論文の内容の要旨

論文題目：

Characterization of murine cytosolic phospholipase $A_2\varepsilon$

和訳 ： マウス 細胞質型ホスホリパーゼ $A_2\varepsilon$ の 解析

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Mammalian cells contain various kinds of phospholipases that hydrolyze phospholipids to various kinds of products to play important roles in cellular physiology. There are four classes of phospholipases: PLA (PLA_1 and PLA_2), PLB, PLC, and PLD, that are grouped according to the bond hydrolyzed on phospholipids substrates. Among them, phospholipase A_2 hydrolyzes the sn-2 (enriched in polyunsaturated fatty acid) ester bond of glycerophospholipid to release fatty acids and lysophospholipids, and both of which have potent biological activity. Furthermore, subcellular organelles have been known to have different lipid compositions. As a result, the action of phospholipase A_2 at specific membrane compartment may affect the physical properties of cellular membrane that may influence cell shape, endocytotic and secretory processes. Phospholipase A_2 is classified into four categories: cytosolic PLA_2 ($cPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$), secretory PLA_2 ($sPLA_2$), and platelet-activating factor acetylhydrolase (PAF-AH). So far, at least 20 gene loci for human PLA_2 are found.

The cytosolic (group IV) phospholipase A_2 family is composed of six intracellular enzymes named $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$, $cPLA_2\delta$, $cPLA_2\varepsilon$, $cPLA_2\zeta$, all of which have different degrees of homology in lipase and C2 domains to each other except $cPLA_2\gamma$, which doesn't contain C2 domain.

$cPLA_2\alpha$ was first identified by purification of the protein, and now is most extensively studied cytosolic PLA_2 enzyme. It is widely expressed in mammalian cells and has Ca^{2+}-dependent activity, substrate preference for arachidonoyl phospholipids. Until now we have known about many biochemical and physiological property of $cPLA_2\alpha$ such as increased expression of $cPLA_2\alpha$ by certain proinflammatory cytokines and growth factors in some cell lines, regulation of $cPLA_2\alpha$ membrane binding and localization by its catalytic and C2 domains, and phosphorylation, novel nuclear localization, and some $cPLA_2\alpha$ binding proteins. Furthermore, to date, there have been many literatures about functional roles of $cPLA_2\alpha$ in various kinds of disease models and physiological processes.

Human cPLA$_2\gamma$ lacks a C2 domain and is constitutively membrane bound by C-terminal CAAX sequence, which has been shown to be farnesylated in mammalian cells. Enzymatic analysis of purified human cPLA$_2\gamma$ has shown that it has approximately 25-100-fold greater lysophospholipase activity than PLA$_2$ activity. It may also function as a transacylase in HEK293 cells when over-expression. In 2006, a principle variant of endogenous human cPLA$_2\beta$, cPLA$_2\beta$3, was identified in a human lung epithelial cell line (BEAS-2B). It has calcium dependent PLA$_2$ activity against PE but not PC and low level lysophospholipase activity. It was constitutively associated with membrane in BEAS-2B cells and localized to mitochondria and early endosomes. In 2010, murine cPLA$_2\beta$ was found to have an increased activity on phosphatidylcholine vesicles by anionic phosphoinositides and cardiolipin.

In 2005, our research group identified three novel cytosolic phospholipase A2s, murine cPLA$_2\delta$, ε and ζ・・ by wide database queries. The preliminary data showed that they have distinct enzymatic properties, tissue distribution and subcellular localization and suggested that they may have different roles in cellular function. Human cPLA$_2\delta$ was found to be expressed in stratified squamous epithelium of the cervix, fetal skin and also associated with psoriasis. In 2010, cPLA$_2\delta$ was reported to have relatively higher phospholipase A$_1$ activity than PLA$_2$ activity. In 2007, cPLA$_2\zeta$ was found to be expressed in mouse lung fibroblasts and to have higher lysophospholipase and PLA$_2$ activity than cPLA$_2\beta$, which could be inhibited by pyrrolidine-2 and Wyeth-1. In addition, EGFP-cPLA$_2\zeta$ could translocate to membrane ruffles and vesicles in response to ionomycin. Preliminarily cPLA$_2\varepsilon$ was reported to be associated with lysosomes and had Ca^{2+}-dependent PLA$_2$ activity. In 2010, purified human cPLA$_2\varepsilon$ protein was found to have low enzymatic activity but tend to increase activity by anionic phosphoinositides on the vesicle membrane. However, its biochemical characteristics and biological role are still largely unknown.

Several phospholipase A$_2$s are expressed in the central nervous system and play roles in the pathophysiologic aspects. There is increasing evidence for the involvement of cPLA$_2\alpha$ in regulation of inflammation, excitatory functions and plasticity in the brain. Cytosolic PLA$_2\alpha$ was suggested to regulate the persistent decrease in the expression of AMPA receptors, and may play a role in cerebellar LTD (long term depression) and motor learning. In the brain, the basal expression and activity of group VI iPLA$_2$s are higher than other PLA$_2$. In human astrocyte A172 cells, iPLA$_2\beta$ is present, and takes a part in lipid remodeling process. iPLA$_2\gamma$ is present in endoplasmic reticulum of the brain and may be involved in oxidant-induced cell death and lipid peroxidation. Mice with iPLA$_2\beta$ deficiency developed normally, but showed severe motor dysfunction due to degeneration of axons.

