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Characterization of a sperm factor for egg activation at fertilization of the newt *Cynops pyrrhogaster*

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Abstract

Eggs of the newt, *Cynops pyrrhogaster*, arrested at the second meiotic metaphase are activated by sperm at fertilization and then complete meiosis to initiate development. We highly purified a sperm factor for egg activation from a sperm extract with several chromatographies. The purified fraction containing only a 45 kDa protein induced egg activation accompanied by an intracellular Ca^{2+} increase when injected into unfertilized eggs. Although injection of mouse phospholipase C (PLC) ζ-mRNA caused a Ca^{2+} increase and egg activation, partial amino acid sequences of the 45 kDa protein were homologous to those of *Xenopus* citrate synthase, but not to PLCs. An anti-porcine citrate synthase antibody recognized the 45 kDa protein both in the purified fraction and in the sperm extract. Treatment with the anti-citrate synthase antibody reduced the egg-activation activity in the sperm extract. Injection of porcine citrate synthase or mRNA of *Xenopus* citrate synthase induced a Ca^{2+} increase and caused egg activation. A large amount of the 45 kDa protein was localized in two lines elongated from the neck to the middle piece of sperm. These results indicate that the 45 kDa protein is a major component of the sperm factor for egg activation at newt fertilization.

Keywords: Amphibian; Fertilization; Egg activation; Citrate synthase; PLCζ

Introduction

For the normal initiation of animal development, the timing of egg activation must be precisely controlled during fertilization. A fertilizing sperm provides a signal for egg activation at the time of sperm–egg binding or fusion. It has been demonstrated in many species that egg activation is caused by a Ca^{2+} increase in the egg cytoplasm (Stricker, 1999; Miyazaki, 2006; Whitaker, 2006). Since the intracellular Ca^{2+} increase is not only necessary, but is also sufficient, for egg activation in many species, it is important to understand the mechanism of egg activation by a fertilizing sperm as it triggers the intracellular Ca^{2+} increase at fertilization.

In vertebrates, two different mechanisms have been proposed for the Ca^{2+} elevation in egg activation (Iwao, 2000a,b). One is that an agonist (ligand) of the sperm membrane binds to a receptor on the egg membrane to transmit a signal for releasing Ca^{2+} through an inositol 1,4,5-trisphosphate (IP3) receptor on the endoplasmic reticulum. In the anuran frog, *Xenopus laevis*, we demonstrated that an intracellular Ca^{2+} increase is induced by external treatment with Arg–Gly–Asp (RGD)-containing peptides (Iwao and Fujimura, 1996), which are known ligands for integrins on the plasma membrane. A protein of the metalloprotease/disintegrin/cysteine-rich (MDC) family (xMDC16) on *Xenopus* sperm membranes is known to be involved in sperm–egg binding (Shilling et al., 1997) and external application of a peptide containing its disintegrin domain, a KTE amino acid sequence, induces egg activation (Shilling et al., 1998). In addition, a protease purified from the newt sperm activates *Xenopus* eggs when applied externally (Iwao et al.,

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1994, 1995; Mizote et al., 1999). A protease, cathepsin B, which has similar substrate specificity to the newt sperm protease, causes egg activation accompanied by the intracellular Ca^{2+} elevation. Recently, we found that a single-transmembrane protein, *Xenopus* uroplakin III (xUPIII), is localized to the lipid/membrane rafts and exposed on the egg surface and that xUPIII is involved in sperm–egg interaction as well as subsequent Src-dependent intracellular events of egg activation (Sakakibara et al., 2005). Furthermore, cathepsin B causes digestion of xUPIII on the egg membrane to induce egg activation accompanied by tyrosine phosphorylation of egg-raft-associated Src kinase, phospholipase Cγ, and xUPIII itself (Sato et al., 1999, 2003; Mahbub Hasan et al., 2005). Thus, in frog fertilization, it is most likely that the signal for egg activation can be transmitted when the sperm binds to the egg surface (Sato et al., 2006). However, in urodele newt, *Cynops pyrrhogaster*, the eggs are resistant to the treatment with RGD-containing peptides (Iwao, 2000a), and a very small percentage of *Cynops* eggs are activated by external treatment with the sperm protease (Iwao et al., 1994), suggesting the involvement of a different mechanism of egg activation in newt fertilization. The signal transduction through the egg receptor on the egg membrane may occur in the activation of invertebrate eggs (Townley et al., 2006), but not in that of mammalian eggs (Miyazaki, 2006; Whitaker, 2006).

