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Molecular Basis of Unique Specificity and Regulation of Group VIA Calcium-Independent Phospholipase A_2 (PNPLA9) and Its Role in Neurodegenerative Diseases

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Abstract

Glycerophospholipids are major components of cell membranes and consist of a glycerol backbone esterified with one of over 30 unique fatty acids at each of the *sn*-1 and *sn*-2 positions. In addition, in some human cells and tissues as much as 20% of the glycerophospholipids contain a fatty alcohol rather than an ester in the *sn*-1 position, although it can also occur in the *sn*-2 position. The *sn*-3 position of the glycerol backbone contains a phosphodiester bond linked to one of more than 10 unique polar head-groups. Hence, humans contain thousands of unique individual molecular species of phospholipids given the heterogeneity of the *sn*-1 and *sn*-2 linkage and carbon chains and the *sn*-3 polar groups. Phospholipase A_2 (PLA_2) is a superfamily of enzymes that hydrolyze the *sn*-2 fatty acyl chain resulting in lyso-phospholipids and free fatty acids that than undergo further metabolism. PLA_2's play a critical role in lipid-mediated biological responses and membrane phospholipid remodeling. Among the PLA_2 enzymes, the Group VIA calcium-independent PLA_2 (GVIA $iPLA_2$), also referred to as PNPLA9, is a fascinating enzyme with broad substrate specificity and it is implicated in a wide variety of diseases. Especially notable, the GVIA $iPLA_2$ is implicated in the sequelae of several neurodegenerative diseases termed "phospholipase A_2-associated neurodegeneration" (PLAN) diseases. Despite many reports on the physiological role of the GVIA $iPLA_2$, the molecular basis of its enzymatic specificity was unclear. Recently, we employed state-of-the-art lipidomics and molecular dynamics techniques to elucidate the detailed molecular basis of its substrate specificity and regulation. In this review, we summarize the molecular basis of the enzymatic action of GVIA $iPLA_2$ and provide a perspective on future therapeutic strategies for PLAN diseases targeting GVIA $iPLA_2$.

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Keywords

phospholipase A_2; GVIA iPLA$_2$; PLA2G6; iPLA$_2\beta$; PNPLA9; neurodegeneration; neurodegenerative diseases

1. Introduction

Glycerophospholipids are major components of cell membranes and primarily function to partition cells and sub-cellular organelles, but they can also be a ready source of substrate for a variety of metabolic enzymes. Glycerophospholipids are composed of a glycerol backbone esterified with fatty acyl chains on its *sn*-1 and *sn*-2 positions and a phosphate group on the *sn*-3 position forming phosphatidic acid. Several head groups including choline, ethanolamine, serine, glycerol, inositol, and various inositol phosphates are esterified at the *sn*-3 phosphate forming polar head groups. Furthermore, in addition to the variation of acyl chains and head groups, the actual linkage at the *sn*-1 acyl chain can be an alkyl ether or a vinyl ether rather than an acyl group (Figure 1). Saturated and monounsaturated fatty acids are preferentially esterified at the *sn*-1 position, and *sn*-2 positions are rich in polyunsaturated fatty acids (PUFAs) (Yamashita et al., 2014). The characteristics and functions of the glycerophospholipids vary depending on the specific combination of acyl chains and head groups. Many 1,000's of unique molecular species are formed in mammals.

Phospholipase A_2 (PLA$_2$) is a superfamily of enzymes that hydrolyze glycerophospholipids at the *sn*-2 position and produces *sn*-2 lysophospholipids and free fatty acids (Dennis et al., 2011) (Figure 1). Lysophospholipids function as lipid mediators and are involved in numerous biological activities acting on a large variety of lysophospholipid receptors (Takagi et al., 2022; Tan et al., 2020). The free fatty acids, especially PUFAs, that are generated are further metabolized into various bioactive lipid mediators, often referred to as oxylipins, responsible for inflammation and resolution (Buczynski et al., 2009; Dennis & Norris, 2015; Funk, 2001; Serhan, 2007). Also, PLA$_2$ is a key enzyme, along with various lysophospholipid acyltransferases (LPLAT), that contributes to the Lands' cycle to maintain the homeostasis and diversity of the *sn*-2 acyl chain composition of membrane glycerophospholipids (Lands, 1957; Pérez-Chacón et al., 2009; Shindou & Shimizu, 2009). Therefore, PLA$_2$s play a central role in lipid-mediated biological activities and membrane glycerophospholipid remodeling and are involved in numerous diseases (Dennis et al., 2011).

The PLA$_2$ superfamily is comprised of six main types of enzymes and each possesses distinct structural features (Dennis et al., 2011). The secreted PLA$_2$s (sPLA$_2$s), the biggest category of the PLA$_2$ superfamily, is now composed of a number of groups (Group I, II, III, V, IX, X, XI, XII, XIII, and XIV) and subgroups. They have been extensively characterized and studied in various snake and bee venoms as well as in a variety of mammalian and human tissues (Murakami et al., 2015). sPLA$_2$s are small enzymes with a molecular mass of less than 20 kDa, requiring mM Ca^{2+} for their catalytic activity and mainly function extracellularly. Group IV cytosolic PLA$_2$s (GIV cPLA$_2$s) are intracellular cytosolic enzymes

containing calcium-binding C2 domains except for one subgroup of GIV cPLA$_2$ (GIVC cPLA$_2$, cPLA$_2\gamma$) (Leslie, 2015). Group VI calcium-independent PLA$_2$s (GVI PLA$_2$s), also known as the patatin-like phospholipase (PNPLA) family, are intracellular enzymes sharing the patatin domain found in a potato protein that has lipase activity (Kienesberger et al., 2009). Platelet-activating factor acetyl hydrolases (PAF-AHs) are named for their hydrolytic activity toward PAF, but also have been named as Lipoprotein-associated PLA$_2$ (Lp-PLA$_2$) for their activity toward oxidized phospholipids found extensively in lipoproteins, and these enzymes are categorized as Group VII and VIII PLA$_2$s (Kono & Arai, 2019). Lysosomal PLA$_2$ (LPLA$_2$, GXV PLA$_2$) is characterized by its localization in the lysosome and late endosome and is active under acidic conditions (Shayman & Tesmer, 2019). Adipose-specific PLA$_2$ (AdPLA$_2$, GXVI PLA$_2$) is a membrane-associated enzyme specifically expressed in white adipose tissue (Duncan et al., 2008). All PLA$_2$ enzymes discovered to date that have Group or Subgroup designations have been assigned to one of these six types based on their structural characteristics. Thus, the PLA$_2$ superfamily is composed of more than fifty unique enzymes (Dennis et al., 2011). Note that some enzymes possess no hydrolytic activity toward the *sn*-2 position of glycerophospholipids (PLA$_2$ activity) despite their name. Also, some enzymes exhibit PLA$_1$, lysophospholipase, triacylglycerol lipase, or transacylase activities. These unique enzymes play a central role in lipid metabolism and contribute to numerous biological events based on their tissue expression, subcellular localization, post-transcriptional regulation, and substrate specificity. Also, note that there are other enzymes that express PLA$_2$ activity besides those classified as being part of the PLA$_2$ superfamily, such as the α/β hydrolase domain (ABHD) proteins that possess a GXSXG lipase consensus motif (Lord et al., 2013; Murakami, 2017, 2019).

A subgroup of GVI iPLA$_2$ (GVIA iPLA$_2$, also referred to as PLA2G6, iPLA$_2\beta$, and PNPLA9) is a versatile PLA$_2$ enzyme involved in various diseases including diabetes, cancer, and various neurodegenerative diseases (Ramanadham et al., 2015). Despite its physiological role, its structure, regulation, and substrate specificity have not been well described. Recently, state-of-the-art techniques have enabled us to elucidate the molecular basis of the enzymatic action of GVIA iPLA$_2$. This review summarizes recent progress in understanding its structure, regulation, substrate specificity, and role in neurodegenerative diseases.

