Neuronal lineage-specific induction of phospholipase C_ε expression in the developing mouse brain

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【学位論文題目】

Neuronal lineage-specific induction of phospholipase Cε expression in the developing mouse brain
(マウス脳の発生におけるホスホリパーゼCεの神経細胞系列特異的な発現誘導)

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Introduction

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphoinositide-specific phospholipase C (PI-PLC) is a key event triggering intracellular signal transduction from various receptor molecules at the plasma membrane by yielding two intracellular second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), which induce activation of protein kinase C (PKC) and mobilization of Ca^{2+} from intracellular stores, respectively. More than 12 members of mammalian PI-PLC isoforms have been identified and classified into five classes, β, γ, δ, ε, and ζ, which are regulated through distinct mechanisms. PLCε was recently identified and characterized by possession of two Ras-associating (RA) domains in its C-terminal region and a CDC25 homology domain in its N-terminal region. The RA domains are responsible for activation of PLCε through direct association with the GTP-bound active forms of the small GTPases Ras, Rap1, and Rap2. The CDC25 homology domain acts as a guanine nucleotide exchange factor (GEF) for Rap1 and thereby amplifies the Rap1-dependent activation of PLCε.

Although the molecular mechanism of the PLCε regulation has been intensively studied, little is known about the function of PLCε in physiological processes such as cell growth and differentiation. To address this point, it is important to understand the spatial and temporal expression patterns of PLCε during embryogenesis and adulthood.

Here, a detailed analysis of the spatial and temporal expression patterns of PLCε in mouse embryos and adults was performed with special emphasis on the central nervous system. The results indicate a specific induction of PLCε in the neuronal lineage.

Methods and Results

Cloning of mouse PLCε cDNA------Two EST clones, mr95e10.r1 and md98g02.r2, in the mouse EST database showed striking homology to the C-terminal region of human PLCε. To obtain a full-length cDNA of mouse PLCε, 5'-RACE was carried out using a mouse E11 Marathon™ embryo cDNA library. The resulting cDNA (registered with the accession number AB076247 in the DDBJ/EMBL/GenBank™) encodes a 2,282-amino acid residue protein possessing the CDC25 homology domain and two RA domains which are characteristic of PLCε, in addition to the catalytic X and Y, and the calcium-dependent lipid-binding C2 domains, which are well conserved among all the PI-PLC isoforms. The protein, expressed as a FLAG-tagged form in COS7 cells and purified, exhibited a hydrolyzing activity toward PIP_2 *in vitro*, demonstrating that it was the mouse counterpart of PLCε.

Regional and temporal expression of the PLCε mRNA during embryogenesis------Northern blotting using RNA extracted from the whole body of mouse embryos indicated that the *PLCε* mRNA expression greatly increased on embryonic day 11(E11) and that the expression further increased in the later embryonic stages. This suggested that the *PLCε* mRNA expression might be associated with development of certain organs.

To further examine the regional expression patterns of the *PLCε* mRNA during embryogenesis, an *in situ* hybridization experiment using the ^{33}P-labeled antisense *PLCε* RNA probe was carried out. On E12.5, the *PLCε* mRNA was highly expressed in the telencephalon, diencephalon and rhombencephalon of the developing brain as well as in the spinal neural tube. The region expressing the *PLCε* mRNA corresponded to the outer zone of the developing brain rather than the region facing the ventricles. On E15.5, PLCε still exhibited similar expression patterns in the brain.