Another possible mechanism has been proposed for mammalian fertilization in which a soluble sperm factor for egg activation enters into the egg cytoplasm after sperm–egg fusion. The injection of cytosolic sperm extracts into unfertilized eggs has been shown to trigger Ca^{2+} oscillation, which is a series of increases in the intracellular Ca^{2+} concentration, in both hamster (Swann, 1990; Parrington et al., 1996) and mouse eggs (Oda et al., 1999), as well as in several invertebrates (Stricker, 1999; Whitaker, 2006) including ascidians (Kyozuka et al., 1998). Recent studies of mammals strongly support the soluble factor model for Ca^{2+} oscillation (Swann and Parrington, 1999). A novel isotype of phosphoinositide-specific PLCζ was isolated and found to be expressed in spermatids (Saunders et al., 2002). Injection of not only PLCζ mRNA, but its recombinant protein, generates Ca^{2+} oscillation in mouse eggs (Saunders et al., 2002; Kouchi et al., 2004). It is estimated that a single mammalian sperm contains sufficient PLCζ protein to induce Ca^{2+} oscillation in a fertilized egg (Saunders et al., 2002; Fujimoto et al., 2004; Yoda et al., 2004). Since PLCζ is present in the sperm extracts obtained from various mammalian sperm (Saunders et al., 2002; Kurokawa et al., 2005; Fujimoto et al., 2004), it is probably the active component in the sperm extracts for the Ca^{2+} oscillation that leads to egg activation in mammalian fertilization. PLCζ may also be involved in egg activation in other vertebrates because it was reported that a chicken homologue of PLCζ (Coward et al., 2005), and sperm extracts from a fish (Coward et al., 2003) and a frog (Dong et al., 2000), can each cause Ca^{2+} oscillation in mouse eggs. This has yet to be established, however, because no detailed characterization of the active molecule in the sperm extracts in non-mammalian vertebrates has been performed.

A fertilizing *Cynops* sperm causes a wave-like increase in the intracellular Ca^{2+} concentration in the egg cytoplasm at fertilization (Yamamoto et al., 1999, 2001). An initial brief Ca^{2+} increase occurs followed by a Ca^{2+} wave that spreads at a velocity of 5.0–6.0 μm/s for about 40 min after fertilization. Injection of sperm extract into unfertilized *Cynops* eggs induces a wave-like Ca^{2+} increase similar to that at fertilization, which results in egg activation: resumption of meiosis, degradation of cyclin B and Mos, and DNA replication followed by abortive cleavage (Yamamoto et al., 2001). This Ca^{2+} increase is both necessary and sufficient for egg activation in *Cynops* eggs because the disruption of this Ca^{2+} increase by injection of the Ca^{2+} chelator, BAPTA, inhibits all activation events at fertilization (Yamamoto et al., 2001) and ionophore A23187 caused egg activation with a Ca^{2+} increase (Yamamoto et al., 1999). The Ca^{2+} increase in *Cynops* eggs is probably induced by a release of Ca^{2+} from an intracellular Ca^{2+} store within the endoplasmic reticulum, via IP3 receptors, since injection of IP3, but not cyclic-ADP ribose, causes a Ca^{2+} increase (Yamamoto et al., 2001). Injection of heparin, an inhibitor of IP3 receptors, prevents Ca^{2+} waves at fertilization in *Cynops* eggs. The sperm factor for egg activation in *Cynops* sperm extract is a heat-labile protein(s) having a molecular weight more than 10 kDa. Although a single newt sperm seems to contain a sufficient amount of sperm factor to activate an egg, its molecular characteristics remain to be investigated.

In the present study, we highly purified from a newt sperm extract a 45 kDa protein that shows egg-activation activity and shares characteristics with a citrate synthase, and we also observed that a large amount of citrate synthase is localized in fibrous structures in the sperm. Porcine citrate synthase as well as mRNA of *Xenopus* citrate synthase induced egg activation, and treatment of the sperm extract with anti-porcine citrate synthase antibody decreased its egg-activation activity. Our findings strongly suggest that this 45 kDa protein is one of the major components of the sperm factor for egg activation at newt fertilization.

Materials and methods

Preparation of eggs, sperm extracts and mRNA

Sexually mature newts, *C. pyrrhogaster*, were collected near Yamaguchi, Japan or purchased from dealers. To induce ovulation, the female was injected 80 IU of human chorionic gonadotropin (HCG, ASUKA Pharmaceutical) every 2 days. Unfertilized eggs were obtained by squeezing the abdomen of the females. The jelly layers were removed with 1.5% sodium thioglycolate (pH 9.5) followed by thorough washing with Steinberg's solution (SB: 58.0 mM NaCl, 0.67 mM KCl, 0.34 mM $CaCl_2$, 0.85 mM $MgSO_4$, 4.6 mM Tris–HCl, pH 7.4). The dejellied eggs were kept in SB more than 2 h before use to remove the artificially activated eggs. Mature sperm were obtained by squeezing the males and suspended in De Boer's solution (DB: 110.3 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, 5.7 mM Tris–HCl, pH 7.4). After washing by centrifugation (350×*g*, 20 min, 4 °C), the precipitated sperm were suspended in intracellular-like medium (ICM: 120 mM KCl, 0.1 mM EGTA, 10 mM Na-β-glycerophosphate, 0.2 mM PMSF, 1 mM DTT, 20 mM HEPES–NaOH, pH 7.5). The sperm suspension was sonicated on ice (50 W, 15 s, 5 times; US50, Nissei, Tokyo) to disrupt sperm plasma membranes and then centrifuged (10,000×*g*, 20 min, 4). The supernatant was collected as a sperm extract and stored −80 °C until use. A cDNA fragment of *X. laevis* citrate synthase (CS, Gene Bank: