Phospholipase Cζ, 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: PLCζ. Since the discovery of PLCζ, 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 PLCζ is present in a non-mammalian vertebrate. Using genomic and cDNA databases, we have identified the cDNA encoding a PLCζ orthologue in the domestic chicken that, like the mammalian isoforms, is a testis-specific gene. The chicken PLCζ cDNA is 2152 bp in size and encodes an open reading frame of 639 amino acids. When injected into mouse oocytes, chicken PLCζ cRNA triggers Ca²⁺ oscillations, indicating that it has functional properties similar to those of mammalian PLCζ. Our findings suggest that PLCζ may have a universal role in triggering egg activation in vertebrates.


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, PLCζ, that has the expected properties of the physiological agent of egg activation. For example, when injected into mouse oocytes, recombinant mouse PLCζ cRNA (Saunders et al. 2002) or protein (Kouchi et al. 2004), triggers Ca²⁺ oscillations similar to those seen at fertilization, whereas immunodepletion of endogenous PLCζ from sperm extracts removes their ability to cause the release of Ca²⁺ (Saunders et al. 2002). PLCζ 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).

PLCζ homologues have been identified in mice, humans and monkeys (Saunders et al. 2002, Cox et al. 2002). However, so far, PLCζ 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 PLCζ 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\(\zeta\) 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\(\zeta\) orthologue in a non-mammalian vertebrate, the domestic chicken.

**Materials and Methods**

**Identification of a chicken PLC\(\zeta\) orthologue in the chicken genome**

Murine and human PLC\(\zeta\) 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\(\zeta\) and had a similarly conserved syntenic location on the genome. The chicken PLC\(\zeta\) gene was predicted using Genewise (Binney et al. 2004).

**Cloning of chicken PLC\(\zeta\) 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\(\zeta\) 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\(\zeta\) (*Macaca fascicularis*; AB070108), human PLC\(\zeta\) (NM_033123), mouse PLC\(\zeta\) (NM_054066). A dendrogram of PLC\(\zeta\) and PLC\(\beta1\) sequences was then constructed using CLUSTALW and unweighted pair-group method with arithmetic mean methodology. Sequences were then analysed using the ExPaSy Molecular Biology Server (Swiss Institute of Bioinformatics; http://ca.expasy.org/).

**Injection of PLC\(\zeta\) cRNA into mouse oocytes**

cRNA was synthesized from linearized full-length chicken PLC\(\zeta\) 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., Downington, PA, USA).

**Results**

**Identification of a chicken PLC\(\zeta\) orthologue in the chicken genome**

Searches of the chicken genome with murine and human PLC\(\zeta\) protein sequences identified a novel PLC-like sequence that exhibited strong sequence similarity to mammalian PLC\(\zeta\). This novel chicken PLC was predicted to be a PLC\(\zeta\) orthologue on the basis of its strong...
sequence similarity to mammalian PLC\(_{\zeta}\) and its conserved syntenic location on the genome. Despite extensive searches, no pleckstrin homology (PH) domain was discernible that was encoded within the chicken PLC\(_{\zeta}\) 5' sequence. The putative chicken PLC\(_{\zeta}\) shares between 55 and 58% amino acid identity and between 70 and 71% similarity to mammalian PLC\(_{\zeta}\) isoforms. A dendrogram was used to demonstrate monophyly among human, macaque, mouse and chicken PLC\(_{\zeta}\) isoforms, with PLC\(_{\delta 1}\) isoforms as outgroup sequences (Fig. 1A).

Further evidence that the chicken gene is a PLC\(_{\zeta}\) orthologue is clearly demonstrated by its genomic location. In the chicken genome, PLC\(_{\zeta}\) is flanked by the same genes as those flanking PLC\(_{\zeta}\) in mammals (Fig. 1B). In mammals, PLC\(_{\zeta}\) 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, PLC\(_{\zeta}\) shares the same back-to-back arrangement with CAPZA3 (Fig. 1B), although a chicken

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**Figure 1** Molecular identification of chicken PLC\(_{\zeta}\). (A) Dendrogram illustrating monophyly among human, macaque, mouse and chicken PLC\(_{\zeta}\) isoforms, with PLC\(_{\delta 1}\) isoforms as outgroup sequences. The topology of this dendrogram is supported by bootstrap values of 100% at all internal nodes. (B) Conserved syteny for genes flanking chicken PLC\(_{\zeta}\) 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, \(\zeta\); CAPZA3, testis-specific capping protein (actin filament) muscle Z-line, \(\alpha\); PEPP2, phosphatidylinositol 3-phosphate-binding PH domain protein 2; AEBP2, Adipocyte enhances (AE) binding protein 2.
sequence similar to the mammalian bidirectional promoter was not discernible.

