The Role of X/Y Linker Region and N-terminal EF-hand Domain in Nuclear Translocation and Ca\textsuperscript{2+} Oscillation-inducing Activities of Phospholipase C\(\zeta\), a Mammalian Egg-activating Factor*  

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Sperm-specific phospholipase C-zeta (PLC\(\zeta\)) causes intracellular Ca\textsuperscript{2+} oscillations and thereby egg activation and is accumulated into the formed pronucleus (PN) when expressed in mouse eggs by injection of cRNA encoding PLC\(\zeta\), which consists of four EF-hand domains (EF1-EF4) in the N terminus, X and Y catalytic domains, and C-terminal C2 domain. Those activities were analyzed by expressing PLC\(\zeta\) mutants tagged with fluorescent protein Venus by injection of cRNA into unfertilized eggs or 1-cell embryos after fertilization. Nuclear localization signal (NLS) existed at 374–381 in the X/Y linker region. Nuclear translocation was lost by replacement of Arg\textsuperscript{376}, Lys\textsuperscript{377}, Arg\textsuperscript{378}, Lys\textsuperscript{379}, or Lys\textsuperscript{381} with glutamate, whereas Ca\textsuperscript{2+} oscillations were conserved. Nuclear targeting was also absent for point mutation of Lys\textsuperscript{299} and/or Lys\textsuperscript{301} in the C terminus of X domain, or Trp\textsuperscript{13}, Phe\textsuperscript{14}, or Val\textsuperscript{18} in the N terminus of EF1. Ca\textsuperscript{2+} oscillation-inducing activity was lost by the former mutation and was remarkably inhibited by the latter. A short sequence 374–383 fused with Venus showed active translocation into the nucleus of COS-7 cells, but 296–309 or 1–19 did not. Despite the presence of these special regions, both activities were deprived by deletion of not only EF1 but also EF2–4 or C2 domain. Thus, PLC\(\zeta\) is driven into the nucleus primarily by the aid of NLS and putative regulatory sites, but coordinated three-dimensional structure, possibly formed by a folding in the X/Y linker and close EF/C2 contact as in PLC\(\delta\), seems to be required not only for enzymatic activity but also for nuclear translocation ability.

PLC\(\zeta\) is a novel isozyme of PLC (the enzyme that hydrolyzes membrane PIP\textsubscript{2} into IP\textsubscript{3} and diacylglycerol) and a strong candidate of the mammalian sperm- or sperm egg fusion and induces Ca\textsuperscript{2+} oscillations as well (7). 3) Ca\textsuperscript{2+} oscillation-inducing ability of sperm extract injected into eggs (4, 8) is lost when pretreated with an antibody against PLC\(\zeta\) (2). 4) PLC\(\zeta\) content in the mouse sperm and the number of Ca\textsuperscript{2+} spikes at fertilization are reduced by transgenic RNA interference of PLC\(\zeta\) (9). 5) PLC\(\zeta\) has such a high Ca\textsuperscript{2+} sensitivity of PIP\textsubscript{2}-hydrolyzing activity that the enzyme can be active in the resting cells at \(\sim 100 \text{nM} \text{Ca}^{2+}\) (7, 10), suitable for the sperm factor as the first stimulus in the egg cytoplasm at fertilization.

Another important property of PLC\(\zeta\) is nuclear translocation ability. PLC\(\zeta\) expressed by RNA injection is accumulated into the formed PN (3, 11, 12). This is consistent with earlier observation that sperm-derived Ca\textsuperscript{2+} oscillation-inducing activity is concentrated into PN formed several hours after fertilization, as examined by transfer of the ooplasm or PN into unfertilized eggs (13). Ca\textsuperscript{2+} oscillations cease at about the time of PN formation (14), but continue without stopping when PN formation was prevented by injection of a lectin, WGA (15). Therefore, it is thought that translocation of the sperm factor or PLC\(\zeta\) into PN plays a key role in cessation of Ca\textsuperscript{2+} oscillations at the interphase of a cell cycle (11, 13, 15).

1,4,5-trisphosphate/IVF, in vitro fertilization; II MII, metaphase of second meiosis; NLS, nuclear localization signal; NTR, nuclear transport receptor; PH domain, pleckstrin homology domain; PIP\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate; PN, pronucleus or pronuclei; sPLC\(\zeta\), short form of PLC\(\zeta\).
Structure-function analysis of PLCζ is implicated, because PLCζ is a biologically important factor and can be practically utilized for artificial egg activation. PLCζ is composed of four EF-hand domains in the N terminus, X and Y catalytic domains, and C2 domain in the C terminus (2), common to other isozymes of PLC (16), but lacks N-terminal PH domain found in PLCβ, γ, δ, and ε (2). A short form of PLCζ, s-PLCζ, is thought to be expressed in the mouse sperm, because mRNA encoding a protein, which lacks three EF-hand domains but is identical to PLCζ in other region, has been found to exist in the mouse testis (AK006672 in EMBL). Both 74- and 65-kDa protein bands are detected by Western blotting of mouse sperm extract using anti-PLCζ antibody (17). We have found that s-PLCζ expressed in mouse eggs has much less Ca²⁺ oscillation-inducing activity and is hardly accumulated in PN (3), suggesting that EF-hand domains are responsible for these important properties. Actually, deletion of N-terminal EF-hand domains results in the loss of Ca²⁺ oscillation-inducing activity (10, 18).

