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Expression of multiple Src family kinases in sea urchin eggs and their function in Ca^{2+} release at fertilization

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ABSTRACT

Egg activation at fertilization in deuterostomes requires a rise in intracellular Ca^{2+}, which is released from the egg's endoplasmic reticulum. In sea urchins, a Src Family Kinase (SpSFK1) is necessary for the PLCγ-mediated signaling event that initiates this Ca^{2+} release (Giusti, A.F., O'Neill, F.J., Yamasu, K., Foltz, K.R. and Jaffe, L.A., 2003. Function of a sea urchin egg Src family kinase in initiating Ca2+ release at fertilization. Dev. Biol. 256, 367–378.). Annotation of the *Strongylocentrotus purpuratus* genome sequence led to the identification of additional, predicted SFKs (Bradham, C.A., Foltz, D.R., Beane, W.S., Amone, M.I., Rizzo, F., Coffman, J.A., Mushegian, A., Goel, M., Morales, J., Geneviere, A.M., Lapraz, F., Robertson, A.J., Kelkar, H., Loza-Coll, M., Townley, I.K., Raisch, M., Roux, M.M., Lepage, T., Gache, C., McClay, D.R., Manning, G., 2006. The sea urchin kinome: a first look. Dev. Biol. 300, 180–193.; Roux, M.M., Townley, I.K., Raisch, M., Reade, A., Bradham, C., Humphreys, G., Gunaratne, H.J., Killian, C.E., Moy, G., Su, Y.H., Ettensohn, C.A., Wilt, F., Vacquier, V.D., Burke, R. D., Wessel, G. and Foltz, K.R., 2006. A functional genomic and proteomic perspective of sea urchin calcium signaling and egg activation. Dev. Biol. 300, 416–433.). Here, we describe the cloning and characterization of these 4 additional SFKs and test their function during the initial Ca^{2+} release at fertilization using the dominant-interfering microinjection method coupled with Ca^{2+} recording. While two of the new SFKs (SpFrk and SpSFK3) are necessary for Ca^{2+} release, SpSFK5 appears dispensable for early egg to embryo transition events. Interestingly, SpSFK7 may be involved in preventing precocious release of Ca^{2+}. Binding studies indicate that only SpSFK1 is capable of direct interaction with PLCγ. Immunolocalization studies suggest that one or more SpSFK and PLCγ are localized to the egg cortex and at the site of sperm–egg interaction. Collectively, these data indicate that more than one SFK is involved in the Ca^{2+} release pathway at fertilization.

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Introduction

A required benchmark of egg activation – the coordinated collection of events that mediate the egg to embryo transition – is an internal rise in free Ca^{2+} (reviewed in Stricker, 1999; Runft et al., 2002; Whitaker, 2006). While multiple Ca^{2+} release pathways are present in deuterostome eggs, the initial rise occurs at the site of sperm–egg interaction, where Ca^{2+} is released from the egg's endoplasmic reticulum (ER) via the production of IP_3 through the action of a phospholipase C (PLC) and subsequent activation of the inositol 1,4,5-trisphosphate receptor (IP_3R). In ascidians and echinoderms, it is the PLCγ form that is responsible for producing the IP_3 (Carroll et al., 1997, 1999; Rongish et al., 1999; Runft et al., 2004; Shearer et al., 1999; Runft and Jaffe, 2000). In the eggs of these invertebrate deuterostomes, as well as those of zebrafish and possibly *Xenopus*, Src family kinase (SFK) activity is required for activation (Giusti et al., 1999b, 2000, 2003; Kinsey, 1997; Kinsey et al., 2003; Kinsey and Shen, 2000; Shen et al., 1999; O'Neill et al., 2004; Runft and Jaffe, 2000; Sato et al., 1999; Wu and Kinsey, 2000). A SFK is thought to directly phosphorylate the regulatory (activating) tyrosines in the SH2C domain of the egg PLCγ (O'Neill et al., 2004; Runft et al., 2004), similar to the mechanism of PLCγ activation in immune cells (Bogin et al., 2007; Braiman et al., 2006; DeBell et al., 2007; Qi and August, 2007).

Src family kinases (SFKs) are modular non-receptor protein-tyrosine kinases that mediate a variety of cellular responses and events including cell growth, differentiation, cell shape, migration and survival (Thomas and Brugge, 1997; Parsons and Parsons, 2004). The SFKs have a conserved domain structure (Supplemental Fig. 1A): a unique region at the N-terminus, a src homology domain 1 (SH1), a src homology domain 3 (SH3), a src homology domain 2 (SH2) and finally the tyrosine kinase domain (TyK) at the C-terminus (Roskoski, 2004, 2005). Most SFKs also have conserved myristylation residues at the N-terminus (M-G-x-x-x-S/R), which target them to the membrane (Resh, 1994). In the "off" state, SFKs are phosphorylated on a C-terminal tyrosine (by a related, C-terminal Src kinase, CSK). An intramolecular interaction between the SFK C-terminal phosphotyrosine and the SH2 domain clamps the activation (A) loop in the kinase

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domain. In the presence of an activator, the SH2 domain releases the C-terminal tyrosine, binding instead to the activator. This releases the clamp on the kinase domain A-loop and phosphorylation on an activating tyrosine pushes the enzyme into full activation (Boggon and Eck, 2004). Overexpression of the SH2 domain serves as a competitor for the upstream activator(s) and acts to inhibit the activation of the endogenous SFK in a dominant-interfering way. This is consistent with how SH2 domains work in general — as specific mediators of protein–protein interactions via recognition of context-specific phosphorylated tyrosines (Koch et al., 1989; Moran et al. 1990; Parsons and Parsons, 2004; Roskoski, 2005).

A sea urchin (*Strongylocentrotus purpuratus*) SFK, designated SpSFK1, was shown to be necessary for Ca^{2+} release at fertilization using the dominant-interfering strategy and further, the SpSFK1 kinase activity profile was rapidly and transiently fertilization-dependent, consistent with a role in triggering initial release of Ca^{2+} (Giusti et al., 2003). There were hints that additional SFKs were represented in the genome and perhaps expressed in sea urchin eggs (Kamel et al., 1986; Sakuma et al., 1997; Onodera et al., 1999), which was not surprising given that the SFKs are a large superfamily, but these cDNAs were refractory to cloning. Subsequently, an arrayed sea star oocyte/egg cDNA library screen yielded three distinct SFKs and two of these, AmSFK1 (the SpSFK1 ortholog) and AmSFK3, were both shown to be necessary for Ca^{2+} release at fertilization (O'Neill et al., 2004). Kinase activity assays revealed that AmSFK3 activates rapidly and prior to AmSFK1 and dosage experiments have led to a model whereby AmSFK3 activates AmSFK1, which then goes on to activate PLCγ.

Annotation of the *S. purpuratus* genome sequence led to the identification of additional, predicted SFKs (Bradham et al., 2006; Roux et al., 2006). Here, we describe the cDNA cloning and characterization of these 4 additional SFKs and test their function during the initial Ca^{2+} release at fertilization using the dominant-interfering microinjection method coupled with Ca^{2+} recording. While microinjection of the SH2 domains of two of the new SFKs (SpFrk and SpSFK3) inhibit Ca^{2+} release, SpSFK5 SH2 had no obvious effect on any aspect of early egg to embryo transition events. Interestingly, SpSFK7 may be involved in preventing precocious release of Ca^{2+}. SpSFK1 can interact directly with PLCγ and initial localization studies suggest that both proteins are present at the site of sperm–egg interaction.

Materials and methods

Animals and gametes

Sea urchins (*Strongylocentrotus purpuratus*) were collected from the Santa Barbara Channel, maintained in 10 °C natural seawater open system tanks and fed a diet of *Macrocystis*. Gametes were collected by injection of 0.5 M KCL as described (Foltz et al., 2004).

Cloning and expression of the SpSFK SH2 domains

Cloning of the full length SpSFK cDNAs is described in the Supplemental materials. The full length cDNA sequences of the SpSFKs have been deposited in the database under the following accession numbers: SpFrk, FJ161081; SpSFK3, FJ161082; SpSFK5, FJ161083; SpSFK7, FJ161084.

