Phospholipase C ζ , the trigger of egg activation in mammals, is present in a non-mammalian species

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Abstract

The activation of the egg to begin development into an embryo is triggered by a sperm-induced increase in intracellular egg Ca²⁺. There has been much controversy about how the sperm induces this fundamental developmental event, but recent studies suggest that, in mammals, egg activation is triggered by a testis-specific phospholipase C: PLC ζ . Since the discovery of PLC ζ , it has been unclear whether its role in triggering egg activation is common to all vertebrates, or is confined to mammals. Here, we demonstrate for the first time that PLC ζ is present in a non-mammalian vertebrate. Using genomic and cDNA databases, we have identified the cDNA encoding a PLC ζ orthologue in the domestic chicken that, like the mammalian isoforms, is a testis-specific gene. The chicken PLC ζ cDNA is 2152 bp in size and encodes an open reading frame of 639 amino acids. When injected into mouse oocytes, chicken PLC ζ cRNA triggers Ca²⁺ oscillations, indicating that it has functional properties similar to those of mammalian PLC ζ . Our findings suggest that PLC ζ may have a universal role in triggering egg activation in vertebrates.

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Introduction

Activation of the egg by the sperm is a fundamental developmental event that frees the egg from its state of cell-cycle arrest, allows metabolism to resume, and acts as a block to polyspermy. In almost all species studied, egg activation is induced by an increase in the concentration of intracellular calcium ions (Ca²⁺) (Stricker 1999). However, the mechanism whereby the sperm triggers the release of Ca²⁺ in the egg has been a subject of controversy over recent decades, with opinion divided as to whether egg activation is initiated by a surface interaction between a sperm ligand and an egg receptor, or by a cytosolic 'sperm factor' that is released into the egg from the sperm during gamete fusion (Evans & Kopf 1998, Swann & Parrington 1999).

Recent studies suggest that the mammalian sperm factor is a sperm-specific phospholipase C, PLC ζ , that has the expected properties of the physiological agent of egg activation. For example, when injected into mouse oocytes, recombinant mouse PLC ζ cRNA (Saunders *et al.* 2002) or protein (Kouchi *et al.* 2004), triggers Ca²⁺ oscillations identical to those seen at fertilization, whereas

immunodepletion of endogenous PLC ζ from sperm extracts removes their ability to cause the release of Ca²⁺ (Saunders *et al.* 2002). PLC ζ also has other distinctive properties, such as its high Ca²⁺ sensitivity (Kouchi *et al.* 2004) and a propensity to accumulate in the pronucleus of the zygote (Larman *et al.* 2004, Yoda *et al.* 2004).

PLC ζ homologues have been identified in mice, humans and monkeys (Saunders *et al.* 2002, Cox *et al.* 2002). However, so far, PLC ζ has not been identified other than in eutherian mammals. Therefore, given the nearly universal conservation of a Ca²⁺ signal at fertilization, a major unanswered question is whether PLC ζ has a role only during egg activation in mammals or whether it has a more universal role. There is indirect evidence to suggest that the latter might be the case: when injected into mouse oocytes, sperm extracts from chicken and the clawed frog, *Xenopus laevis*, trigger Ca²⁺ oscillations similar to those seen at fertilization (Dong *et al.* 2000). We have shown further that a sperm protein extract from tilapia, a commercially important teleost (bony) fish, also triggers Ca²⁺ oscillations when injected into mouse oocytes (Coward *et al.* 2003). In addition, sperm factor activities have been identified in animals as diverse as

marine worms (Stricker 1997) and sea squirts (Kyoizuka *et al.* 1998), although to date there have been no reports that these invertebrate sperm factors can trigger Ca^{2+} oscillations in mouse oocytes. However, it remains far from clear whether such sperm factor activities are functions of a PLC ζ orthologue in these species, or are caused by some completely different signalling protein. In the current study, we demonstrate, for the first time, the existence of a PLC ζ orthologue in a non-mammalian vertebrate, the domestic chicken.