In the present study, we addressed the molecular structure responsible for the nuclear translocation ability in a quantitative manner and in parallel with precise assay of Ca²⁺ oscillation-inducing activity, focusing on the putative NLS region and EF-hand domain region. PLCζ or its mutants fused with a fluorescent protein Venus (19) were expressed by injection of respective cRNA into mouse eggs. Nuclear translocation was investigated in PN of 1-cell embryos after artificial activation by PLCζ or fertilization by the sperm. Translocation was also examined in the nucleus of cultured somatic cells after transfection of cDNA.

**EXPERIMENTAL PROCEDURES**

Preparation of Gametes and Insemination—Mature eggs at MII were obtained from superovulated B6D2F1 mice (see Ref. 20 for details), and freed from cumulus cells by 0.05% hyaluronidase (Sigma). M2 medium was used during egg preparation, RNA injection, [Ca²⁺]i measurement, and observation of eggs or embryos. Twenty to thirty eggs were transferred to a 400-μl drop of M2 medium covered with paraffin oil in a glass-bottomed plastic dish, which was placed on the stage of an inverted fluorescence microscope (TMD, Nikon) and heated at 31–33 °C. Eggs were injected with cRNA (see below). In another experiments, cRNA was injected into 1-cell embryos after IVF. M16 medium was used during egg preparation, RNA injection, [Ca²⁺]i, measurement, and observation of eggs or embryos. 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A

PLCζ-Venus RNA (50 ng/µl)

Fluorescence intensity

Time after RNA injection (h)

--- cytoplasm

--- pronucleus

B

1: Venus alone (10 ng/µl)

2: PLCζ (30 ng/µl)

Fluorescence intensity

Time after insemination (h)

--- cytoplasm

--- pronucleus

C

D210R (50 ng/µl)

Fluorescence intensity

Time after insemination (h)

--- cytoplasm

--- pronucleus
treated with 0.05% hyaluronidase, and after washing, injected with cRNA.

Construction of Plasmids—cDNA encoding full-length PLCζ (647 amino acid residues; see Fig. 1A) (GenBankTM accession number AF435950) or s-PLCζ lacking 110 amino acid residues from the N terminus (AK006672) was prepared using PCR techniques, fused with Venus (19) in the C terminus, and subcloned into pBluescript II SK(+) (see Ref. 21 for details). The methods were the same as described previously (4).

Point replacement of an amino acid or partial deletion of amino acid sequence in cDNA of PLCζ was constructed by GeneTailorTM site-directed mutagenesis system (Invitrogen), using PLCζ-Venus-pBluescript II SK(+) as the template (see Fig. 1A for domain features and Tables for designation). To circumvent unwanted mutations, a region surrounding the targeted amino acid(s) and presenting unique restriction sites was subcloned in the parental vector and verified by DNA sequencing using Applied Biosystems ABI PRISM 310 DNA sequencer.

Amplified fragments were digested with KpnI and SpeI, and resulting fragments were used as templates for domain features and Tables for designation. To evaluate nuclear translocation ability, a region surrounding the target amino acid(s) and presenting unique restriction sites was subcloned in the parental vector and verified by DNA sequencing using PLCζ-Venus-pBluescript II SK(+) (see Ref. 21 for details). The methods were the same as described previously (4).

Judgment of nuclear translocation ability. Evaluation criteria (a) concentration of RNA for injection, (b) induction of repetitive Ca2+ spikes, (c) The time between RNA injection to 1st Ca2+ spikes, (d) Delay (arbitrary unit) at 3 h after RNA injection. A standard concentration of RNA was resolved in 150 mM KCl solution (final concentration, 100–1,000 ng/µl) into MII eggs or 1-cell embryos using a glass micropipette (injected amount, 2–4 pl per egg or embryo of which volume is 200 pl). To make the expression level of RNA injection into 1st Ca2+ spikes more than 200 poly(A) in the 3′-tail (see Ref. 21 for details). Dried RNA was resolved in 150 mM KCl solution (final concentration, ~1.5 µg/µl). RNA was diluted to the range between 10 and 1,000 ng/µl and injected into MII eggs or 1-cell embryos using a glass micropipette (injected amount, ~4 pl per egg or embryo of which volume is 200 pl). To make the expression level of RNA injection was adjusted in such way that fluorescence intensity (F) of Venus in the egg was in the range between 55 and 90 (arbitrary unit) at 3 h after RNA injection. A standard concentration of PLCζ-Venus RNA was 50 ng/µl. RNA concentration used was raised up to 1,000 ng/µl, when extreme overexpression was necessary.

Measurement of Venus Fluorescence—Of 30–40 MII eggs injected with cRNA, 4–9 eggs were left in the same dish and subjected to continuous measurement of F. Fluorescent images of eggs were acquired every 3 min at 31–33 °C, using an EB-
Nuclear Translocation of PLCζ

