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Original Article

Fertilization triggers activation of Fyn Kinase in the Zebrafish egg

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ABSTRACT Fertilization results in the tyrosine phosphorylation of several egg proteins and studies have shown that tyrosine protein kinase activity is required for successful fertilization. The Fyn protein kinase has been detected in eggs of the sea urchin, frog and rat, although measurement of fertilization-induced changes in Fyn kinase activity have only been successful in the sea urchin system. The present study demonstrates the presence of Fyn kinase in the zebrafish egg and the stimulation of this enzyme at fertilization. Activation of Fyn was detected as early as 30 seconds post-fertilization and increased approximately six-fold by 2 minutes post-insemination. The activation of Fyn in the zebrafish egg required sperm and was not observed in spontaneously activated eggs.

KEY WORDS: *fertilization, Fyn kinase, zebrafish, egg.*

Introduction

The fertilizing sperm triggers a series of biochemical events in the egg which serve to establish the block to polyspermy, activate egg metabolism, and trigger re-entry into the cell cycle. Essential control mechanisms during egg activation include the activation of protein phosphorylation cascades involving serine-threonine as well as tyrosine protein kinases (Keller *et al.*, 1980; Satoh and Garbers, 1985; Peaucellier *et al.*, 1988; Ciapa and Epel, 1991; Abassi *et al.*, 2000;). Tyrosine protein kinases are thought to be required at several steps during the egg activation process (Fig. 1). Studies employing dominant-negative fusion proteins (Giusti *et al.*, 1999a,b), and microinjection of exogenous, active kinases (Spivack and Maller, 1985; Unger and Steele, 1992; Giusti *et al.*, 2000) have indicated that Src-family kinases are activated shortly after sperm-egg fusion and function to stimulate production of IP3 and thereby induce a calcium transient which is an essential step required for subsequent development. In addition, studies using chemical PTK inhibitors (Moore and Kinsey, 1995; Wright and Schatten, 1995; Shen *et al.*, 1999; Glahn *et al.*, 1999; Sato *et al.*, 20000) have demonstrated additional roles for PTKs during later events such as pronuclear migration and initiation of DNA synthesis. Identification of the specific PTKs that function during each phase of egg activation has been difficult. Several Src-family PTKs are expressed in eggs (Schartl and Barnekow, 1984; Steele *et al.*, 1990; Sakuma *et al.*, 1997; Onodera *et al.*, 1999; Sato et al., 1999), and the specific contribution of each to egg activation is an important question. The c-Fyn encoded protein tyrosine kinase is expressed in eggs of the sea urchin, frog, and rat, where it is localized to the cortical region as well as to the meiotic and mitotic spindles (Kinsey, 1996; Talmor *et al.*, 1998). Fyn kinase is activated in response to fertilization in the sea urchin egg and seems to play a role in triggering the sperm-induced calcium transient (Kinsey and Shen, 2000), however technical limitations have prevented analysis of fertilization-induced changes in other species.

The objective of the present study was to characterize the distribution of Fyn in the zebrafish egg and determine whether fertilization triggered changes in Fyn kinase activity. This study revealed that Fyn is expressed in the zebrafish egg and is stimulated at fertilization, demonstrating the utility of this vertebrate egg for biochemical analysis of egg activation.

Results

Detection of the Fyn protein kinase in the Zebrafish egg

To demonstrate the expression of c-Fyn in the zebrafish egg, we used combined immunoprecipitation and immune-complex kinase assay analysis. Initial experiments using total egg extracts were not successful due to the large amount of yolk protein present in these eggs. We therefore devised a subcellular fractionation procedure to separate the yolk material from the membrane fraction (microsomal fraction). The fractionation scheme was based

Abbreviations used in this paper: DAG, 1,2 diacylglycerol; EDTA, ethylenediaminetetra-acetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N,N tetra-acetic acid; HEPES, [2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]; IP3, inositol 1,4,5 triphosphate; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; P-Tyr, phospho-L-tyrosine; GPI, glycosylphosphatidylinositol; PTK, protein tyrosine kinase.