Materials and Methods

Identification of a chicken PLC ζ orthologue in the chicken genome

Murine and human PLC ζ protein sequences were used to search the chicken genome database at Ensembl (http://www.ensembl.org/Gallus_gallus/) using BLAST (Altschul *et al.* 1997). The search identified a novel PLC-like sequence that exhibited strong sequence similarity to mammalian PLC ζ and had a similarly conserved syntenic location on the genome. The chicken PLC ζ gene was predicted using Genewise (Birney *et al.* 2004).

Cloning of chicken PLC ζ cDNA

A full-length clone (in pSPORT1 vector) was identified from a cDNA library constructed from testis (domestic White Leghorn cockerel, *Gallus domesticus*; Savolainen *et al.* 2005) and was fully sequenced by MWG Biotech Ltd (UK) to confirm its identity. The full-length nucleotide sequence was translated into a predicted amino acid sequence with the ExPaSy Molecular Biology Server (Swiss Institute of Bioinformatics; <http://ca.expasy.org/>). For purposes of comparison, the putative chicken PLC ζ was initially compared by constructing a multiple sequence alignment using CLUSTALW (Thompson *et al.* 1994), with the following sequences (accession numbers in parentheses): monkey PLC ζ (*Macaca fascicularis*; AB070108), human PLC ζ (NM_033123), mouse PLC ζ (NM_054066). A dendrogram of PLC ζ and PLC δ 1 sequences was then constructed using CLUSTALW and unweighted pair-group method with arithmetic mean methodology. Sequences used in the dendrogram were as follows (accession numbers in parentheses): mouse PLC ζ (NM_054066), human PLC ζ (NM_033123), mouse PLC δ 1 (AAH25798), human PLC δ 1 (AAH50382), chicken PLC δ 1 (XP_418522). The accession number for chicken PLC ζ is AY843531. The domain structure of the putative chicken PLC ζ was investigated using SMART (Schultz *et al.* 1998).

Tissue expression of PLC ζ

A range of tissues (testis, heart, liver, kidney, brain, spleen, smooth muscle and gizzard) were collected from a freshly culled domestic White Leghorn (*Gallus domesticus*) cockerel and were immediately snap-frozen in liquid nitrogen.

Total RNA was prepared from each tissue using an RNeasy Kit (Qiagen, UK) in accordance with the manufacturer's instructions. For PCR analysis, total RNA (5 μg) from each of the harvested tissues was reverse-transcribed into single-stranded cDNA using a Cloned AMV First Strand Synthesis kit (Invitrogen, UK) and amplified with oligonucleotide primers designed to amplify the full-length chicken PLC ζ gene: CZ5 (5'-ATGGAGGAGAACAGATGGTTT-3') and CZ3 (5'-GTAGTACCAGACATACACAAA-3') using the Expand High Fidelity PCR System (Roche Diagnostics, UK). For northern blot analysis, 40 μg total RNA from each harvested tissue were denatured and fractionated on a 1% formaldehyde RNA gel and blotted onto a Hybond-N⁺ membrane (Amersham Biosciences, UK) using a vacuum blotter (BioRad, UK). Membranes were subsequently stained with 0.03% methylene blue/0.2 mol/l sodium acetate to visualize RNA and provide a comparison of lane loading. Membranes were incubated for 20 min at 65 °C with Rapid-Hyb Buffer (Amersham Biosciences, UK) and then overnight at 65 °C with a ³²P-dCTP-labelled probe (full-length cDNA, prepared using CZ5 and CZ3 oligonucleotides). The probe was labelled with a Megaprime II Labelling System (Amersham Biosciences, UK). After hybridization, the membrane was washed with 50 ml 2 × SSC 0.1% SDS at room temperature, followed by two 15-min washes in 50 ml 1.0–0.1% SSC/0.1% SDS at 65 °C as necessary. Hybridization signals were detected using a Typhoon Phosphor Imaging System (Amersham Biosciences, UK).