2. Structure and characteristics of GVIA iPLA$_2$

GVIA iPLA$_2$ is one of the PLA$_2$ enzymes that utilizes a Ser/Asp catalytic dyad for its hydrolysis reaction, as does its plant homolog patatin (Rydel et al., 2003). Also, GVIA iPLA$_2$ possesses not only PLA$_2$ activity but also acyl-CoA thioesterase (Carper et al., 2008; Jenkins et al., 2006), lysophospholipase, and transacylase activity (Lio & Dennis, 1998). Human GVIA iPLA$_2$ is encoded by the *PLA2G6* gene on chromosome 22q13.1 with 16 exons. *PLA2G6* produces several products by alternative splicing, and at least two are enzymatically active proteins. GVIA-1 iPLA$_2$ was first cloned as a 752 amino acid protein with a molecular mass of 85 kDa including eight ankyrin repeats at the N terminus of the catalytic domain and containing the GXS^{465}XG lipase consensus sequence originally identified in the mouse enzyme (Ackermann et al., 1994; Tang et al., 1997). However, this variant was not a full-length protein in humans. In 1998, a longer isoform, an 806 amino

acid protein with a molecular mass of 88 kDa, was identified in human cells as a product of alternative splicing and named GVIA-2 iPLA$_2$ (Larsson et al., 1998; Ma et al., 1999). GVIA-2 iPLA$_2$ contains a 54 amino acid proline-rich insertion from exon 8 disrupting the eighth ankyrin repeat resulting in a longer linker region between the seventh ankyrin repeat and the lipase domain (Fig. 2A) (Ma et al., 1999). Both the long and short variants of GVIA iPLA$_2$ are found in human and rat cells in a tissue-dependent manner (Larsson Forsell et al., 1999; Ma et al., 1999; Winstead et al., 2000). However, GVIA-1 iPLA$_2$ seems to be the only active variant in hamster and mouse cells (Winstead et al., 2000). It was reported that both variants are Ca^{2+}-independent enzymes and function as oligomers (Ma et al., 1999). However, the enzymatic activity of the long variant is upregulated by ATP, whereas the short variant is not (Ma et al., 1999). Since it has been suggested that the ankyrin repeat is important for oligomer formation of the GVIA iPLA$_2$ (Tang et al., 1997), the 54 amino acid insertion may affect its oligomer formation and function. Still, the details have not been well described. Also, it has been suggested that the 54 amino acid insertion induces the membrane localization of GVIA iPLA$_2$ (Larsson Forsell et al., 1999).

In addition to these two enzymatically active variants, several inactive variants have been identified. GVIA Ankyrin-1 and −2 (GVIA Ank-1 and −2) are products of the *PLA2G6* gene, but they are enzymatically inactive due to the termination of translation before the lipase domain (Larsson et al., 1998). GVIA Ank-1 shares the same amino acid sequence from residue 1 to 476 with GVIA-2 iPLA$_2$, which contains the 54 amino acid insertion and is terminated at residue 479. On the other hand, GVIA Ank-2 lacks residues 70 to 143 of the GVIA Ank-1 due to skipping of the second exon and an altered C-terminal 50 amino acids, resulting in a protein of 427 amino acids (Fig. 2A). These variants are enzymatically inactive, but it was reported that they suppress the activity of GVIA iPLA$_2$ by forming a hetero-dimer, suggesting that the ankyrin repeats of GVIA iPLA$_2$ contribute to its oligomerization (Larsson et al., 1998). In 2018, the X-ray crystal structure of GVIA-1 iPLA$_2$ was solved, although several residues were lacking, but it revealed that the GVIA iPLA$_2$ forms a homo dimer through their catalytic domains (Fig. 2B) (Malley et al., 2018). Also, the crystal structure identified an additional ankyrin repeat with a less conserved signature sequence (Fig. 2A). These findings suggested that the GVIA iPLA$_2$ homo dimer forms a hetero oligomer through another coupling protein binding to ankyrin repeats; still further investigation is needed to more precisely define the oligomerization state of active GVIA iPLA$_2$. It was reported that recombinant GVIA iPLA$_2$ protein lacking ankyrin repeats is inactive (Larsson et al., 1998). Therefore, although understanding just how ankyrin repeats function is poorly understood, ankyrin repeats are clearly critical for proper GVIA iPLA$_2$ function.

GVIA iPLA$_2$ is predominantly localized in the cytosol (Gross et al., 1993). Still, it has been reported that GVIA iPLA$_2$ shows multiple subcellular localizations including plasma membrane, endoplasmic reticulum (ER), mitochondria, Golgi, and the nucleus, depending on the cell type and stimulation (Bao et al., 2004; Chiu et al., 2017; Ramanadham et al., 2004; Song, Bao, et al., 2010). The membrane-binding region of the lipase domain of GVIA iPLA$_2$ was identified by hydrogen/deuterium exchange mass spectrometry (HDX-MS) and molecular dynamics (MD) simulations (Bucher et al., 2013; Hsu et al., 2009). These studies showed that the membrane anchoring region formed an amphipathic helix

(residues 710 to 724) which penetrates the surface of the lipid bilayer (Fig. 2C) (Bucher et al., 2013). Furthermore, the MD simulation suggested that the membrane anchoring region also functioned as a lid covering the active site of GVIA iPLA$_2$ in the absence of membrane, which upon membrane association undergoes a conformational change, contributing to the allosteric effect of the membrane (Bucher et al., 2013; Mouchlis & Dennis, 2022).

It is worth mentioning that the crystal structure failed to elucidate details of the membrane anchoring region, indicating the flexibility of the region (Malley et al., 2018). Although the membrane binding region was proposed, the membrane binding region itself does not fully explain the complex localization of GVIA iPLA$_2$. Although the detailed mechanisms of how intracellular localization of GVIA iPLA$_2$ is regulated are still enigmatic, caspase-3 cleavage of GVIA iPLA$_2$ at the N-terminal cleavage site in ankyrin repeats (^{180}DVTD^{183}) induces nucleus association (Ramanadham et al., 2004). Also, it has been reported that GVIA iPLA$_2$ interacts with the ER chaperone calnexin, and the interaction might be through ankyrin repeats (Malley et al., 2018; Song, Rohrs, et al., 2010). These observations indicate that the ankyrin repeats have an essential role in the regulation of subcellular localization of GVIA iPLA$_2$. Indeed, our preliminary data shows that GVIA iPLA$_2$ ankyrin repeat domains that are lacking the lipase domain shows specific subcellular localization. The underlying mechanism of the unique regulation of the subcellular localization needs further investigation.

3. Regulation of GVIA iPLA$_2$

GVIA iPLA$_2$ is expressed ubiquitously in humans. It was reported that the *PLA2G6* gene is regulated by the sterol regulatory element binding protein-1 (SREBP-1) through its sterol regulatory element (Lei et al., 2010). In addition, it has been suggested that a transcription factor ELK1 binds to the promotor region of the *PLA2G6* gene and may regulate its expression according to a database search, although experimental evidence is lacking (Matys et al., 2006; W. Y. Sun et al., 2021). Other than that, the promoter region of the *PLA2G6* gene contains a region homologous to the consensus sequence of several transcriptional factors. Therefore, gene expression of *PLA2G6* might be exquisitely regulated by multiple factors. Although studies on transcriptional regulation of GVIA iPLA$_2$ have not progressed far, understanding the regulation would be helpful in controlling the expression of GVIA iPLA$_2$ to treat related diseases.

