59 expression [staining with mouse anti-hCD59 antibody (5H8) followed by phycoerythrin-conjugated anti-mouse IgG (Biolegend)]. PIGT-KO clone #3 was rescued by transfecting human PIGT-expressing vector pME-puro-3HA-hPIGT. After puromycin selection, the restored population was sorted to obtain PIGT+ cells. PIGT+KO clone #3 was transfected with an empty vector, pME-puro, and the puromycin-resistant population was selected as PIGT− cells. These cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum containing 3 μg/ml puromycin. For flow cytometric analysis, cells were stained with phycoerythrin-conjugated anti-human TNAP (B4-78; isotype, mouse IgG1; Santa Cruz Biotechnology) or anti-human CD73 (AD2, isotype; mouse IgG1; Biolegend) and anti-human CD59 antibody (isotype; mouse IgG1, clone 5H8) followed by phycoerythrin-conjugated goat anti-mouse IgG. Stained cells were analyzed using a MACS QuantVYB analyzer (Miltenyi Biotec).

**Measurement of ALP and CD73 activities in SHSY5Y cell lines**

ALP activities were measured using a Great EscAPe SEAP kit (Takara Bio Inc) in lysates of PIGT+ and PIGT− cells. The ALP activity is expressed in terms of the amount of placental ALP in the kit, which was used as a positive control. CD73 activities were measured by adenosine formation from AMP in lysate from PIGT+ and PIGT− cells. The quantitative method for adenosine using HPLC was described previously in the section “Measurement of hydrolysis of AMP.” The APCP sensitivity of CD73 activity was calculated by subtracting the activity in the presence of 4 μM APCP from the total activity in the absence of APCP.

**Extracellular hydrolysis and uptake of vitamin B₂ and vitamin B₆-dependent PLP and PL production in GPI-deficient cells**

PIGT− and PIGT+ cells were cultured in vitamin B₂-deficient medium for 5 days, followed by culture in medium containing a vitamin B₂ derivative (0.2 μM FMN, FAD, RF, or no vitamin B₂) for 24 h. Vitamin B₂ and B₆ concentrations were measured in medium and cells by HPLC. The HPLC conditions for measurement of FAD, FMN, and RF were the same as described previously for measurement of FMN hydrolysis. For the measurement of PL and PLP, a previously reported HPLC method with a fluorescence detector was used after precolumn derivatization with semicarbazide (40).

**Measurement of mitochondrial function**

Cellular respiration (oxygen consumption rate [OCR]) was assessed using an XFp Extracellular Flux Analyzer (Seahorse Bioscience). Cells were cultured in vitamin B₂-depleted medium for 5 days. Then, 10⁴ cells per well were incubated in poly-L-lysine-coated wells with vitamin B₂-depleted medium (Sigma-Aldrich Inc) or with that supplemented by RF, FAD, or FMN (0.2 μM) for 24 h. The XF Cell Mito Stress Test (Seahorse Bioscience Inc) was used to measure the key parameters of mitochondrial respiration using specific mitochondrial inhibitors and uncouplers: oligomycin (1 μM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (2 μM), and a mixture of rotenone/antimycin A (both 0.5 μM) were injected sequentially following the manufacturer’s instructions. Before drug addition, basal OCR was measured. Oligomycin was injected to inhibit ATP synthase (complex V), and the OCR was recorded. To determine the maximal respiration, the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone was injected. Finally, a mixture of rotenone/antimycin A was injected to inhibit the flux of electrons through complexes I and III and to enable calculation of the spare respiratory capacity. Residual O₂ consumption shows mitochondria-independent O₂ consumption.

**Statistical analysis**

Data are expressed as means ± SD. Statistically significant differences were determined using one-way analysis of
variance followed by Tukey post hoc test or Student t test, with \( p < 0.05 \) or 0.01 as the criterion. Pearson correlation analysis was performed to analyze correlations. Kinetic analyses were performed using Sigma Plot (Systat Software Inc).

**Data availability**

All data are contained within the manuscript.

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**Abbreviations**—Theabbreviations used are: ALP, alkaline phosphatase; AMP, adenosine 5'-monophosphate; ACP, α,β-methyleneadenosine 5'-diphosphate; FAD, flavin-adenine dinucleotide; FMN, flavinmononucleotide; GPI, glycosylphosphatidylinositol; GIP, GPI-anchored protein; HPP, hypophosphatasia; IGD, inherited GPI deficiency; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; GLP, glycerol phosphatidylethanolamine; class C; PIGT, phosphatidylinositol glycan anchor biosynthesis class T; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; PN, pyridoxine; PNP, pyridoxine 5'-phosphate; RF, riboflavin; TNSALP, tissue-nonspecific ALP; OCR, oxygen consumption rate.