Table 2

| PLCζ mutant | Ca2⁺ oscillation-inducing ability | Nuclear translocation ability |
|-------------|----------------------------------|------------------------------|
|             | RNA† | Eggs | Fₐₘ | Ca2⁺ spikes | Delay¢ | RNA† | Eggs | Fₐₘ | Fₚₜ/Fₖₗ | NTº |
|             | ng/µl | no. | min | 1-cell | 6 h | no. | 8 h | 6 h |
| Venus alone | 50 | 7 | 70 | + | 28 | 6 | 175 | 98 | 0.98 | − |
| PLCζΔ2–14 | 60 | 4 | 80 | + | 36 | 6 | 75 | 7 | 0.80 | +++ |
| PLCζΔ2–9 | 50 | 8 | 55 | + | 50 | 4 | 63 | 105 | 1.28 | (+)b |
| PLCζΔ2–19 | 1000 | 5 | 325 | − | 5 | 530 | 1.05 | 1.22 | (+)b |
| PLCζM1–Q19 | 25 | 4 | 2750 | − | 5 | 93 | 0.98 | − |
| E10A,R12A | 55 | 5 | 150 | + | 42 | 35 | 6 | 123 | 1.25 | + |
| R12E,W13A,F14A | 1000 | 4 | 345 | − | 70 | 4 | 82 | 0.75 | − |
| R12E | 45 | 5 | 170 | + | 29 | 20 | 3 | 122 | 1.57 | + |
| W13A | 180 | 8 | 195 | (+)¢ | 68 | 120 | 4 | 72 | 0.88 | 0.80 | − |
| W13F,F14W | 45 | 5 | 260 | (+)¢ | 72 | 15 | 4 | 45 | 0.91 | 0.89 | − |
| W13F | 50 | 5 | 144 | + | 53 | 30 | 4 | 72 | 1.02 | 1.07 | ± |
| F14A | 45 | 4 | 80 | (+)¢ | 115 | 50 | 6 | 65 | 0.87 | 0.79 | − |
| F14W | 45 | 5 | 275 | + | 32 | 12 | 4 | 40 | 1.48 | 2.27 | + |
| W13F,F14W | 45 | 5 | 260 | (+)¢ | 72 | 15 | 4 | 45 | 0.91 | 0.89 | − |
| E16A | 55 | 5 | 240 | + | 30 | 15 | 2 | 88 | 1.17 | 1.25 | + |
| K17A | 55 | 4 | 120 | + | 34 | 40 | 3 | 135 | 1.13 | 1.46 | + |
| K17E | 50 | 4 | 230 | + | 32 | 20 | 3 | 60 | 1.18 | 1.69 | + |
| V18A | 30 | 6 | 80 | + | 55 | 20 | 4 | 40 | 1.48 | 2.27 | + |
| V18A | 60 | 4 | 220 | + | 40 | 20 | 4 | 136 | 0.95 | 0.95 | − |
| Δ2–39 (ΔEF1-tr)i | 50 | 8 | 180 | − | 40 | 7 | 86 | 0.70 | − |
| Δ2–110 (s-PLCζ) | 550 | 4 | 1745 | − | 400 | 6 | 2120 | 0.73 | − |
| Δ2–77 (ΔEF1–2-tr) | 50 | 14 | 115 | − | 50 | 6 | 140 | 0.75 | − |
| Δ2–110 (s-PLCζ) | 250 | 14 | 870 | (+)¢ | 173 | 500 | 5 | 1050 | 0.84 | − |
| Δ2–110 (s-PLCζ) | 50 | 7 | 90 | − | 50 | 9 | 75 | 0.84 | 0.85 | − |
| Δ2–167 (ΔEF1–4-tr) | 250 | 5 | 890 | (+)¢ | 187 | 500 | 4 | 635 | 0.85 | 0.86 | − |
| Δ10–19 | 1000 | 5 | 1640 | − | 20 | 5 | 88 | 0.70 | − |
| Δ45–110 (ΔEF2–3) | 1000 | 4 | 1270 | − | 20 | 4 | 80 | 0.85 | − |
| Δ45–163 (ΔEF2–4) | 1000 | 6 | 1170 | − | 40 | 9 | 70 | 0.73 | − |
| Δ552–610 (ΔC2) | 500 | 9 | 2145 | − | 50 | 8 | 70 | 0.79 | − |
| D542A | 70 | 9 | 300 | + | 28 | 20 | 3 | 92 | 1.41 | 2.73 | + |
| D542R | 50 | 4 | 55 | + | 36 | 70 | 4 | 154 | 1.11 | 1.54 | + |

† Concentration of RNA for injection.
‡ F (mean value) of Venus at 3 h after RNA injection.
§ Induction of repetitive Ca²⁺ spikes.
¢ The time between RNA injection to 1st Ca²⁺ spike (mean value).
†† RNA injection into MII egg or 1-cell embryo.
¶ Ratio of F (cPLCζ vs. cytoplasm) at 6 or 8 h after RNA injection (mean).
¶¶ Judgment of nuclear translocation ability. Evaluation criteria (r = Fₚₜ/Fₖₗ): R < 1.0, negative (−); 1.0 ≤ R ≤ 1.1, faint (+); 1.1 < R ≤ 1.5, positive (+); 1.5 < R ≤ 2.0, fair (+++); 2.0 ≤ R, strong (++++)
∥ Delayed nuclear translocation.
* Delayed Ca²⁺ response (delay time > 60 min).
i EF1-truncated PLCζ.

Table 1. Values at 8 h were also presented to see the progression of nuclear accumulation.

[Ca²⁺], Measurement—Ca²⁺ oscillations were recorded in another optical system by conventional Ca²⁺ imaging method using an image processor. Four to five MII eggs were injected with 50 µM solution of the Ca²⁺-sensitive fluorescent dye fura dextran (Molecular Probes Inc.) together with a cRNA and were subjected to [Ca²⁺], measurement for 9 h after RNA injection. F of fura was measured without interference with that of Venus, by applying 340- and 380-nm UV lights alternatively and by leading emission light through a 400-nm dichroic mirror (DCLP; Omega) and a 500–520-nm bandpass filter. Fluoresscence was detected by an EB-CCD camera (C7190–23; Hamamatsu Photonics). Ca²⁺ images were acquired at intervals of 20 s and processed to calculate Fₚₜ/Fₖₗ later using NIH Image (a public domain image processing software for the Macintosh computer). Formation of the PN and nuclear translocation of a PLCζ mutant were examined 5 and 9 h after RNA injection, respectively.