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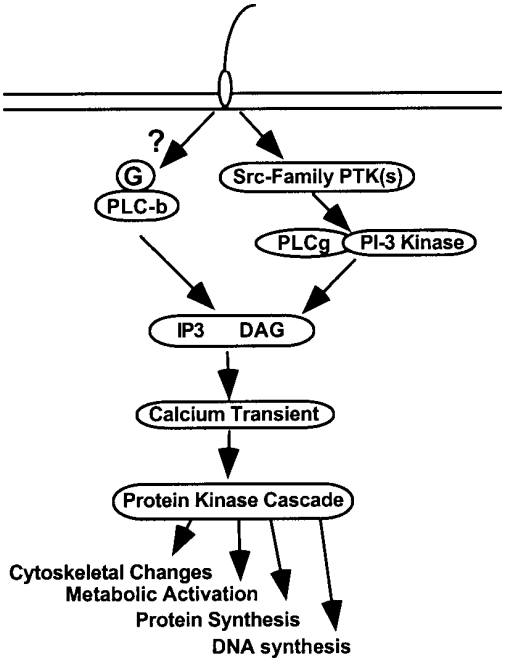

Fig. 1. Proposed mechanism of egg activation. *Sperm-egg fusion triggers activation of phospholipase C activity through two possible mechanisms; one involving G proteins and PLCβ and a second (thought to be the dominant mechanism) involving one or more Src-family PTKs leading to stimulation of PLCγ. Polyphosphoinositide hydrolysis results in the accumulation of a pool of IP3 which triggers calcium release from internal stores. This calcium transient stimulates many biochemical pathways within the egg including a second protein kinase cascade leading ultimately to the activation of the egg.*

on a method for isolating membranes enriched in GPI-linked proteins from oligodendrocytes (Krämer *et al.*, 1999) and allowed resolution of a heavy membrane fraction at the 23% / 35% sucrose interface, and a "light" membrane fraction at the 12% / 23% interface. Analysis of the different fractions isolated from zebrafish zygotes revealed that both the light membrane fraction (12 / 23% interface) as well as the heavy membrane fraction (23/35% interface) were enriched in plasma membrane marker enzymes such as ouabain-inhibitable Na^+ / H^+ dependent ATPase (Table 1).

TABLE 1

MARKER ENZYMES ASSOCIATED WITH ZEBRAFISH EGG MEMBRANE FRACTIONS

	Light Membranes		Heavy Membranes	
Enzyme	Specific Activity	Rel. Sp. Act.	Specific Activity	Rel. Sp. Act.
Na^+/K^+ ATPase	1388 +/- 419	63	700 +/- 223	36
NADPH-Cyt C Reductase (*)	922 +/- 344	21.6	298 +/-106	7.3
Succinate Dehyd.	151+/- 65	0.23	257 +/- 119	0.38
Acid Phosphatase	594 +/- 156	0.34	705 +/- 322	0.46

Enzyme assays were performed as in Materials and Methods and represent the average of three preparations +/- S.D. Values are expressed as nanomoles/h/mg protein except for NADPH-Cyt C reductase (*)which is expressed as micromoles/ hour/ mg protein. The relative specific activity is equal to the specific activity divided by the specific activity of the egg homogenate.

These fractions were also enriched in markers for endoplasmic reticulum (NADPH-cytochrome-c reductase) but not mitochondrial or lysosomal markers (succinate dehydrogenase, acid phosphatase). The particulate fraction recovered from the bottom of the sucrose gradient represented the majority of the cellular protein and contained large quantities of insoluble protein, probably yolk.