Immunohistochemical analysis of the cell-type specificity of PLCε expression------To further define the spatial distribution of the PLCε expression especially in the nervous system, an immunohistochemical analysis using mouse brain sections was performed. To this end, a specific antibody was raised against a synthetic peptide corresponding to the C-terminal 19-amino acid residues of mouse PLCε (KEEKPVGALSSSDTVGYQQ) and affinity-purified. The specificity of this

antibody was confirmed by Western blotting of the extracts of cells expressing PLCε (mouse embryonal carcinoma P19 cells expressing endogenous PLCε and COS7 cells transfected with a PLCε-expression vector) and by immunocytochemistry of the PLCε-expressing COS7 cells. On E10.5, cells in almost all the regions of the neuroepithelium surrounding the fourth ventricle showed weak PLCε expression. Among this uniform expression, cells in the outermost zone stood out with a markedly high level of the PLCε expression. To characterize the cells highly expressing PLCε, the same brain sections were examined for expression of cell-type-specific markers, nestin for the neural stem cells and microtubule-associated protein 2 (MAP2) for the differentiated neurons. Almost all the cells highly expressing PLCε were completely negative for nestin but strongly positive for MAP2. The neuroepithelium of the telencephalic vesicle, which develops later than that of the rhombencephalon, showed a similar uniform and weak expression patterns of PLCε without any high expression in the outermost zone. This neuroepithelium showed uniformly positive staining for nestin but essentially no staining for MAP2. The expression patterns of PLCε in brain sections of the E14.5 embryos, in which more than 60% of the cortical cells had been reported to be positive for neuron-specific markers, were also examined. The nestin-positive neural stem cells were mainly located in the ventricular zone surrounding the lateral ventricle while the MAP2-positive neurons were mainly located in the marginal zone. As observed with the E10.5 embryo, the PLCε-highly positive cells almost coincided with the MAP2-positive cells but not with the nestin-positive cells. These results indicated that the expression of PLCε is induced specifically in the cells committed to the neuronal lineage, which exhibit expression of MAP2 but not nestin.

Induction of PLCε expression during neuronal differentiation of neuroepithelial cells in vitro------Neuroepithelial stem cells were prepared from the forebrains of E10.5 embryos and enriched by forming neurospheres in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). The neurospheres were then induced to differentiate into neurons in the culture medium from which EGF and bFGF had been removed. Immunocytochemical analysis using specific antibodies indicated that the neuroepithelial stem cells were positive for nestin and weakly positive for PLCε, but negative for MAP2. In contract, the differentiated cells showing neuron-like morphologies became highly positive for PLCε and MAP2, but completely negative for nestin. These observations were very similar to those with the embryo brain sections.

The time course of the expression of the *PLCε* mRNA during the early stages of the *in vitro* neuronal differentiation was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) along with those of neural cell-type-specific developmental markers; Mash1, glial fibrillary acidic protein (GFAP), nestin and MAP2. Mash1, a basic helix-loop-helix (bHLH) proneural transcription factor, is known to be expressed transiently in the very early stages of neural differentiation and to trigger neural differentiation. GFAP is specifically expressed in astrocytes. The results of RT-PCR indicated that PLCε is expressed in a similar time course with MAP2, which is later than those of nestin and Mash1. A faint expression of GFAP was detected after MAP2 expression, reflecting a low rate of differentiation into the glial lineage in this culture system. These results further suggested that the expression of PLCε might be induced after commitment into the neuronal lineage.

Analysis of PLCε expression in glial-lineage cells------To examine whether the high expression of PLCε was associated with differentiation into the glial lineage or not, an *in vitro* differentiation of the neuroepithelial stem cells derived from E14.5 mouse embryos, which were capable of differentiation into a mixed population containing both GFAP-positive astrocytes and MAP2-positive neurons, was analyzed. After neural differentiation for 5 days, the cells highly expressing PLCε were negative for nestin but positive for MAP2, which was very similar to the findings with the E10.5 embryo-derived culture. On the other hand, the GFAP-positive astrocytes expressed PLCε much more weakly than the MAP2-positive neurons. These observations held

true in the *in vivo* studies with brain sections. Collectively, the results indicate that the induction of PLCε expression is specifically associated with the differentiation into the neuronal lineage but not with that into the glial lineage.