CCD camera (C7190–23; Hamamatsu Photonics) and an image processor (Argus 50; Hamamatsu Photonics). Excitation light was passed through a 470 – 490-nm bandpass filter and a 20× objective lens. Emitted light was passed through the objective lens, a 510-nm dichroic mirror (DM510; Nikon), and a 520 – 560-nm bandpass filter. Autofluorescence of the egg, probably derived from oxidized flavins (22), was subtracted from total fluorescence to obtain F. Other eggs were kept in another dish and subjected to precise observation at 3, 5, and 8 h after RNA injection using a confocal laser scanning microscope (LSM310, Carl Zeiss) with excitation light of 510 nm. Differential interference contrast images were recorded simultaneously by another sensor for transmitted laser light. All these procedures were also taken in the experiment in which cRNA was injected into 1-cell embryos about 5.5 h after insemination.

Judgment of Nuclear Accumulation—The ratio of F in the PN to that in the cytoplasm (Fₚₜ/Fₖₗ) at 6 h after RNA injection was taken as a parameter for nuclear accumulation. The evaluation criteria were tentatively defined, as indicated in the legend of Table 1. Values at 8 h were also presented to see the progression of nuclear accumulation.
Nuclear Translocation in Cultured Somatic Cells—COS-7 cells cultured on glass coverslips were transfected with cDNA of Venus-tagged PLC\(\varepsilon\) mutants, using FuGENE6 (Roche Diagnostics) (23). Fluorescent cells were observed 9, 24, 48, or 72 h later by confocal microscopy (LSM510META, Carl Zeiss). For [Ca\(^{2+}\)], measurement, cells were loaded with fura-2 acetoxy-methyl ester (fura-2 AM; Molecular Probes) by incubation in 4 \(\mu\)M fura-2 AM for 30 min at 24 °C. [Ca\(^{2+}\)], measurement was performed at 24 °C in Tyrode’s solution 24 and 48 h after transfection.

RESULTS

Ca\(^{2+}\) Oscillation-inducing Activity and Nuclear Translocation Ability of Wild-type PLC\(\varepsilon\)—The domain feature associated with amino acid number of PLC\(\varepsilon\) is illustrated in Fig. 1A. Under the present experimental conditions, expression of PLC\(\varepsilon\) in MII eggs was detected by Venus-derived F from 30 min after injection of 50 ng/\(\mu\)l RNA, increased up to 3–4 h, and attained a steady level (Fig. 2A). The magnitude of expression of PLC\(\varepsilon\)-Venus was compared in F at 3 h after RNA injection (Tables 1 and 2). The first Ca\(^{2+}\) transient was generated 30–40 min after injection of RNA of wild-type PLC\(\varepsilon\) (Fig. 1B). The delay time was a parameter that reflects Ca\(^{2+}\) oscillation-inducing activity of expressed PLC\(\varepsilon\) mutants (Tables 1 and 2); that is, the higher activity shortened the delay time. The second and third Ca\(^{2+}\) spikes occurred at an interval of ~20 min. The interval was shortened up to 10 min for succeeding Ca\(^{2+}\) spikes (Fig. 1B). These Ca\(^{2+}\) oscillations, which are probably caused by continuously produced IP\(_3\) (24), lasted for 3–4 h and suddenly ceased prior to the formation of (female) PN at about 5 h after RNA injection. The higher PLC\(\varepsilon\) activity resulted in earlier termination of Ca\(^{2+}\) oscillations, possibly because of a negative feedback via production of diacylglycerol and subsequent activation of protein kinase C (25), and/or down-regulation of IP\(_3\) receptor type 1 which develops as a result of Ca\(^{2+}\) oscillations, notably ~4 h after fertilization or parthenogenetic activation (26, 27). It should be noted that expressed PLC\(\varepsilon\) was continuously accumulated into the formed PN (Fig. 2A) as described previously (4). F in the PN (F\(_{\text{PN}}\)) became more than twice of F in the cytoplasm (F\(_{\text{C}}\)) 6 h after RNA injection (Table 1). PLC\(\varepsilon\) that entered PN appeared to avoid the large nucleolus, which was identified as a round structure with a clear circumference in the bright field image (Fig. 2A, paired photographs at the right).

Nuclear translocation of PLC\(\varepsilon\) was observed as well, when RNA was injected into the 1-cell embryo in which male and female PN were recognized 5 h after insemination. In the 1-cell embryo, Ca\(^{2+}\) oscillations induced by IVF had already ceased (14, 28), and another series of Ca\(^{2+}\) spikes were induced by expressed PLC\(\varepsilon\) after a long delay of ~80 min and at long intervals of 40–60 min (Fig. 1C). Phosphoinositide signaling pathway and/or IP\(_3\) receptor-mediated Ca\(^{2+}\) release seems to be suppressed in the 1-cell embryo, at the interphase of cell cycle (11). As shown in Fig. 2B (line 2), F\(_{\text{PN}}\) peaked later than F\(_{\text{C}}\) at the early stage after RNA injection, but exceeded the latter at 3.5 h. Subsequently, F\(_{\text{PN}}\) continuously increased and became twice of F\(_{\text{C}}\) at about 8 h. Venus alone was more expressed than PLC\(\varepsilon\)-Venus (Fig. 2B, line 1) because of the smaller molecule. For Venus alone, F\(_{\text{PN}}\)/F\(_{\text{C}}\) was close to 1.0 (photographs of the inset in Fig. 2B; Table 1), indicating free diffusion through nuclear pores.