GVIA iPLA$_2$ binds ATP, and its enzymatic activity is upregulated or protected by ATP in a phosphorylation-independent manner (Ackermann et al., 1994). It has been suggested that ATP increases the thermodynamic stability of the enzyme preventing inactivation since the effect of ATP on the GVIA-1 iPLA$_2$ activity is almost completely compensated for by adding 10% glycerol to stabilize the enzyme *in vitro* (Lio & Dennis, 1998). Later, it was reported that ATP protects the inactivation of GVIA-1 iPLA$_2$ by oxidation of cysteine (Song et al., 2006). The authors showed that ATP and dithiothreitol (DTT) prevent disulfide bond formations leading to inactivation at 37°C. However, since DTT could not completely compensate for the effect of ATP, the authors argued that ATP prevents loss of activity by undefined mechanisms. ATP might also stabilize GVIA-2 iPLA$_2$, but the stabilization cannot fully explain the effect of ATP on GVIA-2 iPLA$_2$ (Lio & Dennis, 1998;

Ma et al., 1999), suggesting that ATP can be an activator for GVIA-2 iPLA$_2$ somehow. GVIA-1 and −2 iPLA$_2$ possess consensus residues for a nucleotide-binding motif GXGXXG (^{485}GGGVKG^{490} for GVIA-2 iPLA$_2$) found in protein kinases in the lipase domain (Hanks et al., 1988). Therefore, it has been thought that ATP binds to the region. However, Malley *et al.* reported that ATP interacts with the ankyrin repeat domain of GVI iPLA$_2$ (sixth ankyrin repeat) by co-crystallization with ATP, although its resolution was insufficient to show the detailed binding mode (Malley et al., 2018). Investigating the detailed ATP binding mode has the potential of revealing novel insights into understanding its activation mechanisms and help develop activators of GVIA iPLA$_2$.

Despite GVIA iPLA$_2$ activity being independent of Ca^{2+}, Ca^{2+}-activated calmodulin (CaM) has been proposed to inhibit its activity by direct binding of calmodulin to GVIA iPLA$_2$ (Wolf & Gross, 1996). It has been suggested that GVIA iPLA$_2$ interacts with CaM through CaM binding motifs such as the 1–9-14 motif (^{676}IRKGQANKVKKLSI^{689} for GVIA-2 iPLA$_2$) and IQ motif (^{755}IQYFRLNPQLGTDI^{768} for GVIA-2 iPLA$_2$) which reside in the lipase domain (Fig. 2A, C) (Jenkins et al., 2001). Recently, it was shown that CaM most likely binds to the 1–9-14 motif, not to the IQ motif, and dimer formation is critical for CaM binding to GVIA iPLA$_2$ using a fluorescent polarization assay (Malley et al., 2018). Also, it has been reported that GVIA iPLA$_2$ forms a signaling complex with calcium/calmodulin-dependent kinase IIβ (CaMKIIβ) (Wang et al., 2005).

It is known that GVIA iPLA$_2$ is regulated by caspase-3 cleavage via several cleavage sites. As described earlier, GVIA iPLA$_2$ is processed into a 63 kDa isoform by caspase-3 at the N-terminal cleavage site and is activated and accumulated at the nucleus, presumably destroying the nuclear membrane upon apoptosis (Ramanadham et al., 2004). In addition, a 70 kDa GVIA iPLA$_2$ truncated at the C-terminal was found in INS-1 insulinoma cells and specifically participated in insulin secretion (Ramanadham et al., 2003). However, the detailed description of truncated GVIA iPLA$_2$s is lacking in other than pancreatic cells.

4. Substrate specificity of GVIA iPLA$_2$

When the murine GVIA iPLA$_2$ was purified and cloned, *in vitro* substrate specificity toward the *sn*-2 acyl chain was investigated (Ackermann et al., 1994; Tang et al., 1997). These later papers independently reported that GVIA iPLA$_2$ showed higher activity toward *sn*-2 palmitic acid (16:0) over arachidonic acid (AA, 20:4) in contrast to cPLA$_2$, which is highly selective for the *sn*-2 AA chain (Clark et al., 1991). Furthermore, Tang *et al.* reported that *sn*-2 linoleic acid (18:2) and oleic acid (18:1) are better substrates for GVIA iPLA$_2$ than AA (Tang et al., 1997). Also, they reported that GVIA iPLA$_2$ showed remarkably higher activity toward the phospholipid with a head group of PA than PC. However, the limitation of available pure glycerophospholipid species and the activity detection methods at the time which included radioactivity and colorimetric methods, prevented determining the detailed *in vitro* substrate specificity of GVIA iPLA$_2$.

The recent evolution of lipid synthetic methodology has resulted in the commercial availability of pure phospholipid molecular species standards and the development of mass spectrometry based lipidomics has enabled us to investigate the detailed *in vitro*

substrate specificity of PLA_2 enzymes toward at least all the major molecular species of glycerophospholipids. In 2018, a lipidomics-based *in vitro* PLA_2 activity assay using HPLC-MS/MS was developed (Mouchlis et al., 2018). The assay can evaluate PLA_2 activity by quantifying the lysophospholipid products of PLA_2 activity toward mixed micelles containing specific glycerophospholipids and the nonionic surfactant (octaethylene glycol monododecyl ether, $C_{12}E_8$). The substrate specificity of the GVIA-2 $iPLA_2$ toward PC containing myristic acid (14:0), 16:0, stearic acid (18:0), 18:1, 18:2, 20:4, and docosahexaenoic acid (DHA, 22:6) on the *sn*-2 position was investigated using this assay.

It was found that phospholipids containing *sn*-2 14:0 and 18:2 were the optimum substrates for the human recombinant GVIA-2 $iPLA_2$ (Mouchlis et al., 2018). To explain this unique *sn*-2 acyl chain specificity, all-atom MD simulations were carried out using a complex of each substrate and the lipase domain of the GVIA $iPLA_2$. The substrates were docked in the active site based on the optimal binding observed in earlier simulations (Mouchlis et al., 2015). The MD simulations suggested that GVIA $iPLA_2$ utilizes two alternative hydrophobic sub-pockets to accommodate the *sn*-2 acyl chain; one is composed of aliphatic hydrophobic residues such as Leu491, Ile494, Ile523, Leu524, Leu564, Met537, and Leu560 suitable for accommodating short saturated acyl chains like 14:0; the other site contained aromatic residues such as Tyr541, Tyr555, and Phe644 that can interact with double bonds in the acyl chain via pi-pi stacking and is suitable for unsaturated acyl chains like 18:2 (Fig. 3A) (Mouchlis et al., 2018). Furthermore, the study revealed that GVIA-2 $iPLA_2$ slightly prefers the head group of PE in the equal molar mixture of major head groups (PC, PE, PS, PG, PA), although the preference was mild.

Thereafter, the more detailed substrate specificity of GVIA-2 $iPLA_2$ was investigated using the same method especially focusing on AA, eicosapentaenoic acid (EPA, 20:5), and DHA precursors of various bioactive oxylipins (Dennis & Norris, 2015; Willenberg et al., 2015). In the study, we reported that GVIA-2 $iPLA_2$ shows significantly higher activity toward *sn*-2 EPA among AA, EPA, and DHA containing PE even in a mixture of phospholipids containing various *sn*-2 acyl chains and that activity toward EPA was comparable to that toward 18:2 (Fig. 3B) (Hayashi et al., 2021). Furthermore, MD-simulations revealed that, in contrast to the case of 14:0 and 18:2, GVIA $iPLA_2$ significantly and stably accommodated EPA in the shallow hydrophobic cavity by adopting both aliphatic and aromatic residues to form a hydrophobic environment fitting the curvature of the EPA chain (Fig. 3C). In contrast, we suggested that hydrophobic subsites for the *sn*-2 acyl chains seemed not to be able to easily retain a DHA chain due to its additional two carbons. This series of studies revealed the detailed *in vitro* substrate specificity of GVIA $iPLA_2$ toward various major glycerophospholipids. We concluded that the geometry of the hydrophobic pocket contributes to the binding pose of the ligands and confers the unique substrate specificity on the GVIA $iPLA_2$ (Hayashi et al., 2021; Mouchlis et al., 2015, 2018). Furthermore, an *ex vivo* study using rat hearts and PLA_2 inhibitors recently reported that GVIA $iPLA_2$ preferentially releases EPA rather than DHA (Manson et al., 2023). This indicates that the *in vitro* specificity and the molecular dynamics features of GVIA $iPLA_2$ have relevance to its *in vivo* functions.

