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Nuclear translocation of phospholipase C-zeta, an egg-activating factor, during early embryonic development ☆

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Abstract

Phospholipase C-zeta (PLCζ), a strong candidate of the egg-activating sperm factor, causes intracellular Ca^{2+} oscillations and egg activation, and is subsequently accumulated into the pronucleus (PN), when expressed in mouse eggs by injection of RNA encoding PLCζ. Changes in the localization of expressed PLCζ were investigated by tagging with a fluorescent protein. PLCζ began to translocate into the PN formed at 5–6 h after RNA injection and increased there. Observation in the same embryo revealed that PLCζ in the PN dispersed to the cytoplasm upon nuclear envelope breakdown and translocated again into the nucleus after cleavage. The dynamics was found in the second mitosis as well. When RNA was injected into fertilization-originated 1-cell embryos or blastomere(s) of 2–8-cell embryos, the nuclear localization of expressed PLCζ was recognized in every embryo up to blastocyst. Thus, PLCζ exhibited alternative cytoplasm/nucleus localization during development. This supports the view that the sperm factor could control cell cycle-dependent generation of Ca^{2+} oscillations in early embryogenesis.

Keywords: Phospholipase C-zeta; Egg activation; Mammalian egg; Sperm factor; Nuclear translocation; Cell cycle; Early embryonic development

Egg activation at fertilization is induced by a dramatic increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), common to all species examined to date [1]. Mammalian eggs show a long-lasting series of repetitive $[Ca^{2+}]_i$ rises due to Ca^{2+} release from the endoplasmic reticulum mainly through inositol 1,4,5-trisphosphate (IP_3) receptors [2]. These 'Ca^{2+} oscillations' are thought to be caused by a cytosolic sperm factor driven into the ooplasm upon sperm-egg fusion [3,4], since they are produced by injection of sperm extract [4] or a spermatozoon into the egg [5]. The sperm-derived Ca^{2+} oscillation-inducing activity is concentrated into the pronucleus (PN) formed several hours after fertilization, as examined by transfer of the ooplasm or PN into unfertilized eggs [6]. Ca^{2+} oscillations cease at about the time of PN formation [7] and resume at the nuclear envelope breakdown (NEB) prior to the first cleavage [8], suggesting that the redistribution of the sperm factor may turn on and off Ca^{2+} oscillations [8,9].

A strong candidate of the sperm factor is a sperm-specific subtype of IP_3-producing enzyme phospholipase C, PLCζ [10]. Fertilization-like Ca^{2+} oscillations are induced by PLCζ expressed in mouse eggs after injection of its RNA [10,11] or by injection of recombinant PLCζ protein [12]. Interestingly, expressed PLCζ that activated the egg is subsequently accumulated into the PN [11,13]. Thus, the nuclear translocation ability of PLCζ

☆ *Abbreviations:* $[Ca^{2+}]_i$, intracellular calcium concentration; DIC, differential interference contrast; F, fluorescence intensity; IP_3, inositol 1,4,5-trisphosphate; MII, metaphase of second meiosis; NEB, nuclear envelope breakdown; PLCζ, phospholipase C-zeta; PN, pronucleus.

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as a sperm factor candidate could be a key factor for regulation of cell cycle-dependent Ca^{2+} oscillations. We investigated cytoplasm/nucleus redistribution of PLCζ during second meiosis, and first and second mitosis of the mouse embryo, by expressing PLCζ tagged with a fluorescent protein 'Venus' [14] after injection of RNA. The nuclear translocation ability of PLCζ was also followed during development up to blastocysts.

Materials and methods

Preparation of gametes. Mature eggs were collected from the oviducts of superovulated B6D2F1 female mice and freed from cumulus cells by brief treatment with 0.05% hyaluronidase (Sigma, St. Louis, MO) in M2 medium [15]. 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, Tokyo, Japan). Sperm from the cauda epididymides were incubated in M16 medium at 37 °C (5% CO_2 in air) for several hours for capacitation and acrosome reaction [15].

Construction of plasmid. All methods followed the previous study [11]. Briefly, PLCζ cDNA was cloned from cDNA library originated from mouse testis mRNAs and was amplified by PCR. It was subcloned into pBluescript II SK(+) (Stratagene, La Jolla, CA) using *Eco*RI and *Xba*I sites. PLCζ cDNA in the plasmid was amplified, and its fragments were digested with *Kpn*I and *Spe*I, and ligated to the *Kpn*I and *Spe*I sites of pBluescript II SK(+). cDNA of Venus [14] (from Dr. A. Miyawaki; Brain Science Institute, RIKEN) in a plasmid was amplified, and its fragments were digested with *Spe*I and *Not*I, and ligated to the *Spe*I and *Not*I sites of PLCζ-pBluescript II SK(+).