The nuclear translocation of PLC\(\varepsilon\) expressed in the 1-cell embryo after IVF served as a control for mutants that had quite low or no Ca\(^{2+}\) oscillation-inducing activity and were incapable of activating the egg. For example, the mutant in which Asp210 in the X catalytic domain (see Fig. 1A) was replaced with arginine (D210R) was defective in Ca\(^{2+}\) oscillation-inducing activity even when overexpressed (Fig. 1D and Table 1), as shown previously (2). Nuclear accumulation of D210R took place (Fig. 2C), but it was substantially slower, compared with that of wild-type PLC\(\varepsilon\) (Table 1). F in the nucleolus was comparable to that in the nucleoplasm 12 h after RNA injection (Fig. 2C, paired photographs at the right). Some fraction of PLC\(\varepsilon\) may to be accumulated to the nucleolus after a long delay.

Nuclear Localization Signal in PLC\(\varepsilon\)—According to-NLS sequence searched from data base, PLC\(\varepsilon\) possesses two regions containing a cluster of basic amino acid residues (lysine and arginine), which is found in many nuclear proteins (29). One is in the C terminus of the X domain from 299–308 (KKKLRKMK) and another is in the X/Y linker region from 374 to 381 (KKKRRKMK). Paired mutation in Lys\(_{377}\) and Lys\(_{379}\) of PLC\(\varepsilon\) has been shown to prevent nuclear import (30). These residues correspond to Lys\(_{379}\) and Lys\(_{381}\) of PLC\(\varepsilon\). The Ca\(^{2+}\) oscillation-inducing activity was lost by replacement of Lys\(_{379}\) or Lys\(_{381}\) with acidic amino acid, glutamate, or both Lys\(_{379}\) and Lys\(_{381}\) with neutral amino acid, alanine (Table 1, K379E & K381E, C, Δ2–19).

It has been shown (11) that K377E lacks nuclear translocation ability and that Ca\(^{2+}\) oscillations induced by RNA encod-
Expression level of these mutants might be higher than that in the previous work (11), and the feedback inhibition on Ca\(^{2+}\) oscillations described above might predominantly operate in our experiments. Nuclear accumulation was negative for R376E, K377E, and K379E (Table 1; Fig. 4A for K379E). Replacement of Lys\(^{377}\) with alanine showed prominent accumulation in PN, similar to wild-type PLC\(\zeta\) (Table 1). Accumulation was faint for R378E and K381E (see Legend of Table 1 for the evaluation criteria), positive but delayed for K374E, and positive for V373E, K375E, M380E, and I382E (Table 1). Thus, Arg\(^{376}\), Lys\(^{377}\), Arg\(^{378}\), Lys\(^{379}\), and Lys\(^{381}\) are essential for the nuclear translocation ability. To examine whether the NLS sequence is autonomously functional, a short fragment of Lys\(^{374}\)–Ala\(^{383}\) fused with Venus was co-expressed with Venus-free PLC\(\zeta\) F\(_{\text{PN/F}}\_c\) was close to 1.0, comparable to that of Venus alone (Table 1); that is, positive accumulation was not detected in PN.

Effects of Truncation at EF-hand Domain Region—Four EF-hand domains were defined by referring to those of PLC\(\delta1\), as indicated in Fig. 1A (18). s-PLC\(\zeta\) which is expressed in the mouse testis and lacks EF1–3 from the N terminus, could induce Ca\(^{2+}\) oscillations only when it was extremely overexpressed (\(\Delta 2–110\) in Table 2). However, the first Ca\(^{2+}\) transient appeared after a long delay of 3 h from the instance of RNA injection (Fig. 3B). The Ca\(^{2+}\) oscillation-inducing activity of s-PLC\(\zeta\) was at least two orders of magnitude lower than that of full-length PLC\(\zeta\) estimated from F at the time of the first Ca\(^{2+}\) transient. Repetitive Ca\(^{2+}\) spikes were generated at long intervals of 30–40 min, and stopped at about the time of PN formation 5–6 h after the occurrence of the first Ca\(^{2+}\) transient (Fig. 3B). No nuclear accumulation of expressed s-PLC\(\zeta\) was observed (Fig. 4B), even when RNA at a high concentration was injected into the 1-cell embryo (Table 2).

No Ca\(^{2+}\) spike occurred for truncation of the N terminus up to EF4 (\(\Delta 2–167\); \(\Delta 1–104\)-tr) even when \(\Delta 1–104\)-tr was

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**FIGURE 4. Lack of nuclear translocation of PLC\(\zeta\) mutants.** A, K379E. B, s-PLC\(\zeta\), and C, \(\Delta 2–19\).
extremely overexpressed (Table 2). A mutant of shorter truncation up to EF2 (Δ2–77; ΔEF1–2-tr) exhibited delayed Ca\(^{2+}\) oscillations, comparable to ΔEF1–3-tr. Even shorter truncation up to EF1 (ΔEF1–tr) was incapable of inducing any Ca\(^{2+}\) spike (Table 2). Thus, EF1 plays an important role in the Ca\(^{2+}\) oscillation-inducing activity. The reason for discrepancy between ΔEF1-tr and ΔEF1–2-tr or ΔEF1–3-tr is unclear, but EF2 apparently serves as an inhibitory factor in the absence of EF1. None of these truncation mutants underwent nuclear accumulation (Table 2), indicating that the EF-hand region is also important for nuclear translocation.