As shown in Fig. 1, glycerophospholipids can possess alkyl ether linkages or vinyl ether linkages instead of an ester linkage in their *sn*-1 position, and this affects the biological activities (Dean & Lodhi, 2018). The former is a precursor to PAF (1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) when the sn-2 chain is converted to an acetyl group. The latter exists ubiquitously as a component of cell membranes and contributes to the formation of lipid raft microdomains (Pike et al., 2002; Rodemer et al., 2003), membrane trafficking, and cell differentiation (Dean & Lodhi, 2018; Hossain et al., 2016; Komljenovic et al., 2009; Teigler et al., 2009; Tsukahara et al., 2006).

We have investigated the effect of the *sn*-1 acyl chain linkage using the *in vitro* assay and reported that GVIA-2 iPLA$_2$ showed lower activity toward *sn*-1 alkyl ether phospholipids compared to *sn*-1 ester and vinyl ether phospholipids by up to a factor of two (Fig. 3C) (Hayashi et al., 2022). However, no significant difference was observed between the activity toward ester and vinyl ether phospholipids. Detailed explanations for how the GVIA iPLA$_2$ distinguishes the *sn*-1 acyl chain linkage are unknown, but the previous MD simulation showed that Lys729 in the active site of GVIA iPLA$_2$ frequently interacts with the *sn*-1 acyl chain linkage (Fig. 3E). Therefore, the *sn*-1 acyl chain linkage is likely to contribute to the binding affinity of a substrate in the active site. Also, PLA$_2$ extracts a single substrate from the glycerophospholipid pool to hydrolyze the substrate (Mouchlis et al., 2015). MD simulations revealed how a single glycerophospholipid is pulled into GVIA iPLA$_2$ active site, and several residues interact with the *sn*-1 linkage upon the extraction (Fig. 3D). Therefore, although further investigation is needed to determine if and how the *sn*-1 acyl chain linkage might play a role in the substrate extraction by GVIA iPLA$_2$. Notably, the study also revealed that the *sn*-1 acyl chain linkage does not significantly affect the *sn*-2 acyl chain specificity and that the GVIA iPLA$_2$ prefers steric acid at the *sn*-1 position. Also, consistent with the previous report, the GVIA iPLA$_2$ possesses hydrolase activity toward PAF, although its activity was far lower than that toward longer *sn*-2 acyl chains (Hayashi et al., 2022; Tang et al., 1997).

Although the detailed *in vitro* substrate specificity of GVIA iPLA$_2$ has been clarified, sometimes there is a gap between *in vitro* specificity and observations in *in vivo* and *ex vivo* studies. DHA-containing glycerophospholipids are highly abundant in the brain (Brenna & Diau, 2007; C. T. Chen et al., 2009), and several studies reported that *PLA2G6* knockout mice show impaired DHA metabolism in the brain (Basselin et al., 2010; Beck et al., 2011; Cheon et al., 2012). However, *in vitro* specificity indicated that at least GVIA-2 iPLA$_2$ shows inferior activity toward DHA compared to AA and EPA in equivalent phospholipids (Hayashi et al., 2021; Mouchlis et al., 2018). Also, our *in vitro* study demonstrated that GVIA-2 iPLA$_2$ slightly prefers glycerophospholipids with 18:0 rather than 16:0 at the *sn*-1 position (Hayashi et al., 2022; Mouchlis et al., 2018). However, it has been demonstrated that GVIA iPLA$_2$ preferentially hydrolyzes glycerophospholipids which have 16:0 in the *sn*-1 position in certain cultured cells and *PLA2G6* knockout mice (Deng et al., 2016; Gil-de-Gómez et al., 2014; Murakami et al., 2005). Substrate availability might be one of the reasons for the gap between *in vitro* and *in vivo* observations. These studies suggested that GVIA iPLA$_2$ hydrolyzes these substrates somewhat, even if they are a poorer substrate for GVIA iPLA$_2$ *in vitro,* depending on substrate availability. Indeed, it was reported that GVIA iPLA$_2$ preferentially hydrolyzed plasmalogen over ester lipids as a source of AA in

certain cells upon thrombin stimulation; however, GVIA iPLA$_2$ utilized ester phospholipids in the plasmalogen-deficient cells without any changes in the AA release. (Beckett et al., 2007; Gaposchkin et al., 2008). Perhaps some unknown factors alter the substrate specificity of GVIA iPLA$_2$, such as interaction with other proteins, post-transcriptional modifications, and alternative splicing. So far, we have been focused on the *in vitro* specificity of the more abundant GVIA-2 iPLA$_2$. Thus, it may be worth investigating further the specificity of the GVIA-1 iPLA$_2$ or the caspase-3 cleaved forms in case they play larger roles in specific brain regions or sub-organelles. Of course, other PLA$_2$'s, such as sPLA$_2$'s may exist in brain and play a role in releasing DHA.

In summary, by taking advantage of new lipidomics and computational techniques, we have been able to establish the *in vitro* substrate specificity of the human Group VIA-2 iPLA$_2$ and its underlying molecular mechanism. However, some open questions remain. One is determining the activity of GVIA iPLA$_2$ toward the numerous PI monophosphate isomers as well as PIP$_2$ and PIP$_3$, for which the lipidomics-based PLA$_2$ assay will have to be modified in order to detect their more polar lysophospholipid products. Another is the specificity of GVIA iPLA$_2$ toward oxidized phospholipids. It has been reported that GVIA iPLA$_2$ expresses a protective role against oxidative stress by hydrolyzing the oxidized acyl chains (Cabo et al., 2006; Kinghorn et al., 2015). However, due to the lack of availability of pure substrates for the *in vitro* assay, the specificity and molecular interactions remain unclear. Furthermore, we previously showed that GVIA iPLA$_2$ can hydrolyze cardiolipins, which are important mitochondria-specific phospholipids whose metabolism is linked to the pathogenesis of Barth syndrome (Hsu et al., 2013; Malhotra et al., 2009). However, the detailed activity toward various cardiolipin molecular species and its binding modes in the active site have not been investigated.

5. Role of GVIA iPLA$_2$ in neurodegenerative diseases

GVIA iPLA$_2$ is expressed ubiquitously in humans, including the brain, and widely exists in different brain regions such as the hypothalamus, hippocampus, cerebral cortex, midbrain, and striatum (Molloy et al., 1998). Various neurodegenerative diseases that are believed to result from mutations in the *PLA2G6* gene have been reported, and now, they are recognized as *PLA2G6*-associated neurodegeneration (PLAN) diseases (Guo et al., 2018). PLAN diseases includes heterogeneity pathology and a phenotype in several neurodegeneration diseases including infantile neuroaxonal dystrophy (INAD), atypical neuroaxonal dystrophy (ANAD), and adult-onset dystonia-parkinsonism (DP). INAD appears during the first 6 months to 3 years of life and rapidly progresses to neurodegenerative disease characterized by brain iron accumulation. In contrast, ANAD appears in early childhood to the juvenile phase, and is also characterized by brain iron accumulation. DP is an adult-onset neurodegenerative disease which appears between 20 to 40 years of age. The PLAN-related mutated regions are widespread in the GVIA iPLA$_2$, including in the ankyrin repeats, and the specific mutation affects the specific symptoms and age of onset (Guo et al., 2018; Morgan et al., 2006; Paisan-Ruiz et al., 2009). Also, mutations of the *PLA2G6* gene are recognized as a critical risk factor for Parkinson's disease and identified as a disease locus PARK14 (Paisan-Ruiz et al., 2009; Tomiyama et al., 2011; Yoshino et al., 2010).

