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PYK2: A calcium-sensitive protein tyrosine kinase activated in response to fertilization of the zebrafish oocyte

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ABSTRACT

Fertilization begins with binding and fusion of a sperm with the oocyte, a process that triggers a high amplitude calcium transient which propagates through the oocyte and stimulates a series of preprogrammed signal transduction events critical for zygote development. Identification of the pathways downstream of this calcium transient remains an important step in understanding the basis of zygote quality. The present study demonstrates that the calcium-calmodulin sensitive protein tyrosine kinase PYK2 is a target of the fertilization-induced calcium transient in the zebrafish oocyte and that it plays an important role in actin-mediated events critical for sperm incorporation. At fertilization, PYK2 was activated initially at the site of sperm–oocyte interaction and was closely associated with actin filaments forming the fertilization cone. Later PYK2 activation was evident throughout the entire oocyte cortex, however activation was most intense over the animal hemisphere. Fertilization-induced PYK2 activation could be blocked by suppressing calcium transients in the ooplasm via injection of BAPTA as a calcium chelator. PYK2 activation could be artificially induced in unfertilized oocytes by injection of IP3 at concentrations sufficient to induce calcium release. Functionally, suppression of PYK2 activity by chemical inhibition or by injection of a dominant-negative construct encoding the N-terminal ERM domain of PKY2 inhibited formation of an organized fertilization cone and reduced the frequency of successful sperm incorporation. Together, the above findings support a model in which PYK2 responds to the fertilization-induced calcium transient by promoting reorganization of the cortical actin cytoskeleton to form the fertilization cone.

Introduction

Fertilization triggers a series of preprogrammed biochemical steps in the oocyte, which function to establish a block to polyspermy, activate zygote metabolism, and initiate resumption of the meiotic cell cycle and zygote development. An early step in this process is the transient elevation of intracellular free calcium. This fertilization-induced calcium transient is common to oocytes of all species examined including organisms as primitive as the sponge and as complex as mammals (Runft et al., 2002; Whitaker, 2006). While species differences exist in the subcellular distribution, timing, and biochemical triggers for these calcium transients, the downstream responses are similar among species. Studies in a variety of models have demonstrated that members of the calcium-calmodulin-dependent kinase family play a critical role in meiosis resumption and metabolic activation (Chang et al., 2009; Miyazaki and Ito, 2006; Ozil et al., 2005). However other calcium sensitive proteins such as synaptotagmin and calcineurin are activated at fertilization and play important roles as well (Leguia et al., 2006;Nishiyama et al., 2007). In addition to pathways regulated directly by calcium, multiple downstream protein kinase-mediated pathways are stimulated indirectly following the calcium transient. Protein tyrosine kinase-mediated events result in phosphorylation of cytoskeleton-associated proteins in the oocyte cortex (McGinnis et al., 2007; Sharma and Kinsey, 2006; Wright and Schatten, 1995) in species ranging from sea urchins to mouse. *Src*-family kinases (SFKs) including Fyn kinase were recently shown to become localized to the oocyte cortex in marine invertebrates (Sharma and Kinsey, 2006; Townley et al., 2009), zebrafish (Sharma and Kinsey, 2006, 2008), frogs (Sakakibara et al., 2005) and mammals (Levi et al., 2010). Fyn kinase was also found to function in maintenance of cortical cytoskeleton polarity in mouse oocytes and to contribute to the process of pronuclear congression (Luo et al., 2010). In the zebrafish oocyte, SFK activation and tyrosine phosphorylation of proteins associated with the cortical actin layer was initiated at the fertilization cone and propagated through the entire oocyte cortex in a manner temporally correlating with the calcium transient (McGinnis et al.,

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2011; Sharma and Kinsey, 2006). However, our inhibitor studies (unpublished) revealed that suppression of SFK activity did not abolish the fertilization-induced wave of tyrosine kinase signaling suggesting that other PTKs may be part of the response to fertilization.

Our analysis of the PTKs that respond during egg activation revealed the presence of a calcium-calmodulin (Kohno et al., 2008) dependent protein tyrosine kinase activity in the zebrafish oocyte plasma membrane which we now have identified as proline-rich tyrosine kinase-2 (Pyk2), (also referred to as; cell-associated kinase-beta (CAK-β), calcium-dependent tyrosine kinase (CADTK), related adhesion focal tyrosine kinase (RAFTK)). PYK2 is a member of the focal adhesion kinase (FAK) family of protein kinases. Like FAK, PYK2 is activated initially through a range of stimuli including growth factors, cytokines, integrin ligation and G-coupled receptor agonists (Orr and Murphy-Ullrich, 2004). PYK2 is unique in that full activation requires participation of calmodulin and elevated intracellular calcium (Wu et al., 2006) and, in many cases, participation of a *Src*-family kinase (Cheng et al., 2002; Sorokin et al., 2001). PYK2 and FAK are cytoplasmic protein tyrosine kinases that transduce signals from cell surface receptors to cytoplasmic pathways through their unique capability as scaffolding proteins as well as through their kinase activity. The 129 kDA PYK2 protein consists of multiple protein interaction domains reviewed (Schlaepfer et al.,1999) such as an N-terminal Ezrin, Radixin, Moesin (ERM) homology domain that includes a calmodulin binding site. The single catalytic site is followed by two proline-rich domains that include SH3 and SH2 binding sites critical to docking functions of the protein. The C-terminal Focal Adhesion Targeting (FAT) domain functions during docking interactions with cytoskeletal components such as paxillin and talin. These diverse protein interaction domains enable PYK2 to integrate a wide variety of extracellular and intracellular signals to regulate Rho activity (Okigaki et al., 2003) and implement actin cytoskeleton functions relating to cell–cell junction turnover (Sun et al., 2011), cell process formation (Gil-Henn et al., 2007) and phagocytosis (Owen et al., 2007). PYK2 can also function in more complex events such as mechanical and osmotic stress response (Koh et al., 2001; Klemm et al., 2010; Takahashi et al., 2003), proliferation (Cox et al., 2006), and survival (Hanks et al., 2003).