**Effects of Mutation at EF1**—As aforementioned results lead us to predict the significant role of EF1, effects of modification of the N terminus and EF1 on nuclear translocation ability were precisely analyzed. Δ2–4 had no significant effect (Table 2). For Δ2–9, however, nuclear accumulation was delayed and recognized 8 h after RNA injection (Table 2). Ca\(^{2+}\) oscillations were also affected in the prolongation of the delay time to ~50 min.

Surprisingly, both Ca\(^{2+}\) oscillation-inducing ability and nuclear accumulation ability were lost for Δ2–14 or Δ2–19 (Table 2; Fig. 3C and Fig. 4C). Deletion of Glu\(^{10}\)–Gln\(^{19}\) associated with intact N terminus (Δ10–19) deprived both abilities. Replacement of Glu\(^{10}\) and Arg\(^{12}\) with alanine had no significant effect, but substitution of three residues Arg\(^{12}\), Trp\(^{13}\), and Phe\(^{14}\) caused loss of both abilities (Table 2). With precise analysis of the two residues having an aromatic side chain, Trp\(^{13}\) and Phe\(^{14}\), W13A or F14A could induce Ca\(^{2+}\) oscillations, but the delay time was prolonged; that is, the activity is substantially lowered. W13A or F14A could not translocate to PN, whereas R12E had normal activities (Table 2). When tryptophan was replaced with phenylalanine (W13F) or the two residues were exchanged (W13F & F14W), the onset of Ca\(^{2+}\) oscillations was substantially delayed, and active nuclear import was lost (Table 2). Both abilities were preserved upon replacement of phenylalanine with tryptophan. Thus, Trp\(^{13}\) is essentially necessary, and Phe\(^{14}\) is replaceable with tryptophan.

S16A, K17A, or K17E was ineffective. In contrast, V18A showed no nuclear translocation ability, even when extremely overexpressed in 1-cell embryos. V18A induced Ca\(^{2+}\) oscillations, but the delay time was significantly prolonged (Table 2). Thus, Trp\(^{13}\), Phe\(^{14}\), and Val\(^{19}\) are critical for nuclear translocation ability and are necessary to keep normal Ca\(^{2+}\) oscillation-inducing activity as well. The region between Glu\(^{10}\) and Gln\(^{19}\) functionally looks like an NLS. However, the sequence Met\(^{1}\)–Gln\(^{19}\) fused with Venus showed no positive accumulation into PN (Table 2).

**Effects of Deletion of EF-hand Domains**—It is interesting to examine whether nuclear translocation takes place for a mutant in which EF1 is connected to the catalytic domain by deleting EF2–4 (Δ45–163; ΔEF2–4). This mutant turned out to have no nuclear translocation ability and Ca\(^{2+}\) oscillation-inducing ability (Table 2). Similarly, a mutant in which EF1 was connected to EF4 by deleting Asp\(^{542}\)–Met\(^{110}\) (Δ45–110; s-PLCζ) preceeded by EF1) lacked both abilities. Thus, EF1 is incapable of causing nuclear translocation without EF2–4; that is, the structure of EF-hand domain region as a whole is necessary.

**Effects of Deletion or Modification of C2 Domain**—Deletion of the C2 domain has been shown to cause the loss of PIP\(_2\)-hydrolyzing activity in vitro (10) and Ca\(^{2+}\) oscillation-inducing ability (18). In the present experiment, deletion mutant of the C2 domain (Δ522–625; ΔC2) had no nuclear translocation ability (Table 2). Point mutation was constructed at Asp\(^{542}\), because it corresponds to one of the putative Ca\(^{2+}\)-ligating aspartates in the C2 domain of all four PLCζ subtypes (31). Replacement of Asp\(^{542}\) with alanine or arginine did not affect nuclear translocation ability as well as Ca\(^{2+}\) oscillation-inducing activity (Table 2).

**Ca\(^{2+}\) Oscillations and Nuclear Translocation in Cultured Somatic Cells—Ca\(^{2+}\) oscillation-inducing activity and nuclear accumulation ability of PLCζ and its mutants tagged with Venus were investigated in COS-7 cells 24–72 h after transfection of PLCζ-Venus cDNA.**

![Graph](image-url)

**FIGURE 5. Ca\(^{2+}\) oscillations in PLCζ-expressed COS-7 cells.** A, negative control of non-transfected cells. B, Ca\(^{2+}\) oscillations in COS-7 cells 24 h after transfection of PLCζ-Venus cDNA. C, no Ca\(^{2+}\) oscillation in cells 24 h after transfection of D210R cDNA. Records from three representative cells are shown in each panel.
72 h, PLCζ was accumulated in the nucleoplasm as well as nucleoli (Fig. 6C).

For D210R, FN was clearly higher than FC (Fig. 6D). Accumulation into nucleoli was observed to the lesser extent, compared with wild-type PLCζ. K377E completely lacked nuclear translocation ability (Fig. 6E). ΔEF1-tr was hardly accumulated in the nucleoplasm (Fig. 6F), indicating that truncation of the N terminus causes remarkable suppression of nuclear translocation. The putative NSL sequence Lys374–Ala383 fused with Venus was clearly accumulated into both nucleoplasm and nucleoli (Fig. 6G), when compared with Venus alone (Fig. 6A). In contrast, the sequence Met1–Gln19 or Glu296–Val309 fused with Venus showed comparable FN and FC (Fig. 6, H and I), indicating no positive accumulation into the nucleus.