In order to detect Fyn kinase, immune-complex kinase assays were performed on subcellular fractions prepared from 1000 zygotes collected 30 minutes post-insemination. Fyn kinase activity was present in these subcellular fractions (Fig. 2, upper panel), as indicated by autophosphorylation of the 59 kDa Fyn protein. Western blot analysis of these samples is shown in the bottom panel where the 59 kDa Fyn protein is present just above the heavy chain of IgG representing the antibody. Phosphoamino acid analysis demonstrated that this 59 kDa protein was phosphorylated only on tyrosine (Fig. 3) indicating that contamination by Ser/Thr kinases was very low. Both the light and heavy membrane fractions contained Fyn protein and enzyme activity.

Comparison of the level of Fyn kinase activity in each fraction relative to the amount of Fyn protein detected by western blot revealed that the light membrane fraction had the highest relative specific activity. The cytosol and particulate fractions contained a small fraction of the total Fyn protein and enzyme activity, detectable only after prolonged exposure of the X-ray film. Most of the Fyn associated with membranes was solubilized by detergent and the detergent insoluble components of each membrane fraction revealed low but detectable levels of Fyn protein and kinase activity representing less that 5% of that in the membrane fractions (not shown). These results indicate that the majority of the Fyn kinase activity in the egg is associated with the light membrane fraction which is enriched in plasma membrane and endoplasmic reticulum.

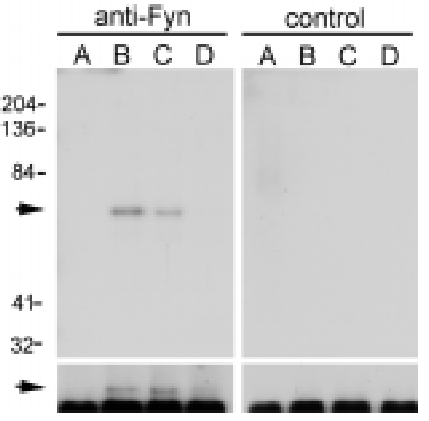

Fig. 2. Detection of Fyn kinase in Zebrafish zygotes. *Fertilized eggs were fractionated on a sucrose density gradient as described in Materials and Methods. Subsequently, samples of each fraction containing 10 μg protein were solubilized and the detergent soluble extracts were incubated with anti-Fyn antibody, or with normal rabbit IgG (control), followed by protein A-Sepharose. Kinase assays were performed as described in Materials and Methods and* **(Upper panel)** *autophosphorylation was detected by autoradiography.* **(Lower panel)** *Western blot analysis of these immunoprecipitates was also performed by incubation of blots with the anti-Fyn antibody followed by chemiluminescence detection. The fractions analyzed were; cytosol (A), light membrane fraction (B), heavy membrane fraction (C), particulate fraction (D).*

Effect of fertilization on the activity of Fyn

In order to determine whether fertilization triggered changes in Fyn kinase activity in the zebrafish egg, samples of 200 eggs were fractionated before and at 2 minutes post-insemination and the activity of Fyn in subcellular fractions was monitored by immune-complex kinase assay. As seen in Fig. 4, very little kinase activity was detected in samples prepared from unfertilized eggs. However, samples prepared 2 minutes post-insemination exhibited very significant levels of Fyn kinase activity. The activity in the light membrane fraction was slightly higher than that of the heavy membrane fraction. The relative amount of Fyn protein in each immunoprecipitation was estimated by quantification of Western blots prepared from the same samples (lower panel) and it can be seen that similar levels of Fyn protein were associated with the light and heavy membrane fractions from both fertilized and unfertilized eggs. A small quantity of Fyn was detected by Western blot in the particulate fraction (D) from both unfertilized and fertilized eggs, but no enzyme activity was detected in this fraction.

Our experimental design did not include washing of the eggs to remove supernumerary sperm, since we needed to homogenize eggs within seconds after fertilization. This raises the possibility that the endogenous Fyn carried by the sperm present in the fertilized eggs could account for the higher level of Fyn kinase activity after fertilization, especially since sperm are known to contain significant PTK activity. The fertilized eggs could contain up to 1.5 μg of sperm protein at the onset of immunoprecipitation. In order to measure Fyn kinase activity in sperm, 2.5μg of sperm protein was solubilized in immunoprecipitation buffer and Fyn kinase activity was immunoprecipitated and assayed as described above. We found that Fyn activity was not detectable in immunoprecipitates from this quantity of sperm.