**References**

1. Rindi, G., Patrini, C., and Poloni, M. (1981) Monophosphate, the only phosphoric ester of thiamin in the cerebro-spinal fluid. *Experientia* **37**, 975–976

2. Hustad, S., Ueland, P. M., and Schneede, J. (1999) Quantification of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in human plasma by capillary electrophoresis and laser-induced fluorescence detection. *Clin. Chem.* **45**, 862–868

3. Vasilaki, A. T., McMillan, D. C., Kinsella, J., Duncan, A., O’Reilly, D. S. J., and Talwar, D. (2010) Relation between riboflavin, flavin mononucleotide and flavin adenine dinucleotide concentrations in plasma and red cells in patients with critical illness. *Clin. Chim. Acta* **411**, 1750–1755

4. Talwar, D., Quasim, T., McMillan, D. C., Kinsella, J., Williamson, C., and O’Reilly, D. S. J. (2003) Optimization and validation of a sensitive high-performance liquid chromatography assay for routine measurement of pyridoxal 5-phosphate in human plasma and red cells using pre-column semicarbazide derivatisation. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **792**, 333–343

5. Ueland, P. M., Uvilk, A., Rios-Avila, L., Midtun, O., and Gregory, J. F. (2015) Direct and functional biomarkers of vitamin B6 status. *Annu. Rev. Nutr.* **35**, 33–70

6. Akiyama, T., Hayashi, Y., Hanaoka, Y., Shibata, T., Akiyama, M., Tsuchiya, H., et al. (2017) Pyridoxal 5'-phosphate, pyridoxal, and 4-pyridoxic acid in the paired serum and cerebrospinal fluid of children. *Clin. Chim. Acta* **472**, 118–122

7. Luong, K. V. Q., and Nguyen, L. T. H. (2005) Adult hypophosphatasia and a low level of red blood cell thiamine pyrophosphate. *Ann. Nutr. Metab.* **49**, 107–109

8. Millán, J. L. and Whyte, M. P. (2016) Alkaline phosphatase and hypophosphatasia. *Calcif Tissue Int.* **98**, 398–416

9. Barile, M., Giancaspro, T. A., Leone, P., Galluccio, M., and Indiveri, C. (2016) Riboflavin transport and metabolism in humans. *J. Inherit. Metab. Dis.* **39**, 545–557

10. Akiyama, T., Selhub, J., and Rosenberg, I. H. (1982) FMN phosphatase and FAD pyrophosphatase in rat intestinal brush borders: role in intestinal absorption of dietary riboflavin. *J. Nutr.* **112**, 263–268

11. Daniel, H., Binninger, E., and Rehner, G. (2012) Cellular function and molecular structure of ecto-nucleotidases. *Partur. Signal. 8*, 437–502

12. Millán, J. L. (2006) Alkaline phosphatases. *Partur. Signal. 2*, 335–341

13. Okuda, J. (1958) Metabolism of flavin nucleotides. I. Of flavin nucleotides in digestive juice. Decomposition. *Chem. Pharm. Bull.* **6**, 662–665

14. Okuda, J. (1958) Metabolism of flavin nucleotides. II. Decomposition of flavin nucleotides in the small intestine. *Chem. Pharm. Bull.* **6**, 665–669

15. Lee, R. S. F., and Ford, H. C. (1988) 5'-Nucleotidase of human placental trophoblastic microvilli possesses cobalt-stimulated FAD pyrophosphatase activity. *J. Biol. Chem.* **263**, 14878–14883

16. Picher, M., Burch, L. H., Hirsh, A. J., Spychala, J., and Boucher, R. C. (2003) Ecto 5'-nucleotidase and nonspecific alkaline phosphatase: two AMP-hydrolyzing ectoenzymes with distinct roles in human airways. *J. Biol. Chem.* **278**, 13468–13479

17. Misumi, Y., Ogata, S., Ohkubo, K., Hirose, S., and Ikehara, Y. (1990) Primary structure of human placental 5'-nucleotidase and identification of the glycolipid anchor in the mature form. *Eur. J. Biochem.* **191**, 563–569

18. Kozenkova, A., de Du, M. H., Cuniasse, P., Ny, T., Hoylaerts, M. F., and Millán, J. L. (2004) Residues determining the binding specificity of uncompetitive inhibitors to tissue-nonspecific alkaline phosphatase. *J. Bone Miner. Res.* **19**, 1862–1872

19. Bhattachary, S., Freundlieb, M., Pippel, I., Meyer, A., Abdelrahman, A., Fiene, A., et al. (2015) α,β-Methylene-ADP (AOPCP) derivatives and analogues: development of potent and selective ecto-5'-nucleotidase (CD73) inhibitors. *J. Med. Chem.* **58**, 6248–6263

20. Anderson, B. B., Fulford-Jones, C. E., Child, J. A., Beard, M. E., and Bateman, C. J. (1971) Conversion of vitamin B6 compounds to active forms in the red blood cell. *J. Clin. Invest.* **50**, 1901–1909