The mouse oocyte expresses certain Src-family and FAK-family kinases at levels much higher than most other cell types (McGinnis et al., 2011) suggesting that these two kinase families play important roles in the function of mammalian oocytes and possibly oocytes of other species as well. We have previously detected FAK kinase activity in zebrafish zygotes and early embryos (Tsai et al., 2005), but that study made use of antibodies targeting the C-terminal domain of FAK which are unable to detect the closely related PYK2 kinase. As a result, the potential role of PYK2 in zebrafish fertilization has remained an unsolved question. The present study has examined the subcellular distribution and activation of PYK2 kinase during fertilization of the zebrafish oocyte with the objective of defining this novel element of the calcium-regulated pathways that are activated by the fertilizing sperm. The results demonstrate that PYK2 is a novel component of the response of the oocyte during fertilization. It was found to be activated initially at the micropyle and subsequently throughout the entire oocyte cortex in response to the fertilization-induced calcium transient. Functional analysis revealed that PYK2 was involved in cytoskeletal rearrangements necessary for sperm incorporation. The discovery of this new component of the oocyte activation pathway provides an important link between the fertilization-induced calcium transient and the changes in the actin cytoskeleton which occur in response to fertilization.

Materials and methods

Oocytes

Oocytes were collected from mature *Danio rerio* and maintained in filtered (0.2 μM) salmon ovarian fluid (Siripattarapravat et al., 2009) or Hank's-BSA (Westerfield, 2007) at 28 °C, while sperm were maintained on ice in sperm extender solution (Lee et al., 1999). Fertilization was accomplished by mixing the sperm (5 μl) with the eggs, then activating the sperm by addition of 1 ml of aquarium water. After the eight cell stage, embryos were transferred into Tubingen E3 medium for culture (Brand et al., 2002). The effect of PYK2 inhibitors AG17 and AG82 (EMD Millipore, Billerica, MA) and PF04594755 (a generous gift from Pfizer, Inc. Groton, CT) on the capacity of oocytes to be fertilized was determined by fertilization assays. The above inhibitors were added to groups of 20–25 oocytes maintained in 35 mM culture plates containing Hanks-BSA and incubated for 45 min. Oocytes were then washed twice with Hanks-BSA, the Hanks-BSA was removed, and 2.5 μl of a 10% vol/vol sperm suspension was added followed by 1 ml of aquarium water to activate sperm motility. Development was allowed to occur to the pronuclear stage 15 min post-insemination (m.p.i.) or to the early cleavage stage (90 m.p.i) and zygotes were fixed in a solution of 4% paraformaldehyde, 0.1% glutaraldehyde, sucrose (4% wt/vol), NaH_2PO_4 (50 mM) pH 7.2. The animal pole of each zygote was then dissected free with a scalpel and stained with DAPI in sucrose (4% wt/vol), NaH_2PO_4 (50 mM) pH 7.2, glycine (10 mM), and NP40 (0.5%). Successful fertilization was assayed by imaging the animal caps by fluorescence microscopy at 20× and quantifying the number of oocytes with 2 pronuclei (indicative of sperm penetration) or the number of blastomere nuclei (indicative of successful cleavage).

Membrane preparation and western blot analysis

To overcome the difficulties of analyzing the yolk-rich zebrafish oocyte, western blot detection was performed using the plasma membrane fraction isolated via density gradient centrifugation (Wu and Kinsey, 2004). Samples were prepared from groups of 500–600 oocytes collected at different times after fertilization using solutions that contained the phosphatase inhibitors sodium orthovanadate (100 μM) and phenylarsine oxide (40 μM) (Sigma Aldrich, St. Louis, MO.). Samples containing 10 μg protein were resolved by electrophoresis on a 10% SDS gel and transferred to Nytran+membranes (EMD Millipore, Billerica, MA) in transfer buffer containing 0.1% SDS. After blocking in 5% dried milk (BioRad Labs, Hercules, CA) containing the above phosphatase inhibitors, blots were probed with a rabbit polyclonal antibody directed against a region of the C-terminal domain of human PYK2 ((P3902) Sigma Aldrich, St Louis, MO), or with phosphorylation site-specific antibodies anti-PYK2-PY^{402}, or anti-PYK2-PY^{579} (Biosource, Grand Island, NY) and detected with peroxidase-coupled anti-rabbit IgG (Sigma Aldrich, St. Louis, MO) then imaged via chemiluminescence. Band intensity was quantified by densitometric scanning and analyzed with Metamorph 7.1 software (Molecular Devices, Sunnydale, CA).

Immunofluorescence microscopy

Zebrafish eggs, and zygotes were prepared for immunofluorescence as previously described (Sharma and Kinsey, 2006). Where possible, the chorion was dissected away manually prior to permeabilization and blocking, however, in cases where the fertilization cone was to be imaged, the chorion was left in place to avoid destruction of this fragile structure and instead, the fixed