Murine cPLA$_2\varepsilon$ was identified by mouse genome search and reported that cPLA$_2\varepsilon$ was expressed in the brain, heart and skeletal muscle detected by northern

blotting. To confirm its endogenous protein and investigate its biological role, polyclonal antibody was generated. Endogenous protein was detected in mouse brain, heart, and skeletal muscle by immunoprecipitation. Immunohistochemical study showed that it was expressed in neurons rather than astrocytes, a result compatible with mRNA expression detected by RT-qPCR. In addition, primary neuron culture system was used for analysis and showed that the expression of $cPLA_2\varepsilon$ was first increased then decreased during neuron/brain maturation. Furthermore, knockdown of $cPLA_2\varepsilon$ gene was conducted, and revealed that the neurons with $cPLA_2\varepsilon$ knockdown have longer total dendritic length than those with scrambled control. Therefore, murine $cPLA_2\varepsilon$ might have a unique role in dendritic development during brain development.

PLA_2 enzymes principally catalyze hydrolysis at the sn-2 position to generate a lysophospholipid and a free fatty acid, although they may also possess PLA_1, lysophospholipase, transacylase, and lysophospholipid acyltransferase activities. In addition to generation of lipid signaling molecules, in recent years, cytoplasmic PLA_2 enzymes are also associated with various intracellular trafficking events directly or indirectly, such as the formation of membrane tubules from the Golgi complex and endosomes, and membrane fusion events in the secretory and endocytic pathways. The discovery and characterization of the SNARE family of proteins has led to a detailed understanding of protein-mediated membrane fusion. It is possible that phospholipid-modifying enzymes also play a role in membrane fusion to achieve maxima fusion efficiencies by modifying phospholipid composition of membranes. PLA_2 activity has been reported to play a role in the fusion of endosomes in vivo and in vitro. Cytosolic $cPLA_2\alpha$ is required for the formation of the traffic-dependent intercisternal tubules and for intra-Golgi transport but not peri-Golgi vesicles. Recently, some phospholipases A_2 were found to locate at some organelles such as early endosomes or cytoplasmic vesicles. But its exact functions on these vesicles are still unknown.

Subcellular localization of $cPLA_2\varepsilon$ was also examined and showed that $cPLA_2\varepsilon$ was mostly co-localized with endosomes and lysosomes, not with Golgi apparatus, endoplasmic reticulum and mitochondria. In addition, centrifuged fractionation was also performed and revealed that $cPLA_2\varepsilon$ was mainly associated with the membrane. As a result, it might imply that $cPLA_2\varepsilon$ have a part in endosomal fusion and regulation.

Living cells contain various kinds of intracellular organelles, which have distinct structures and functions. They play important roles in numerous cellular events, such as cell proliferation, differentiation, maintenance, and death. However, little is known about these organelles' functions in neurons, which have extremely polarized cellular shapes. The endosomal-lysosomal system is a highly dynamic system of acidified cytoplasmic organelles that have essential roles in cellular functions. In nervous system, endocytic activity is particularly high at nerve terminals, dendritic domains and the retrograde

translocation of vesicles to endosome/lysosome related compartments that might be one part of signaling communication. Endosomal pathway to lysosomal degradation is critical for dendrite development in a recent genetic study using Drosophila DA sensory neurons. The functions of recycling endosomes (REs) in neuritogenesis have been investigated by many studies using methods to block RE trafficking. Moreover, overexpression of protrudin induces neurite-like structures through its binding to Rab11, indicating protruding-rab11 system has roles in the membrane recycling required for neurite extension.

Murine cPLA$_2\varepsilon$ was present at the membranes of endosomal/lysosomal systems with interaction with Rab11 proteins revealed by immunoprecipitation, which suggested that cPLA$_2\varepsilon$ might be a participant in the endosome-mediated regulation of neuronal development. In addition, the recycling endosomes are also reported to be associated with neuronal plasticity and important for supplying postsynaptic AMPA-type glutamate receptors during LTP (long term potentiation). Therefore, it is possible as well that murine cPLA$_2\varepsilon$ might associated with the turnover of receptors in neurons through the endosomal system.

Cytosolic phospholipase A$_2$s (except cPLA2γ), have a C2 domain that mediate protein translocation from cytosol to membranes while stimulation with Ca^{2+} ion in the buffer such as cPLA$_2\alpha$, cPLA$_2\beta$3, cPLA$_2\delta$, cPLA$_2\zeta$. However, some of them may be constitutively associated with membrane regardless of calcium ion presence or not, for instance, cPLA$_2\beta$1.

We found that murine cPLA$_2\varepsilon$ was mainly associated with the membrane fraction and showed no significant difference between the presence and absence of Ca^{2+} in the homogenizing buffer. This result was consistent with the phenomenon that murine cPLA$_2\varepsilon$ only had enzymatic activity when Triton X-100 was added in the assay buffer, and increased enzymatic activity when the membrane of vesicles contained anionic phosphoinositides. The differences of cellular localization and enzymatic activity among PLA$_2$ isoforms may be due to the several different amino acid residues in the C2 and catalytic domains. It implies that murine cPLA$_2\varepsilon$ may have another unique way for its localization via protein-protein or protein-lipid interaction in addition to Ca^{2+} mediated membranous binding.

In summary, the endogenous cPLA$_2\varepsilon$ protein is confirmed in mouse tissues such as the brain, heart and skeletal muscle. In the mouse brain, it is expressed in neurons with characteristic endosomal/lysosomal subcellular localization and might serve as a factor associated with the dendritic development. Moreover, it might also possess a distinct mechanism (Ca^{2+}-independent) to regulate its membranous association. Since the neurodegenerative disease is often associated with endosomal functions, cPLA$_2\varepsilon$ might be suspected to have some roles in neuronal degeneration.