Injection of PLC ζ cRNA into mouse oocytes

cRNA was synthesized from linearized full-length chicken PLC ζ using the mMessage Machine kit (Ambion, Austin, TX, USA) in accordance with the Manufacturer's instructions. Outbred MF1 mice (Harlan, Bicester, UK) were first super-ovulated with injections of pregnant mares serum gonadotropin and human chorionic gonadotropin and ovulated oocytes harvested. Microinjection of cRNA constructs and fura2 dextran were then carried out as described previously (Madgwick *et al.* 2004). Injected oocytes were imaged for resultant changes in intracellular Ca^{2+} on a heated stage fitted to a Nikon TE300 inverted microscope equipped for epi-fluorescence (Jones & Nixon 2000). Images were acquired using a Sony Interline CCD camera controlled by MetaFluor software (Universal Imaging Corp., Downingtown, PA, USA).

Results

Identification of a chicken PLC ζ orthologue in the chicken genome

Searches of the chicken genome with murine and human PLC ζ protein sequences identified a novel PLC-like sequence that exhibited strong sequence similarity to mammalian PLC ζ . This novel chicken PLC was predicted to be a PLC ζ orthologue on the basis of its strong

sequence similarity to mammalian PLCζ and its conserved syntenic location on the genome. Despite extensive searches, no pleckstrin homology (PH) domain was discernible that was encoded within the chicken PLCζ 5' sequence. The putative chicken PLCζ shares between 55 and 58% amino acid identity and between 70 and 71% similarity to mammalian PLCζ isoforms. A dendrogram was used to demonstrate monophyly among human, macaque, mouse and chicken PLCζ isoforms, with PLCδ1 isoforms as outgroup sequences (Fig. 1A).

Further evidence that the chicken gene is a PLCζ orthologue is clearly demonstrated by its genomic location. In the chicken genome, PLCζ is flanked by the same genes as those flanking PLCζ in mammals (Fig. 1B). In mammals, PLCζ is located back-to-back with another testis-specific gene, *CAPZA3*, with which it appears to share a bidirectional promoter containing a putative cAMP responsive element modulator protein recognition site (Hurst *et al.* 1998). In chickens, PLCζ shares the same back-to-back arrangement with *CAPZA3* (Fig. 1B), although a chicken

Figure 1 Molecular identification of chicken PLCζ. (A) Dendrogram illustrating monophyly among human, macaque, mouse and chicken PLCζ isoforms, with PLCδ1 isoforms as outgroup sequences. The topology of this dendrogram is supported by bootstrap values of 100% at all internal nodes. (B) Conserved syntenic arrangement of genes flanking chicken PLCζ in chicken (chromosome 1 [chr 1]) and human (chromosome 12 [chr 12]). Gene abbreviations: *PIK3C2G*, phosphoinositide-3-kinase, class 2, gamma polypeptide; *PLCZ1*, phospholipase C, ζ1; *CAPZA3*, testis-specific capping protein (actin filament) muscle Z-line, α3; *PEPP2*, phosphatidylinositol 3-phosphate-binding PH domain protein 2; *AEBP2*, Adipocyte enhancer (AE) binding protein 2.

sequence similar to the mammalian bidirectional promoter was not discernible.

Cloning of chicken PLC ζ cDNA

A full-length clone was identified from a cDNA library constructed from chicken testis tissue (Savolainen *et al.* 2005). Sequencing of the cDNA clone showed that it was 2152 bp in size and coded for an open reading frame of 639 amino acids. This cDNA sequence was identical to our prediction from the genome, and different in coding exons 1 and 7 compared with a RefSeq prediction (accession XM_416413.1). Chicken PLC ζ has a predicted binding affinity (pI) of 8.53 and a molecular mass of 72.53 kDa. Phylogenetic analysis involving chicken, mouse and human PLC ζ isoforms demonstrated that the chicken isoform was the most divergent, as expected (Fig. 1A). The domain structure of chicken PLC ζ exhibits the same organization as mammalian PLC ζ isoforms: a catalytic X-Y domain and a C2 domain, but no PH domain (Fig. 2). Catalytically important residues and a putative phosphoinositide (PI) binding site are also shown in Fig. 2.