In 2006, Morgan *et al.* identified 44 unique mutations, including 32 missense mutations in the *PLA2G6* gene from patients with INAD and neurodegeneration with brain iron accumulation (NBIA) (Morgan et al., 2006). The mutations were widely spread along the sequence of the GVIA iPLA$_2$, including in the ankyrin repeat and the catalytic domain as well as in a 54 amino acid insertion in GVIA-2 iPLA$_2$. Since these mutations were found in both INAD and NBIA, mutations of *PLA2G6* have been thought to result in childhood onset of neurodegenerative diseases. In 2009, two individual missense mutations in the *PLA2G6* gene were discovered in patients with adult-onset DP (Paisan-Ruiz et al., 2009; Sina et al., 2009). Since then, considerable attention has been paid to the *PLA2G6* gene in the adult onset of neurodegenerative diseases in addition to those with childhood-onset, such as INAD and ANAD. Many of these mutations are likely to result in a loss of activity. Indeed, systemic *PLA2G6* null and mutated mice show neurodegenerative phenotypes with degeneration of axons and dendrites in neuronal cells (Malik et al., 2008; Shinzawa et al., 2008; Wada et al., 2009). However, it is still an open question as to why *PLA2G6* mutations correlate with the pathology and phenotype of neurodegeneration diseases. Interestingly, some of the mutations found in DP, such as R741W, were also found in INAD patients (Morgan et al., 2006; Paisan-Ruiz et al., 2009).

In mice studies, although the mutated GVIA iPLA$_2$ expressed in the mice show null PLA$_2$ activity, the phenotype and the age of onset were different from that of GVIA iPLA$_2$ protein null mice (Malik et al., 2008; Shinzawa et al., 2008; Wada et al., 2009), suggesting that existence of inactive protein might also play a role in the brain. Interestingly, it has been reported that some mutations of GVIA iPLA$_2$ found in patients with DP, R741Q, R747W, and R632W, showed comparable or higher PLA$_2$ and PLA$_1$ activity compared to the wild-type enzyme, but similar mutations found in patients with INAD did not (Engel et al., 2010). Thus, a significant number of mutations of *PLA2G6* associated with various neurodegeneration diseases have been reported, but the role of each mutant in the pathogenesis and phenotype is still enigmatic.

GVIA iPLA$_2$ has been recognized as an enzyme responsible for membrane remodeling and maintaining membrane lipid homeostasis (Balsinde et al., 1995, 1997). Therefore, the cause of PLAN has been thought to relate to membrane homeostasis (Malik et al., 2008). As described earlier, GVIA iPLA$_2$ is a responsible enzyme for the metabolism of DHA in the brain (Basselin et al., 2010; Beck et al., 2011; Cheon et al., 2012). Cheon *et al.* compared the lipid compositions in the brain of wild-type and *PLA2G6* knockout mice and reported that the knockout mice impair DHA metabolism in the brain (Cheon et al., 2012). Also, it is known that DHA plays a critical role in neuronal cell survival and health (G. Y. Sun et al., 2018). Therefore, loss of GVIA iPLA$_2$ function appears to cause various neurological disorders via impairment of DHA metabolism in the brain. Furthermore, these authors also reported a comprehensive comparison of the esterified fatty acid composition in the various lipid species in the brain and observed differences in the compositions. Therefore, the impairment of brain phospholipid composition is thought to be a cause of PLAN diseases. In addition, many functions of GVIA iPLA$_2$ related to the pathogenesis of PLAN have been reported so far.

It is well known that Lewy body formation is one of the key pathogenesis findings in Parkinson's disease, and Lewy body's are mainly formed with presynaptic protein α-synuclein (Deramecourt et al., 2006; Mahul-Mellier et al., 2020). Indeed, Lewy body accumulation in the brain from PLAN patients has been observed (Paisán-Ruiz et al., 2012). A study using *PLA2G6* knockout mice showed the expression level of α-synuclein increases in mitochondria and suggested that α-synuclein stabilizes mitochondrial membranes in *PLA2G6* deficient neurons (Beck et al., 2016). Further, a study in Drosophila revealed that GVIA iPLA$_2$ deficiency accelerates α-synuclein accumulation and that the accumulation was restored by oleic acid supplementation, which resulted in the normalization of brain lipid composition (Mori et al., 2019). These studies indicate that GVIA iPLA$_2$ plays a critical role in Lewy body formation through its membrane remodeling function.

More recently, it was reported that GVIA iPLA$_2$ prevents ferroptotic cell death, which is triggered by iron-dependent lipid peroxidation by hydrolyzing oxidized phospholipids, and that a *PLA2G6* mutation induces ferroptotic neuronal cell death leading to PLAN (D. Chen et al., 2021; W. Y. Sun et al., 2021). The authors reported that the R747W mutant, which relates to DP, attenuates the activity of GVIA iPLA$_2$ toward oxidized phospholipids such as 15-hydroperoxy-arachidonoyl-phosphatidylethanolamine without significant effect on the activity toward AA metabolism and promotes ferroptotic cell death leading to Parkinsonian phenotype (W. Y. Sun et al., 2021). Therefore, the activity of GVIA iPLA$_2$ toward oxidized phospholipids is involved in ferroptosis and is also critical in the pathogenesis of PLAN. Notably, the study shows for the first time that the mutation alters the substrate specificity of GVIA iPLA$_2$, although the mutation shows a minimal effect on the basal PLA$_2$ activity. The authors suggested by taking advantage of MD simulations that the R747W mutation reduces the accessibility of the substrates to the active site and the association with the membrane.

By taking advantage of the D331Y mutation, which resides in ankyrin repeats of GVIA iPLA$_2$, found in Asian patients with autosomal-recessive early-onset parkinsonism (AREP) (Lu et al., 2012; Shi et al., 2011), several studies reported the functions of GVIA iPLA$_2$ in the neurons. The D331Y mutant shows less than 50% of PLA$_2$ activity compared to a normal GVIA iPLA$_2$ (Chiu et al., 2019). Chiu *et al.* reported that D331Y knock-in mice exhibited mitochondrial dysfunction, mitophagy impairment, and ER stress (Chiu et al., 2019). Further, another group reported using patient-delivered induced pluripotent stem cells (iPSC) from dopaminergic neurons possessing a homozygous D331Y mutant that the cell exhibited similar PD-related cascade events, including ER stress as in the former report (Ke et al., 2020). In contrast, although this observation was in pancreatic β cells, GVIA iPLA$_2$ upregulates ER stress and apoptosis through the production of proinflammatory cytokines, and inhibition of GVIA iPLA$_2$ by a specific inhibitor prevents β cell death (Ali et al., 2013; Lei et al., 2014). This opposite role of GVIA iPLA$_2$ in ER stress can be attributed simply to the difference in cell type, but the details remain unclear. Since the mutation is in the ankyrin repeats near the putative ATP binding residues, the specific function of ankyrin repeats might contribute to the effect. Interestingly, it has been demonstrated that DHA supplements improved motor dysfunction of D331Y homozygous knock-in mice, suggesting DHA can be critical for the biological event related to the D331Y mutation (Yeh et al., 2021).

Among the GVI iPLA$_2$s, GVIB iPLA$_2$ (iPLA$_2\gamma$) is also known to be implicated in neurodegenerative diseases (Mancuso et al., 2009). It has been reported in animal studies that GVIB iPLA$_2$ is involved in mitochondrial lipid metabolism and increases and prevents mitochondrial lipid peroxidation similar to several mutations in the GVIA iPLA$_2$ (Chao et al., 2018; Mancuso et al., 2009). GVIB iPLA$_2$ is an enzyme that consists of 782 amino acids and possesses both PLA$_2$ and PLA$_1$ activity (Mancuso et al., 2000; Yan et al., 2005). Both GVIA and GVIB iPLA$_2$ are specifically inhibited by (*S*)- and (*R*)-bromoenol lactone (BEL), respectively, and for each the specific optical isomer is critical to the inhibition (Jenkins et al., 2002). GVIB PLA$_2$ lacks the ankyrin repeat domain, and the homology between GVIA and GVIB is low. However, it was observed in the AlphaFold-predicted structure of the GVIB iPLA$_2$ that the three-dimensional structure of the catalytic domain of GVIB iPLA$_2$ is well aligned with that of GVIA iPLA$_2$ (RMSD was 1.23) (Fig. 4) (Jumper et al., 2021; Varadi et al., 2022). Therefore, it is possibile that GVIB iPLA$_2$ shares some functions and substrate specificity with the GVIA iPLA$_2$ although further structural and biochemical investigations are needed.