Discussion

The observations shown here indicate that the induction of PLCε is associated specifically with the differentiation into the neuronal lineage. The high expression of PLCε persists still after terminal differentiation into the neurons, and is observed in almost all the regions harboring the mature neurons. This suggests that PLCε may be involved in a more general aspect of neuronal differentiation or neuronal functions compared to PLCβ4 and PLCβ1 exhibiting region- or cell-type-specific expression.

The mechanism of the neuronal lineage-specific induction of PLCε remains to be clarified. Since the induction of the high expression of PLCε apparently takes place after the expression of Mash1, it may be regulated by proneural bHLH transcription factors which promote neuronal differentiation and inhibit glial differentiation. In addition to determination of the neuronal fate, these factors are important for specification of the regional identity of neurons by their region-specific expression in the neural stem cells. Since no regional specificity is observed in the expression pattern of PLCε, it is unlikely that a single such factor is directly responsible for the induction of the PLCε expression.

The function of PLCε in neuronal differentiation as well as in the mature neurons remains to be clarified. Considering that the activity of PLCε is regulated by association with Ras and Rap, it may play a role in intracellular signaling from the receptors for FGFs and various neurotrophic factors, that are known to be involved in the neural development. For instance, FGF9 action of inducing differentiation of the retinal pigment epithelium into the neural retina can be mimicked by an exogenous expression of the constitutively active mutant of Ras, indicating that Ras plays a crucial role in determination of the neuronal fate. Although the action of FGFs and nerve growth factor (NGF) leading to the induction of neuronal differentiation requires the activation of the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway, the activation of the Ras-Raf-MAPK pathway alone was reported to be insufficient to induce neuronal differentiation. Indeed, a study with conditionally immortalized hippocampal neurons suggested an alternative Ras-dependent pathway, not leading to the MAPK activation, was implicated in FGF-induced neuronal differentiation. Thus, it is possible that the Ras/Rap1-PLCε pathway may mediate a part of the FGF-induced neural differentiation signal.

The importance of Ras-mediated signaling pathways in the activity of mature neurons has been suggested by gene knockout experiments of Ras and its regulators, GEFs and GTPase-activating proteins (GAPs). Homozygous knockout of the Ha-*ras* gene resulted in potentiation of long-term potentiation (LTP). Heterozygous knockout of the *neurofibromatosis type1* gene encoding a GAP for Ras and that of the *Ras-GRF1* gene encoding a Ras-GEF resulted in the learning deficits due to the hyper activation of Ras and the abnormality in LTP, respectively. Up to now, the downstream effector of Ras responsible for these neuronal phenotypes has not yet been identified. PLCε may be a good candidate because PKC and calcium/calmodulin-dependent kinases, whose activities are regulated by diacylglycerol and Ca^{2+}, respectively, are involved in LTP. In addition, the Rap1-specific GEF activity of PLCε may also contribute to its neuronal function because Rap and Ras were reported to oppositely control the synaptic plasticity of hippocampal neurons by regulating the trafficking of the AMPA-sensitive glutamate receptor into the excitatory synapses. Further studies, such as that employing the gene knockout technology, will reveal the function of PLCε in neuronal development and neuronal activities.

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論文題目	Neuronal lineage-specific induction of phospholipase Cε expression in the developing mouse brain （マウス脳の発生におけるホスホリパーゼCεの神経細胞系列特異的な発現誘導）		
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ホスホイノシチド特異的ホスホリパーゼC（PI-PLC）によるホスファチジルイノシトール4, 5-二リン酸（PIP_2）の加水分解は、様々な細胞膜受容体を介する細胞内情報伝達の引き金になっている。この反応からジアシルグリセロールとイノシトール1, 4, 5-三リン酸が産生され、前者はプロテインキナーゼCの活性化、後者は細胞内カルシウムの動員を引き起こす。哺乳動物 PI-PLC は、現在までに約 12 種類が同定され、各々異なった機構で活性調節を受けるβ, γ, δ, ε, ζの5つのクラスに分類されている。PLCεは最近当研究室にて発見された分子種であり、その Ras 結合（RA）ドメインにて低分子量G蛋白質である Ras, Rap1, Rap2 と GTP 依存性に結合して活性化を受ける。さらに PLCεは、Rap1 に対してグアニンヌクレオチド交換因子として作用する CDC25 類似ドメインを持ち、このドメインの作用を介して Rap1 依存性 PLCε 活性化を自己増幅する機能を有する。本研究者は、PLCεの生体内機能を解明する第一歩として以下の実験を行った。