Tissue expression of PLC ζ

To investigate the tissue expression of chicken PLC ζ , we first searched chicken 'expressed sequence tag' (EST) cDNA databases. ESTs (e.g. accession numbers CN232708, CN231906) corresponding to the chicken PLC ζ were identified as originating from testis, but were not identifiable from other tissues. This is consistent with a sperm-specific pattern of expression for chicken PLC ζ , as seen for mammalian PLC ζ . To confirm whether this were the case, we next analysed total RNA for PLC ζ transcripts in a panel of chicken tissues using RT-PCR and northern blot assays. Analysis of the RT-PCR product showed a band at the predicted size (2152 bp) only with testis cDNA and the positive control (Fig. 3A). Sequencing confirmed that the 2152 bp band was indeed chicken PLC ζ . Northern analysis further confirmed that chicken PLC ζ was present only in testis, and not in other tissues, and showed that the chicken PLC ζ mRNA conformed to its predicted size (Fig. 3B).

Injection of PLC ζ cRNA into mouse oocytes

A key question is whether chicken PLC ζ is functionally equivalent to its mammalian orthologue. Studying its ability to cause the release of Ca²⁺ after injection into a chicken oocyte is problematic, because imaging with fluorescent Ca²⁺-sensitive dyes in the thin layer of cytoplasm at the animal pole where the sperm fuses (Gilbert 2003) is technically challenging. Instead, we chose to assay the ability of chicken PLC ζ to cause the release of Ca²⁺ in a mouse oocyte, because previous studies had demonstrated that recombinant mouse, human and monkey PLC ζ cRNA triggered Ca²⁺ spiking in mouse oocytes (Cox *et al.* 2002, Saunders *et al.* 2002). Chicken

PLC ζ cRNA (1.4–0.02 μ g/ μ l, injected at 0.03–0.1% of oocyte volume) was microinjected into mature mouse oocytes to determine its ability to induce Ca²⁺ spiking. The procedure of injection into groups of oocytes lasted between 10 and 15 min. When injections were complete and imaging started, it was apparent that all oocytes had initiated Ca²⁺ spiking (Fig 4A, $n = 17$). The Ca²⁺ spiking frequency, expressed as the interspike interval at the lowest dose (0.02 μ g/ μ l) was 3.75 ± 1.14 min ($n = 5$). This high frequency spiking is similar to that reported with human PLC ζ at the same dose (4.21 ± 1.79 min; Cox *et al.* 2002) and similar to that which we observed with 0.01 μ g/ μ l human PLC ζ cRNA (Fig. 4B; $n = 5$). As a consequence of the Ca²⁺ spiking, which is the necessary and sufficient trigger for full oocyte activation (Hyslop *et al.* 2004), oocytes injected with chicken PLC ζ cRNA and monitored for several hours went on to extrude second polar bodies and form pronuclei (75%; $n = 12$).

Discussion

In the present study, our principal aim was to identify whether a PLC ζ orthologue was present in a non-mammalian species. Until now, PLC ζ has been identified only in mice (Saunders *et al.* 2002), monkeys and humans (Cox *et al.* 2002). We chose to investigate the chicken because it is one of the few non-mammalian vertebrate species to have had its genome fully sequenced and annotated, and also because birds are phylogenetically closer to mammals than are other vertebrate classes such as fish and amphibians (International Chicken Genome Sequencing Consortium 2004, Schmutz & Grimwood 2004).