More than 100 unique pathogenic mutations on *PLA2G6* have been reported so far (Guo et al., 2018). However, many of the reports do not characterize the effect of the mutation on the GVIA iPLA$_2$ enzyme, and how they contribute to the pathogenesis. Therefore, further molecular and biochemical studies are needed in order to better understand the etiology and pathogenesis of PLAN.

6. Conclusions and future directions

We have reviewed the emerging evidence for a central role of GVIA iPLA$_2$ in several important neurodegenerative diseases (Fig. 5). However, despite the recent understanding of the physiological function of GVIA iPLA$_2$, and our new understanding of the mechanism of action and specificity of GVIA iPLA$_2$ at the molecular level, there is still much to learn about the function of GVIA iPLA$_2$. By taking advantage of new lipidomics and computational approaches, the detailed *in vitro* substrate specificity and the enzymes mechanism of action have been revealed, although there are still many details that require further investigation, such as iPLA$_2$ specificity toward oxidized phospholipids. With the solution of the crystal structure of GVIA-1 iPLA$_2$ in 2018, the understanding of iPLA$_2$ structural features should be accelerated and help further understanding of GVIA iPLA$_2$ function. Hopefully, the advances in the basic science underlying iPLA$_2$ action summarized here will lead to new understanding and therapeutic strategies to prevent and/or treat *PLA2G6*-associated diseases, including PLAN.

In particular, the normalization or up-regulation of GVIA iPLA$_2$ should be a straightforward and reasonable strategy to treat or prevent PLAN. By taking advantage of adeno-associated virus (AAV), the delivery of the normal *PLA2G6* gene was attempted, and it was reported that a single dose of AAV carrying the *PLA2G6* gene to *PLA2G6* associated INAD mice significantly improved life span and neuronal viability in the brain (Iankova et al., 2021; Whaler, 2018). Besides, the development of pharmacological chaperons or activators for GVIA iPLA$_2$ mutants can be attempted. Furthermore, computational approaches should be helpful in screening small molecules. However, activation and stabilization mechanisms

of GVIA iPLA$_2$ have not been well enough understood yet to design small molecules. Therapeutic advances of *PLA2G6*-associated diseases will clearly require further study on the detailed molecular mechanism and substrate specificity, and new strategies for activation/inactivation of GVIA iPLA$_2$ are needed.

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Abbreviations:

AA	arachidonic acid
ATP	adenosine triphosphate
CaM	calmodulin
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
GVIA iPLA$_2$	Group VIA calcium-independent PLA$_2$
PLA$_2$	phospholipase A$_2$
PLAN	Phospholipase A$_2$-associated neurodegeneration
PUFAs	polyunsaturated fatty acids

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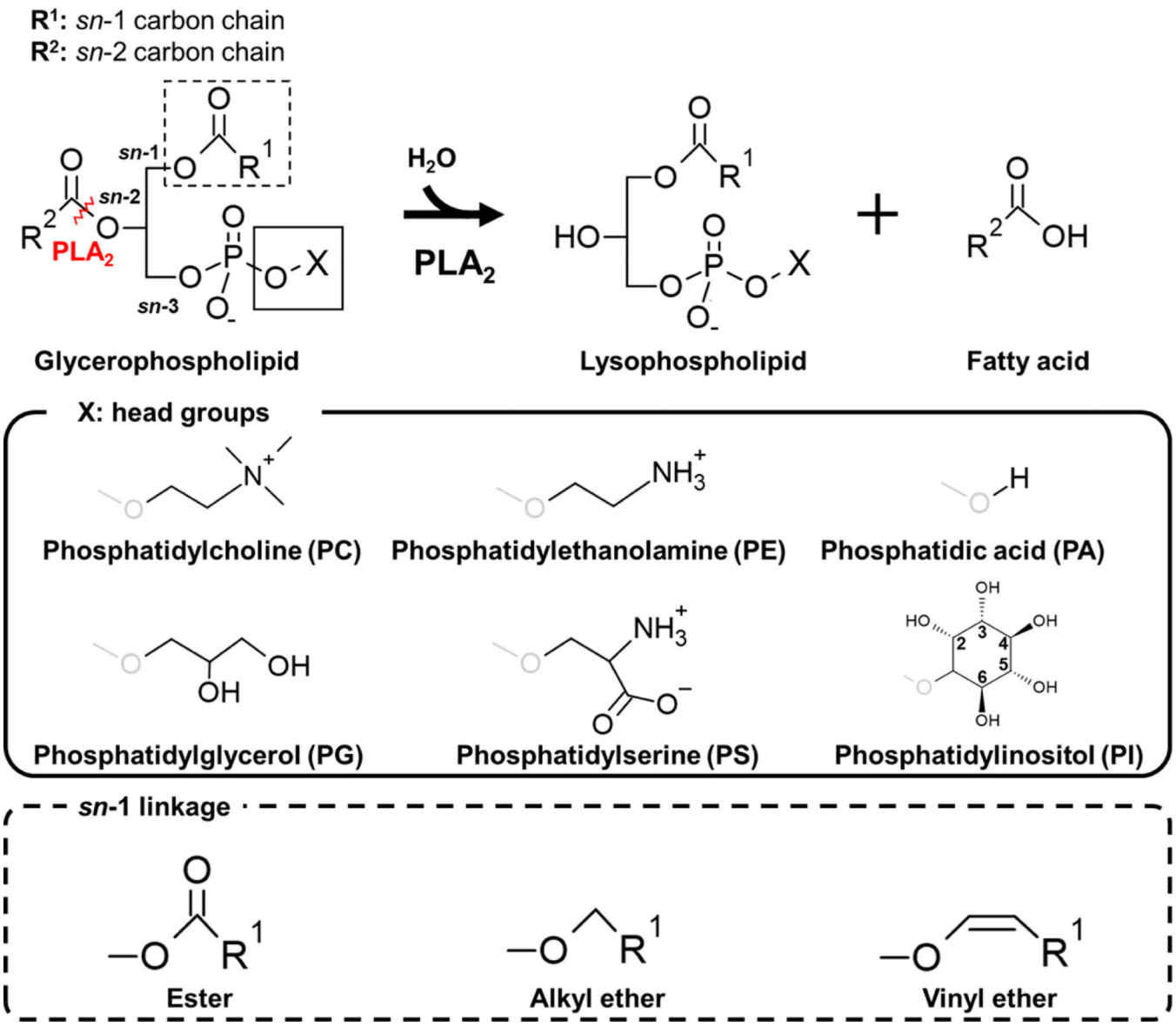

Fig. 1. Enzymatic action of PLA_2 on a variety of glycerophospholipid structures.

Note that phosphatidylinositol (PI) can contain one or more phosphates on each of the inositol backbone positions shown giving rise to numerous additional molecular species.