**Cloning of chicken PLCζ 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 PLCζ has a predicted binding affinity (pI) of 8.53 and a molecular mass of 72.53 kDa. Phylogenetic analysis involving chicken, mouse and human PLCζ isoforms demonstrated that the chicken isoform was the most divergent, as expected (Fig. 1A). The domain structure of chicken PLCζ exhibits the same organization as mammalian PLCζ 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 PLCζ**

To investigate the tissue expression of chicken PLCζ, we first searched chicken ‘expressed sequence tag’ (EST) cDNA databases. ESTs (e.g. accession numbers CN232708, CN231906) corresponding to the chicken PLCζ 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 PLCζ, as seen for mammalian PLCζ. To confirm whether this were the case, we next analysed total RNA for PLCζ 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 PLCζ. Northern analysis further confirmed that chicken PLCζ was present only in testis, and not in other tissues, and confirmed that the 2152 bp band was indeed chicken PLCζ cDNA and the positive control (Fig. 3A). Sequencing conformed 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 PLCζ has a predicted binding affinity (pI) of 8.53 and a molecular mass of 72.53 kDa. Phylogenetic analysis involving chicken, mouse and human PLCζ isoforms demonstrated that the chicken isoform was the most divergent, as expected (Fig. 1A). The domain structure of chicken PLCζ exhibits the same organization as mammalian PLCζ 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.

**Injection of PLCζ cRNA into mouse oocytes**

A key question is whether chicken PLCζ is functionally equivalent to its mammalian orthologue. Studying its ability to cause the release of Ca\(^{2+}\) after injection into a chicken oocyte is problematic, because imaging with fluorescent Ca\(^{2+}\)-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 PLCζ to cause the release of Ca\(^{2+}\) in a mouse oocyte, because previous studies had demonstrated that recombinant mouse, human and monkey PLCζ cRNA triggered Ca\(^{2+}\) spiking in mouse oocytes (Cox et al. 2002, Saunders et al. 2002). Chicken PLCζ cRNA (1.4–0.02 μg/μl, injected at 0.03–0.1% of oocyte volume) was microinjected into mature mouse oocytes to determine its ability to induce Ca\(^{2+}\) 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 Ca\(^{2+}\) spiking (Fig 4A, n = 17). The Ca\(^{2+}\) spiking frequency, expressed as the interspike interval at the lowest dose (0.02 μg/μl) was 3.75 ± 1.14 min (n = 5). This high frequency spiking is similar to that reported with human PLCζ at the same dose (4.21 ± 1.79 min; Cox et al. 2002) and similar to that which we observed with 0.01 μg/μl human PLCζ cRNA (Fig. 4B; n = 5). As a consequence of the Ca\(^{2+}\) spiking, which is the necessary and sufficient trigger for full oocyte activation (Hyslop et al. 2004), oocytes injected with chicken PLCζ 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 PLCζ orthologue was present in a non-mammalian species. Until now, PLCζ 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 PLCζ 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 PLCζ clone also confirmed the absence of a PH domain. This supports its identification as a PLCζ orthologue, as the lack of a PH domain distinguishes PLCζ from PLCβ 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 PLCζ 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 PLCζ. CAPZA3 appears to be a retrogene that has inserted into the genome next to PLCζ and, presumably, in the process acquired the same testis-specific pattern of expression as PLCζ. Thirdly, phylogenetic analysis showed that chicken PLCζ is clearly more similar to mammalian PLCζ isoforms than it is to chicken PLCζ1. Finally, chicken PLCζ appears to have a testis-specific
Figure 2  Comparison of chicken PLCζ with mammalian isoforms: multiple amino acid sequence alignment of chicken PLCζ with monkey PLCζ, human PLCζ and mouse PLCζ. 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).
pattern of expression, and recombinant chicken PLCζ cRNA is able to trigger Ca\(^{2+}\) oscillations in mouse oocytes and cause egg activation, just like the mammalian PLCζ 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 PLCζ orthologue in chicken, and the fact that it appears to have functional properties similar to those of mammalian PLCζ 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 PLCζ orthologue or to a different signalling protein. Our identification of a chicken orthologue of PLCζ with a pattern of expression and properties similar to those of mammalian PLCζ suggests that PLCζ may also be present in the sperm of fish and frogs, and may have a universal role during egg activation in vertebrates.

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Isolation and characterization of chicken PLCζ


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