The novel PLC-like molecule that we identified appears to be a chicken PLC ζ orthologue as judged by the following features. Firstly, it has discrete domains characteristic of a PLC such as an EF-hand domain, X and Y catalytic domains and a C2 domain, along with several catalytically important residues, but no PH domain that is discernible encoded within its genomic sequence. Sequence analysis of a full-length chicken PLC ζ clone also confirmed the absence of a PH domain. This supports its identification as a PLC ζ orthologue, as the lack of a PH domain distinguishes PLC ζ from PLC δ isoforms (Cox *et al.* 2002, Saunders *et al.* 2002). Secondly, in the chicken genome our PLC-like molecule is clearly flanked by the same genes as those flanking mammalian PLC ζ isoforms and thus also probably shares a bidirectional promoter with the testis-specific gene, *CAPZA3* (Hurst *et al.* 1998, Yoshimura *et al.* 1999, Miyagawa *et al.* 2002), as is the case with mammalian PLC ζ . *CAPZA3* appears to be a retrogene that has inserted into the genome next to PLC ζ and, presumably, in the process acquired the same testis-specific pattern of expression as PLC ζ . Thirdly, phylogenetic analysis showed that chicken PLC ζ is clearly more similar to mammalian PLC ζ isoforms than it is to chicken PLC δ 1. Finally, chicken PLC ζ appears to have a testis-specific

Figure 2 Comparison of chicken PLC ζ with mammalian isoforms: multiple amino acid sequence alignment of chicken PLC ζ with monkey PLC ζ , human PLC ζ and mouse PLC ζ . Identical residues are indicated by black shading, conserved residues by dark grey shading, and similar residues by light grey shading. †Catalytically important residues; ‡residues vital for Ca²⁺ binding; *residue mutated by Saunders *et al.* (2002) leading to an inability to release Ca²⁺; #residues forming a putative phosphoinositide binding site (residues 303–310). Sequences of closely spaced bold dots represent the following: EF-hand (residues 48–105), X domain (residues 163–308), Y domain (residues 392–508) and the C2 domain (residues: 528–653).

Figure 3 Tissue expression of chicken PLC ζ . (A) Testis-specific expression of chicken PLC ζ as detected by RT-PCR. (B) Testis-specific expression of chicken PLC ζ as detected by northern blot analysis. Molecular weights are indicated on the vertical axes. T, testis; H, heart; Li, liver; B, brain; Sp, spleen; SM, smooth muscle; Gz, gizzard; (–ve, negative PCR control (distilled water); +ve, positive PCR control (chicken PLC ζ cDNA clone).

pattern of expression, and recombinant chicken PLC ζ cRNA is able to trigger Ca^{2+} oscillations in mouse oocytes and cause egg activation, just like the mammalian PLC ζ isoforms (Cox *et al.* 2002, Saunders *et al.* 2002).

The great commercial importance of chickens means there is great interest in identifying causes of subfertility in this species. Currently, very little is known about the mechanism of egg activation in chickens, despite the fact that defects in this process could be a cause of subfertility in cockerels. Our discovery of a PLC ζ orthologue in chickens, and the fact that it appears to have functional properties similar to those of mammalian PLC ζ and may thus have a similar role, not only is important as a step towards understanding the mechanism of egg activation in this species, but also has potential value as an important molecular marker of male fertility in cockerels.

These findings also have more general relevance for our understanding of the mechanism of egg activation in vertebrates as a whole. Previous studies have shown that an unidentified factor in chicken, frog and fish sperm can trigger Ca^{2+} oscillations when injected into mouse oocytes (Dong *et al.* 2000, Coward *et al.* 2003). However, it was not clear from those studies whether this ability to cause the release of Ca^{2+} was attributable to a PLC ζ orthologue or to a different signalling protein. Our identification of a chicken orthologue of PLC ζ with a pattern of expression and properties similar to those of mammalian PLC ζ suggests that PLC ζ may also be present in the sperm of fish and frogs, and may have a universal role during egg activation in vertebrates.

Figure 4 Ability of chicken PLC ζ to release Ca^{2+} in the mouse oocyte: Ca^{2+} oscillations in mouse oocytes microinjected with cRNA prepared from chicken or human PLC ζ cDNA clones. (A) 0.02 $\mu\text{g}/\mu\text{l}$ chicken PLC ζ cRNA. (B) 0.01 $\mu\text{g}/\mu\text{l}$ human PLC ζ cRNA. The volume microinjected was 0.03–0.1% of that of the oocyte.

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