DISCUSSION

NLS Sequence—The present study demonstrated the site or region of the PLCζ molecule responsible for the nuclear translocation ability by quantitative assay in mouse embryos and confocal microscopy of cultured somatic cells. NLS sequence was identified as the residues 374–381 in the X/Y linker region. Addition of the sequence Lys374–Ala383 to Venus (27-kDa protein), which diffuses through nuclear pores (FPN/FC = 1.0) caused active nuclear import of Venus in COS-7 cells. Nuclear translocation of PLCζ-Venus (74 + 27 kDa) relies primarily on the NLS in which basic amino acids, Arg376, Lys377, Arg378, Lys379, and Lys381, were essential. NLS is thought to be a binding site of the nuclear transport receptor (NTR). The residues 371–381 are well conserved in mouse, rat, human, monkey, pig, and cow (NCBI data bank).

Difference in Nuclear Distribution of PLCζ between Pronucleus and Nucleus—The active nuclear import of Venus fusion with K374-A383 did not occur in PN, unlike in the nucleus of COS cells. Additional import signal in PLCζ may be required for translocation into PN (see below). Another difference existed in the finding that PLCζ was hardly accumulated to the large nucleolus of PN, while Lys374–Ala383 as well as PLCζ was localized in nucleoli of a COS cell. Lys374–Ala383 involving a cluster of basic amino acids may serve as a nucleolar localization signal (32, 33). In the nucleolus of PN, however, the presumptive nucleolar localization signal receptor might not be expressed.

In COS cells, expressed PLCζ was little accumulated in the nucleoplasm at 48 h after transfection, while strongly concentrated to the nucleolus (Fig. 6B). The rate of nucleolar accumulation may be much higher than the rate of net nuclear import. At 72 h, PLCζ was accumulated in the nucleoplasm (Fig. 6C) probably after saturation in nucleoli. This preferential nucleolar localization was less marked for D210R (Fig. 6D), suggesting that the rate of nucleolar targeting could be enhanced by Ca2+ oscillations which are produced by PLCζ but not by D210R in COS cells (Fig. 5).

Import and Export Signals—PLCδ1 is a PLC isoyme similar to PLCζ (2) (38% identity and 49% similarity in 647 amino acid residues of PLCζ), although the PH domain is present in PLCδ1 but absent in PLCζ. PLCδ1 is not accumulated to PN (3) or the nucleus (34). PLCδ1 has a nuclear export signal at a leucine-rich sequence in EF1 (34) and an import signal at lysine-rich sequence in the C terminus of X domain and the X/Y linker of PLCδ1 (30). Import and export are balanced (30). I31-C43 in EF1 of PLCζ may correspond to the export signal sequence of PLCδ1. Lys299 and Lys301 (Lys299/Lys301) in the C terminus of X domain of PLCζ were found to be responsible for nuclear import as in PLCδ1 (30). However, the region itself is not NLS, since the sequence E296-V309 fused with Venus did not show active nuclear import in COS cells. Lys299 and Lys301 (Lys299/Lys301) are thought to be a supplemental component enabling nuclear translocation.
X Catalytic Domain—Point mutation of Asp$^{210}$ (D210R), Lys$^{299}$ (K299E) and Lys$^{301}$ (K301E) in the X catalytic domain caused the loss of Ca$^{2+}$ oscillation-inducing activity. Asp$^{210}$ corresponds to Asp$^{343}$ of PLC$_\beta1$, which is involved in a Ca$^{2+}$ binding site responsible for the enzymatic activity (16, 35). D210R was able to undergo nuclear accumulation, although its rate was substantially lowered. Lys$^{299}$/Lys$^{301}$ is close to one of the residues interacting with the substrate, PIP$_2$, if considered in analogy with PLC$\delta1$ (35). Hence they could affect Ca$^{2+}$ oscillation-inducing activity. Lys$^{299}$/Lys$^{301}$ are also close to the X/Y linker, hence they could affect nuclear translocation activity as well.

EFI Domain—Ca$^{2+}$ oscillations and nuclear translocation are not prerequisite for each other, as they are dissected by point mutation in NLS and Asp$^{210}$. The two independent abilities are affected in a parallel manner by mutational modifications in EF-hand domains and C2 domain. EF1 is an important domain for both abilities. According to the ProDom EF-hand pattern (36), Asp$^{20}$—Ile$^{31}$ in EF1 is the Ca$^{2+}$-binding loop sequence. Ca$^{2+}$ oscillations and nuclear translocation are little affected by replacement at the x and z positions of the loop (D20A and G24A) (18). Thus, the Ca$^{2+}$ binding site in EF1 does not play a critical role in these abilities. Δ2–14, Δ2–19, or Δ10–19 is defective in both abilities. Point mutation in hydrophobic amino acids Trp$^{13}$, Phe$^{14}$, and Val$^{18}$ caused the loss of nuclear translocation and prolonged the onset of Ca$^{2+}$ oscillations. Trp$^{13}$ and Phe$^{14}$ are common to mouse, rat, human, monkey, pig, and cow. The residue 18 is Val$^{18}$ in mouse, pig, and cow, and Ile$^{18}$ in rat, human, and monkey. Thus, these hydrophobic residues are conserved. Although both Trp$^{13}$ and Phe$^{14}$ have an aromatic side chain, Trp$^{13}$ was not replaceable with phenylalanine while Phe$^{14}$ was replaceable with tryptophan. It seems that tryptophan at the exact positions in a hydrophobic moiety is the essential requirement. The sequence Glu$^{10}$—Gln$^{19}$ fused with Venus did not show active translocation into the nucleus of COS-7 cells. The sequence corresponds neither to any known NLS nor to a non-classical NLS found in phospholipids scramblase 1 to interact with importin $\alpha$ (37). Thus, the region is not NLS, but may necessary to take appropriate conformation for nuclear translocation of PLC$_\zeta$ (see below).