RNA and polyadenylation. Fragments of plasmids digested with *Not*I were used as templates for in vitro transcription. RNA was synthesized by T7 polymerase using T7 mMESSAGE mMACHINE Kit (Ambion, Austin, TX). To facilitate RNA translation in the egg, RNA was added with more than 200 poly(A) in the 3′ tail [16] in the presence of 200 μM ATP and 15 U/μl yeast-derived poly(A) polymerase (Amersham, Piscataway, NJ) in a buffer containing (mM) 50 KCl, 20 Tris–HCl (pH 7.0), 0.7 $MnCl_2$, 0.2 EDTA, supplemented with 800 U/ml RNasin (Promega, Madison, WI), 100 μg/ml BSA, and 10% glycerol. Dried RNA was resolved in 150 mM KCl solution (~1.5 μg/μl).

RNA injection. RNA (20 ng/μl) encoding PLCζ-Venus was injected into 40–45 eggs within 30 min using a glass micropipette in M2 medium (injected amount, ~5 pl; RNA in the egg, ~0.5 ng/μl). Eggs were cultured in M16 medium at 37 °C (5% CO_2 in air) until observation at defined stages. RNA (50 ng/μl) was also injected into a 1–8-cell embryos which were previously inseminated and cultured in M16.

Measurement of Venus fluorescence. Venus-derived fluorescence images of embryos were acquired continuously every 10 min at 37 °C or recorded by a single shot at a defined time, using an EB-CCD camera (C7190-23; Hamamatsu Photonics, Hamamatsu, Japan) and an image processor (Argus 50; Hamamatsu Photonics), or using a cooled digital camera (DP70; Olympus, Tokyo, Japan). Excitation light was passed through a 490–510 nm bandpass filter and a 20× objective lens. Emitted light was passed through the objective lens, a 525 nm dichroic mirror, and a 530–560 nm bandpass filter. Bright field images were photographed by the digital camera. Precise observation was done by a confocal laser scanning microscope (LSM510, Carl Zeiss, Oberkochen, Germany) with excitation light of 510 nm, and differential interference contrast (DIC) images were recorded simultaneously by another sensor for transmitted laser light.

Results and discussion

Accumulation of expressed PLCζ into the PN

Fig. 1 shows changes in the fluorescence intensity (F) of Venus in three representative eggs kept in M2 medium after injection of PLCζ-Venus RNA at the metaphase of the second meiosis (MII). F in the cytoplasm (broken line) became detectable from 40 to 50 min after RNA injection, increased up to 4 h, and then attained a steady level or gradually declined. Eggs were activated by repetitive $[Ca^{2+}]_i$ rises caused by PLCζ [8] via production of IP_3 and IP_3-induced Ca^{2+} release. The PN having a large nucleolus was recognized by in the bright field at about 5 h (inset A), although the time may be slightly later than PN formation. It should be noted that F in the PN (solid line) gradually increased and separated from that in the cytoplasm ~30 min before the PN was recognized (arrows in Fig. 1).

F in the PN was greatly elevated during recording up to 18 h (Fig. 1, inset B). However, 1-cell embryos kept in M2 medium (buffered mainly with Hepes) never underwent NEB. In addition, long-term irradiation every 10 min will perturb normal development. Since ~90% of RNA-injected eggs developed to 2-cell embryos during culture in M16 medium (buffered with $NaHCO_3$) without measurement of F, eggs were incubated in M16 in several dishes (five eggs each), and one dish was subjected to a single shot alone at a given time. In these 'developing' 1-cell embryos, F in the PN as well as that of the cytoplasm increased up to 9 h after RNA injection and then declined with time (Fig. 1), although PLCζ was still clearly seen in the PN at 14 h (inset C). In developing embryos, PLCζ may be gradually degraded in the cytoplasm.

Dispersion of PLCζ from the nucleus to cytoplasm after NEB

The PN became invisible in the bright field between 15.5 and 16.5 h after RNA injection, indicating that NEB occurred. F in the PN was lost at this stage (Fig. 1, inset D). A faintly fluorescent oval area was discernible in the central region. Since similar images were observed upon expression of Venus-free PLCζ (not shown), it is autofluorescence, probably derived from oxidized flavins of mitochondria [17] which are accumulated in the perinuclear region of the 1-cell embryo undergoing the first mitosis and the 2-cell embryo [18]. The autofluorescence disturbed distribution analysis of PLCζ. The first cleavage occurred at 16–18 h during culture in M16, but it was often blocked or embryos were fragmented during continuous recording under microscope.

To allow higher F of Venus as well as normal development, RNA was injected into fertilized eggs at 6 h