先ずマウス PLCεの全長 cDNA を単離して塩基配列を決定し、PLCεの全アミノ酸配列を予測した。マウス PLCεは 2282 アミノ酸残基からなり、全 PLC が共有する触媒ドメインの X, Y, C2 ドメインと、PLCεに特徴的な CDC25 類似ドメインと2つの RA ドメインを有していた。

マウス個体全体から抽出した RNA を用いたノーザンブロット法により、約 9 kb の長さの PLCε mRNA の強い発現が胎生 11 日目（E11）頃から始まり、胎生後期にはさらに増強する事がわかった。次に、各発生過程のマウスの凍結切片に対し、^{33}P 標識アンチセンス RNA プローブを用いて *in situ* ハイブリダイゼーションを行ない、E12.5 では終脳胞、間脳胞、菱脳胞および脊髄神経管にわたって PLCε mRNA の特異的な強い発現を認めた。発現部位は、これらの組織の最外層領域に対応した。E15.5 でも、E12.5 と発現パターンは同様であった。さらに、蛍光標識した抗マウス PLCε特異的抗体を用いた免疫組織染色法により、脳における PLCε 発現の部位特異性を詳細に解析した結果、E10.5 の第四脳室周囲の神経上皮全体にわたって PLCεの弱い発現が認められたが、その中で最外層の細胞に非常に強い発現が観察された。同じ切片を神経幹細胞特異的マーカー nestin 並びに分化した神経細胞のマーカー MAP2 と共染色したところ、PLCε 強陽性細胞は MAP2 強陽性細胞と一致し、nestin 陽性細胞とは全く一致しなかった。E14.5 でも、同様の結果が得られた。

次に、E10.5 マウス胎児脳から調製した神経幹細胞の試験管内神経分化系を用いて解析した。分化誘導直後の神経幹細胞は、一様に nestin 陽性、MAP2 陰性で PLCε を弱く発現していた。しかし、分化誘導 1 日後に現れた神経細胞様形態を示す細胞では、PLCεの発現が著明に誘導され、MAP2 強陽性で nestin 陰性となった。さらに、逆転写－ポリメラーゼ連鎖反応（RT-PCR）法を用いて、試験管内神経分化の過程での種々の mRNA の発現の時間経過を調べたところ、PLCεの発現時期は nestin と塩基性 helix-loop-helix 型転写因子 Mash1 の発現より遅れ、MAP2 のそれと同じであった。これらの結果は、胎児脳切片を用いた免疫組織染色実験の結果とよく一致した。さらに、グリア細胞系列への分化に際しては、PLCεの強発現が起こらないことが示された。

以上の結果から、PLCεの発現が神経幹細胞の神経細胞系列への分化に際して特異的に誘導されることが証明された。PLCεは Ras や Rap1 によって活性制御を受けていることから、線維芽細胞増殖因子や神経成長因子などの様々な神経分化調節因子の受容体からの細胞内情報伝達において作用していると推測された。また、Ras が海馬における長期増強 (LTP) や神経細胞分化の調節に係っているとの報告があり、PLCεがこのような機能に関わる Ras 標的蛋白質である可能性がある。

本研究は、哺乳動物における Ras/Rap 標的蛋白質 PLCεについて、その発生過程における組織特異的発現様式を研究したものであるが、PLCεが神経幹細胞の神経細胞系列への分化に際して特異的に発現誘導される事を発見し、PLCεの神経系における機能について重要な知見を得たものとして価値ある集積であると認める。よって、本研究者は、博士（医学）の学位を得る資格があると認める。