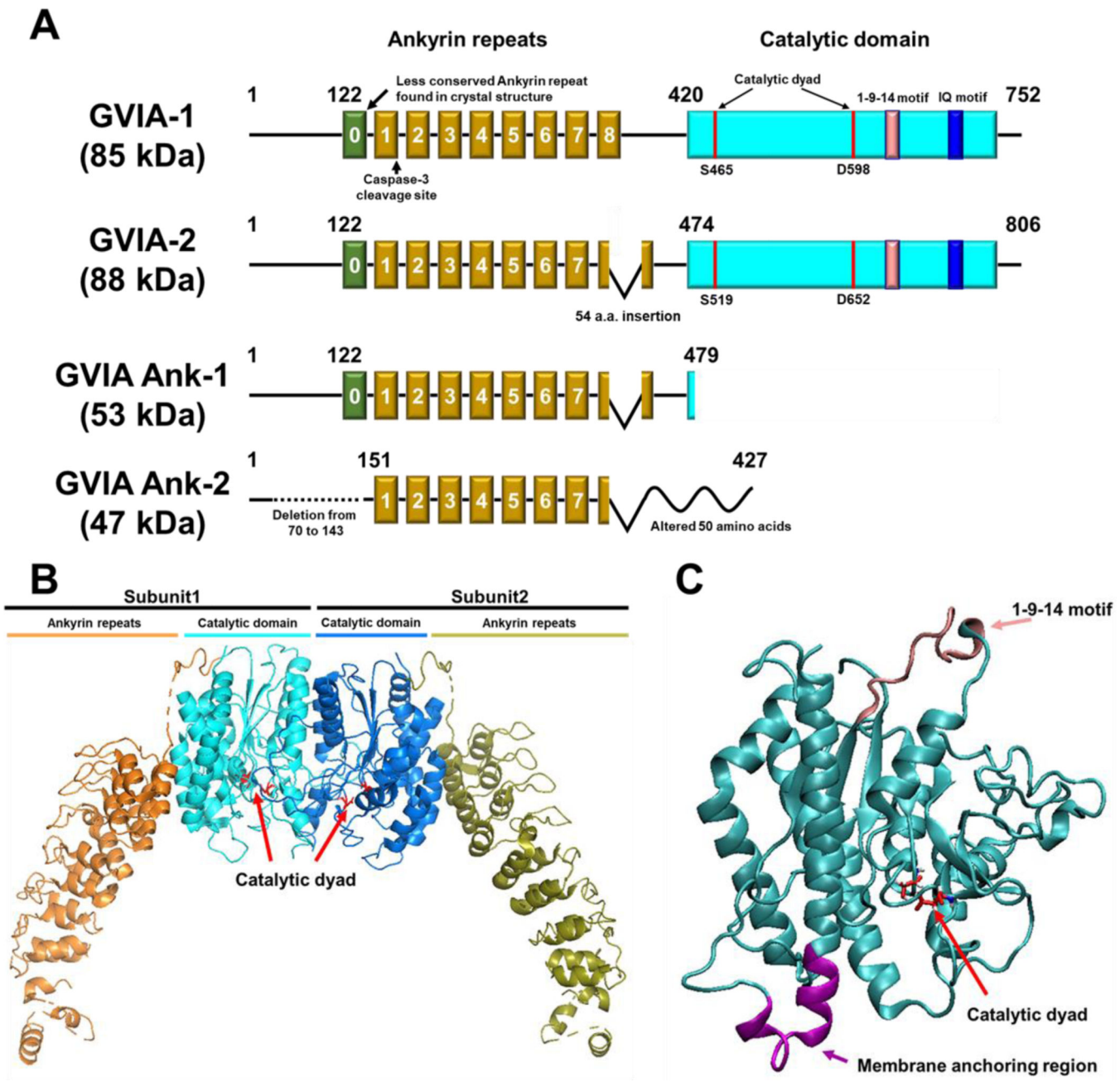

Fig. 2. Structure of GVIA iPLA$_2$.

(A) Schematic presentation of major GVIA iPLA$_2$ splicing variants. **(B)** X-ray crystal structure of short isoform of GVIA iPLA$_2$ dimer with 3.95 Å resolution from (Malley et al., 2018) (Protein Data Bank (PDB) ID: 6AUN). **(C)** Homology model of the catalytic domain of GVIA iPLA$_2$ reproduced from (Mouchlis et al., 2015).

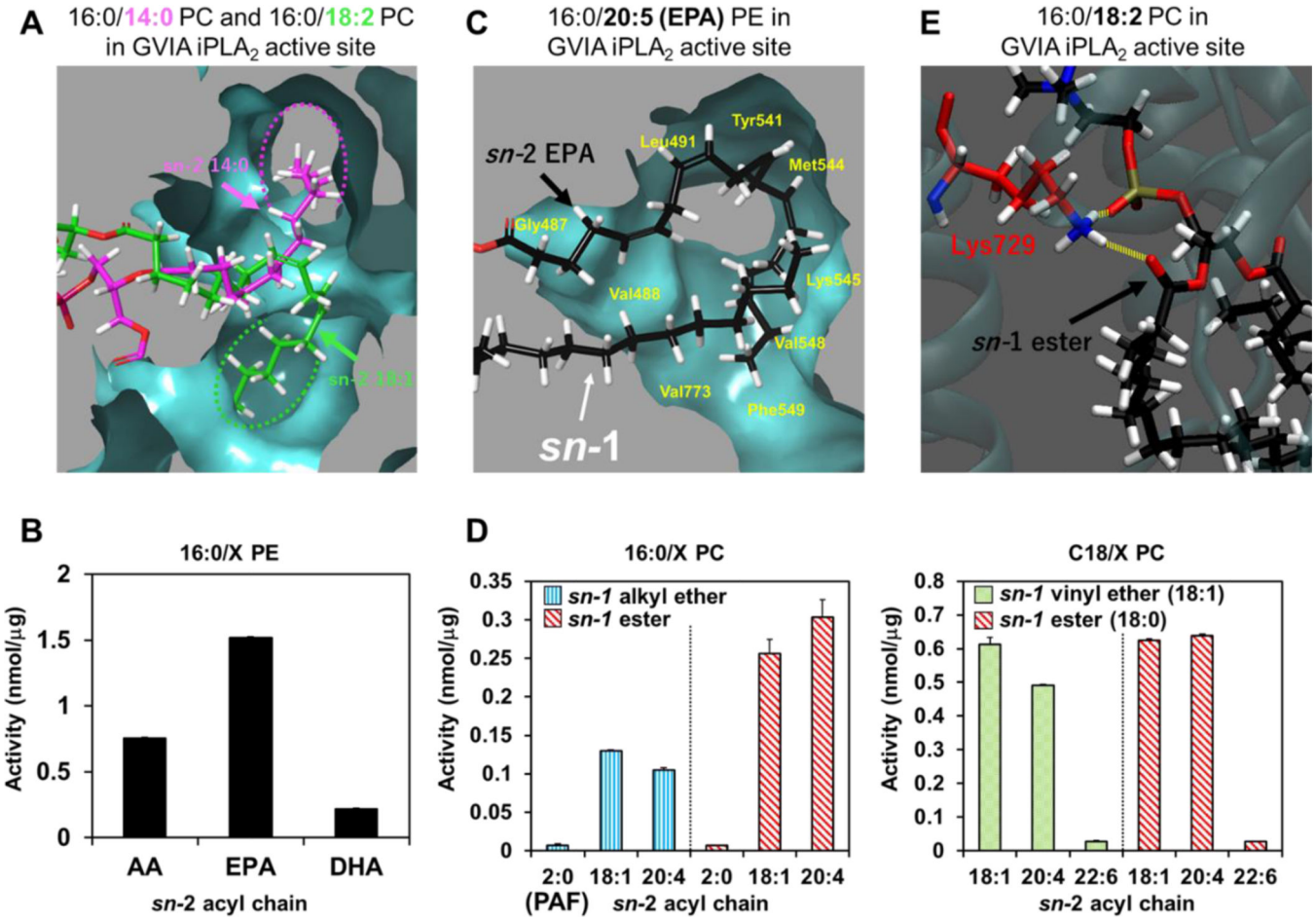

Fig. 3. Substrate specificity of GVIA iPLA$_2$.

(A) Optimal binding mode of *sn*-2 acyl chains of 16:0/14:0 PC (pink) and 16:0/18:2 PC (green) in the hydrophobic subsites of GVIA iPLA$_2$ (cyan surface) after 1 μsec MD simulation. Pink and green dashed lines indicate hydrophobic pockets for *sn*-2 14:0 and 18:1 chains, respectively. The image was newly generated based on the result previously reported (Mouchlis et al., 2018). **(B)** The substrate specificity of GVIA-2 iPLA$_2$ toward *sn*-2 AA, EPA, and DHA in an equal molar mixture of 16:0/20:4 PE, 16:0/20:5 PE, and 16:0/22:6 PE. **(C)** Optimal binding mode of *sn*-2 EPA chain in the hydrophobic subsite of GVIA iPLA$_2$ after 1 μsec MD simulation. The image was newly generated based on the result previously reported (Hayashi et al., 2021). **(D)** The activity of GVIA iPLA$_2$ toward *sn*-1 alkyl ether or ester 16:0 *sn*-2 X PC, where X is 2:0, 18:1, or 20:4 (left panel) and *sn*-1 vinyl ether or ester C18 *sn*-2 X PC, where X is 18:1, 20:4, or 22:6 (right panel). The figures are adapted from our previous report (Hayashi et al., 2022). **(E)** Hydrogen-bonding interaction between Lys729 and *sn*-1 ester linkage of 16:0/18:2 PC in the GVIA iPLA$_2$ active site. The image was newly generated based on the result previously reported (Mouchlis et al., 2015).