SpSFK SH2 domains were assigned using the program ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) to make the initial multiple alignment using HumanSrc (GenBank accession number P12931), AmSFK1 (GenBank accession number AAS01047), AmFrk (GenBank accession number AAS01046), AmSFK3 (GenBank accession number AAS01045) and SpSFK1 (GenBank accession number NP_999783) aligned to SpFrk, SpSFK3, SpSFK5 and SpSFK7. The cloning and expression of the AmSFK SH2 domains is described in O'Neill et al. (2004) and the SpSFK1 SH2 domain is described in Giusti et al. (2003). Primers were designed to flank the SH2 domains from each full length SpSFK clone using AnnHyb v4.934 (http://bioinformatics.org/annhyb). An EcoRI restriction site was built into the 5′ end and a Sal1 restriction site was built into the 3′ end to facilitate in-frame directional cloning. The primers used to amplify the SH2 domains were:

SpFrkSH2 (fwd 5′GAATTCAATCTTTAGAATCAGAACCGTGGT3′/rev 5′ GTCGACGGCTTTTCAATCAACACACACGG3′);
SpSFK3SH2 (fwd 5′GAATTCTATTGCAATCGGAAGGTTGGT3′/rev5′ GTCGACTTCTGTCTCGGGCAGGC3′);
SpSFK5SH2 (fwd 5GAATTCCtTTAGAGGTAGAAGAATGG3′/rev 5′ GTCGACTGTTTGGGGCAGGG3′);
SpSFK7SH2 (fwd 5′GAATTcACGCAGAAGACTGGTACT3′/rev 5′ GTCGACGGGTTCTCCTTGGGACA3′).

Amplified products were cloned initially into the TOPO vector (Invitrogen, Inc.). Inserts were then directionally cloned into the GST expression vector pGEX6P-2 (Invitrogen, Inc.), with the GST located on the N-terminus of each SH2 domain. The constructs were sequenced (Iowa State Sequencing Facility) to confirm correct orientation, maintenance of open reading frame, and proper amino acid sequence.

For expression, the GST fusion constructs were transformed into *E. coli* BL21star (DE) cells (Invitrogen, Inc.). Cultures (1 L total in 3×~330 mL flask) were grown in selective media until they reached (OD_{600} 0.6–0.9) then were induced by adding IPTG (0.5 mM final concentration) at 25 °C for 1 h. Cells were harvested and resuspended in 1× PBS, pH 7.4, 1% TX-100, protease inhibitor cocktail I (CalBiochem, Inc., San Diego, CA) and PEFA bloc (Fluka-Sigma-Aldrich, Inc., Buchs, Switzerland). Cells were lysed using a French pressure cell and the clarified supernatant was incubated for 1 h with 6 mL of a 50% slurry of glutathione agarose beads (Amersham, Inc., Piscataway, NJ). GST fusion proteins were eluted with 50 mM reduced glutathione in 100 mM Tris, pH 8.0, then extensively spin-dialyzed against 1× PBS, pH 7.4 and concentrated for injections using 30 K size exclusion microconcentrators (Millipore, Inc., Billerica, MA). Determination of protein concentration was via the BCA method (Pierce Thermo-Fisher Scientific, Inc., Rockford, IL) and purity was assessed by SDS-PAGE. Aliquots were frozen in liquid N_2 and stored at −80 °C until use.

Microinjection of recombinant GST fusion proteins

Quantitative microinjections were made using mercury-filled micropipettes (Jaffe and Terasaki, 2004). *S. purpuratus* eggs (dejellied) were attached to a poly-L-lysine-coated coverslip, inverted over an injection slide (Kiehart, 1982) and observed with an upright microscope. All injections were made in filtered sea water. Injection volumes were 3% of the egg volume (8 pL for a 270-pL *S. purpuratus* egg).

Calcium measurements

The desired concentration of each SFKSH2 GST fusion protein was mixed with 333 μM calcium green 10 kDa dextran (CG-dex; Molecular Probes, Eugene, OR). The final concentration in the cytoplasm of the egg for each SH2 domain was either 25 μM or 2.5 μm and was 10 μM for the CG-dex. Experiments were performed at 15 °C and injected eggs were fertilized, after resting for at least 10 min, by pipetting 1 μl of a 1:1000 dilution of acrosome-reacted sperm into the injection chamber. This differs from the method used by Giusti et al. (2003) in which eggs retained their jelly coats and the seawater in the chamber was exchanged with a suspension of dilute sperm. CG-dex fluorescence was measured using a 40× objective on an upright microscope (Olympus Bx60; Olympus; Center Valley, PA) connected to a photodiode and recorded using Scope software (AD Instruments, Grand Junction, CO) as previously described (Giusti et al., 2003; Runft et al., 2004; O'Neill et al., 2004). Means, standard deviations and