Four EF-hand Domains—Besides the presence of EF2–4, the C2 domain was necessary for both abilities. Deletion of EF2–3 or EF2–4 resulted in the loss of both abilities, even if EF1 was present. Truncation of EF-hand domains from the N terminus

**FIGURE 7.** Schematic drawing of a model for functional structure of PLC$\zeta$. A, model of wild-type PLC$\zeta$ having Ca$^{2+}$ oscillation-inducing ability and nuclear translocation ability. B–D, model to explain the results for PLC$\zeta$ mutants.
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showed that Ca^{2+} oscillations were barely induced after a long delay by extremely overexpressed s-PLCζ (ΔEF1–3-tr), but were never produced by ΔEF1–4-tr. Thus, s-PLCζ is considered to be the minimal structure required for Ca^{2+} oscillation-inducing activity. EF3 is responsible for high Ca^{2+} sensitivity of PIP_2-hydrolyzing activity in vitro of PLCζ, as shown previously (18). Deletion of C2 domain caused the loss of both abilities, although the abilities were conserved upon point mutation of Asp^{542} in C2 of PLCζ corresponding to the putative Ca^{2+}-lignating aspartate of PLCδ subtypes (31). A chimera formed by replacing the region from X domain to the C terminus of PLCζ with that of PLCδ1 has no detectable PLC activity (18). Taken together, the PLC activity of PLCζ seems to be derived from highly coordinated structure of EF-hand region and C2 domain.

A Model of Functional Structure—We tried to imagine a model of molecular structure to explain present results, as schematically drawn in Fig. 7. Crystal analysis of three-dimensional structure of PLCδ1 has shown that it is folded at the X/Y linker region in such a way that C2 domain in the C terminus makes extensive contact with EF-hand domains in the N terminus and the catalytic domain, forming the catalytic core (16, 38). PLCζ is supposed to take the three-dimensional structure basically similar to that of PLCδ1. In wild-type PLCζ association of EF1 with C2 is considered to be essential to take a compact form as the active conformation (Fig. 7A). The hydrophobic residues in the N terminus of EF1 may play an important role in EF1-C2 interaction. The site for substrate binding is given by close apposition of X and Y catalytic domains. The X/Y linker (309–385) is a flexible region protruding from the catalytic domains. Lys^{299}/Lys^{301} in X domain is located close to the N terminus of the X/Y linker, and NLS (374–381) is located at the C terminus of the X/Y linker, so that the two regions are likely to be neighboring each other. We postulate that K299/K301 is a component of the NTR binding site and that PLCζ-NTR association is accomplished by binding at both Lys^{374}–Lys^{381} and Lys^{309}/Lys^{301} regions (Fig. 7A).

ΔEF-tr or mutation in the N terminus of EF1 will cause dissociation of EF-hand domains from C2 domain. It is deduced that this conformation change may prevent the close apposition of X and Y, and thereby, perturb substrate binding (Fig. 7B). The change may also disturb NTR binding to both Lys^{374}–Lys^{381} and Lys^{309}/Lys^{301} region which became substantially distant each other. Thus, both Ca^{2+} oscillation-inducing ability and nuclear targeting ability are lost.

From results presented in Table 2, EF2 apparently serve as an inhibitory factor in the absence of EF1. EF2 might enhance the dissociation between EF3–4 and C2 (Fig. 7B). When EF2 was deleted, the region of EF3–4 or EF4 could be substantially closer to C2 than in ΔEF1-tr, yielding a slight Ca^{2+} oscillation-inducing activity (Fig. 7C). As ΔEF1–4-tr is incapable of forming the catalytic core (Fig. 7D), it has no Ca^{2+} oscillation-inducing activity. Nuclear translocation ability is lost by deletion of any EF-hand domains.

Biological Significance—A biological significance of translocation of the sperm factor or PLCζ into PN is postulated to be turning Ca^{2+} oscillations off at the entrance of the interphase in the first cell cycle (11, 15, 39). At the transition from G2 to M phase, Ca^{2+} spikes resume upon nuclear envelope breakdown prior to the first cleavage and then disappear at the 2-cell stage (11, 39). Artificially expressed PLCζ that entered the PN disperses into the cytoplasm upon nuclear envelope breakdown and is accumulated again into the nuclei of the 2-cell embryo (11, 12). Thus, cytoplasm/nucleus shuttling of PLCζ is thought to be related to turning off and on Ca^{2+} oscillations in a cell cycle stage-dependent manner.

A phosphoinositide signaling pathway is known to exist in the nucleus (40). For example, PLCβ1 translocates into the nucleus during G2/M transition in immature mouse oocytes and participates in germinal vesicle breakdown via diacylglycerol and protein kinase C (41, 42). Nuclear accumulation of PLCζ might have some roles other than regulating Ca^{2+} oscillations in early embryonic development.

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