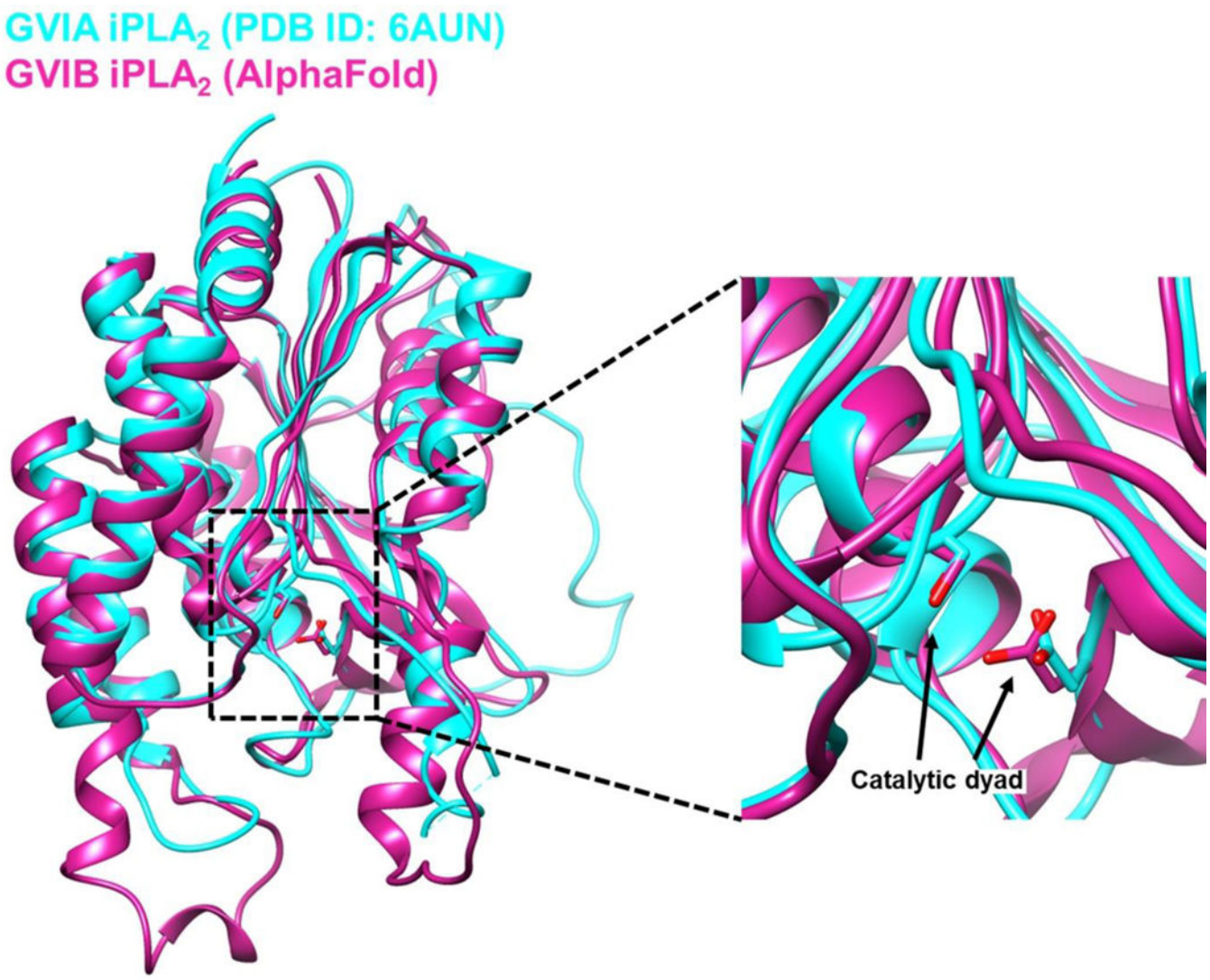

Fig. 4. Alignment of the three-demensional structure of the GVIA and GVIB iPLA$_2$ catalytic domain.

Crystal structure of GVIA iPLA$_2$ (PDB ID: 6AUN, cyan) and AlphaFold-predicted structure of GVIB iPLA$_2$ (purple) were aligned.

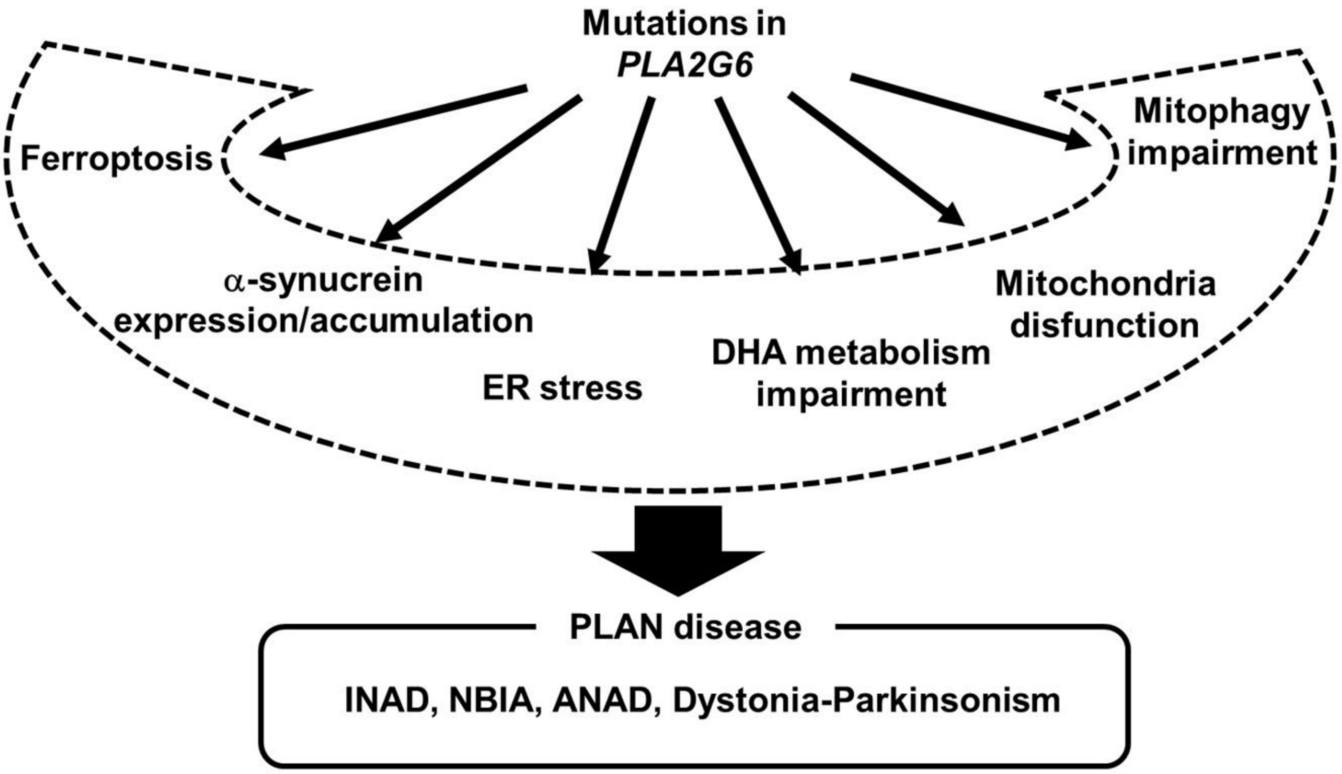

Fig. 5. Schematic illustration of the relationship between mutations in the *PLA2G6* gene and PLAN diseases.