21. Anderson, B. B., Saary, M., Stephens, A. D., Perry, G. M., Lersundi, I. C., and Horn, J. E. (1976) Effect of riboflavin on red-cell metabolism of vitamin B6. *Nature* **264**, 574–575

22. da Silva, V. R., Russell, K. A., and Gregory, J. F. (2012) *Vitamin B6, Present Knowledge in Nutrition*, 10th Ed., Wiley-Blackwell, Hoboken, New Jersey: 307–320
25. Udhayabanu, T., Manole, A., Rajeshwari, M., Varalakshmi, P., Houlden, H., and Ashokkumar, B. (2017) Riboflavin responsive mitochondrial dysfunction in neurodegenerative diseases. J. Clin. Med. 6, 52
26. Akiyama, T., Kubota, T., Ozono, K., Michigami, T., Kobayashi, D., Takeyari, S., et al. (2018) Pyridoxal 5′-phosphate and related metabolites in hypophosphatasia: effects of enzyme replacement therapy. Mol. Genet. Metab. 125, 174–180
27. Cruz, T., Gleizes, M., Balayssac, S., Mornet, E., Marsal, G., Millán, J. L., et al. (2017) Identification of altered brain metabolites associated with TNAP activity in a mouse model of hypophosphatasia using untargeted NMR-based metabolomics analysis. J. Neurochem. 140, 919–940
28. Tarailo-Graovac, M., Sinclair, G., Stockler-Ipsiroglu, S., van Allen, M., Rozmus, J., Shyr, C., et al. (2015) The genotypic and phenotypic spectrum of PIGA deficiency. Orphanet J. Rare Dis. 10, 23
29. Kuwayama, R., Suzuki, K., Nakamura, J., Aizawa, E., Yoshioka, Y., Ikawa, M., et al. (2022) Establishment of mouse model of inherited PIGO deficiency and therapeutic potential of AAV-based gene therapy. Nat. Commun. 13, 3107
30. Ishida, M., Maki, Y., Ninomiya, A., Takada, Y., Campeau, P., Kinoshita, T., et al. (2022) Ethanolamine-phosphate on the second mannose is a preferential bridge for some GPI-anchored proteins. EMBO Rep. 23, e54352
31. Tremblay-Laganière, C., Maroothan, R., Nguyen, T. T. M., Karimiani, E. G., Kirmani, S., Akbar, F., et al. (2021) PIGG variant pathogenicity assessment reveals characteristic features within 19 families. Genet. Med. 23, 1873–1881
32. Kuki, I., Takahashi, Y., Okazaki, S., Kawawaki, H., Ehara, E., Inoue, N., et al. (2013) Vitamin B6-responsive epilepsy due to inherited GPI deficiency. Neurology 81, 1467–1469
33. Tanigawa, J., Nabatame, S., Tominaga, K., Nishimura, Y., Maegaki, Y., Kinosita, T., et al. (2021) High-dose pyridoxine treatment for inherited glycophosphatidylinositol deficiency. Brain Dev. 43, 680–687
34. Gregory, I. F., and Ink, S. L. (1987) Identification and quantification of pyridoxine-β-glucoside as a major form of vitamin B6 in plant-derived foods. J. Agric. Food Chem. 35, 76–82
35. Stagg, J., Divisekera, U., McLaughlin, N., Sharkey, J., Pommey, S., Denoyer, D., et al. (2010) Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. Proc. Natl. Acad. Sci. U. S. A. 107, 1547–1552
36. Allard, B., Longhi, M. S., Robson, S. C., and Stagg, J. (2017) The ectonucleotidases CD39 and CD73: novel checkpoint inhibitor targets. Immunol. Rev. 276, 121–144
37. Wang, L., Fan, J., Thompson, L. F., Zhang, Y., Shin, T., Curiel, T. J., et al. (2011) CD73 has distinct roles in nonhematopoietic and hematopoietic cells to promote tumor growth in mice. J. Clin. Invest. 121, 2371–2382
38. Akimoto, M., Sato, Y., Okubo, T., Todo, H., Hasegawa, T., and Sugiyabayashi, K. (2006) Conversion of FAD to FMN and riboflavin in plasma: effects of measuring method. Biol. Pharm. Bull. 29, 1779–1782
39. Coburn, S. P., Mahuren, J. D., Jain, M., Zubovic, Y., and Wortsman, J. (1998) Alkaline phosphatase (EC 3.1.3.1) in serum is inhibited by physiological concentrations of inorganic phosphate. J. Clin. Endocrinol. Metab. 83, 3951–3957
40. Kobayashi, D., Yoshimura, T., Johno, A., Ishikawa, M., Sasaki, K., and Wada, K. (2015) Decrease in pyridoxal-5′-phosphate concentration and increase in pyridoxal concentration in rat plasma by 4′-O-methylpyridoxine administration. Nutr. Res. 35, 637–642

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