Timing of Fyn activation at fertilization

Studies in the sea urchin system have demonstrated that c-Fyn is activated within minutes of fertilization. To determine whether the zebrafish egg exhibited a similar response, we quantified Fyn kinase activity in egg subcellular fractions at different times after fertilization. The autophosphorylation activity was quantified by densitometric scanning of each autoradiograph, and was normalized to the relative amount of Fyn protein estimated by scanning a western blot of the same sample. The results presented in Fig. 5 demonstrate the effect of fertilization on the Fyn kinase activity in the light membrane fraction. C-Fyn was almost completely inactive in the unfertilized egg but a statistically significant (P=0.02) activation was detected at 30 seconds post insemination. By 120 seconds after addition of sperm, Fyn kinase was activated over six-fold. Similar results were observed in the heavy membrane fraction (not shown) although the overall Fyn activity was in this fraction was less than that in the light membrane fraction.

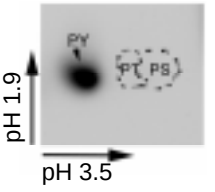

Fig. 3. Detection of P-Tyr in Fyn immunoprecipitates autophosphorylated *in vitro*. *The reaction products of sample B and C (Fig. 2) were resolved by SDS-PAGE, transferred to Immobilon-P, subjected to limited hydrolysis in 6N HCl, then analyzed by two dimensional thin layer electrophoresis and autoradiography. The position of phosphoamino acid standards is indicated by the dashed lines. PY = P-Tyr.*

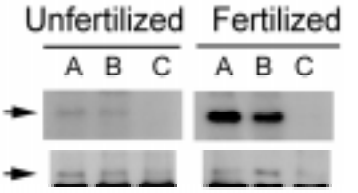

Fig. 4. Effect of fertilization on Fyn kinase activity. *Fyn immunoprecipitates prepared from subcellular fractions of eggs before and at 2 minutes post-insemination were analyzed by* **(upper panel)** *immune-complex kinase assay and autoradiography and by* **(lower panel)** *Western blot with an antibody to Fyn as described in Materials and Methods. The position of the 59 kDa Fyn protein is indicated by the arrow. Samples are: light membranes (A), heavy membranes (B) and particulate fraction (C).*

Effect of Spontaneous Activation: If the zebrafish egg is not fertilized promptly, it undergoes spontaneous activation shortly after exposure to aquarium water resulting in a cortical reaction similar to that induced by the sperm (Lee *et al.*, 1999). In order to determine whether Fyn kinase was stimulated during spontaneous activation, immunoprecipitates were prepared from unfertilized and spontaneously activated eggs five minutes after exposure to aquarium water, then analyzed for Fyn kinase activity. As seen in Fig. 5 (black fill), the Fyn immunoprecipitated from the spontaneously activated egg exhibited a low level of activity similar to that from unfertilized eggs.

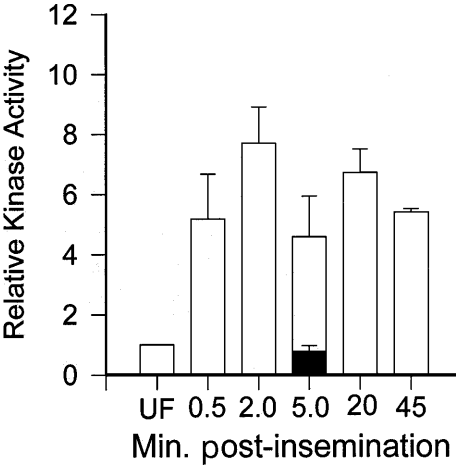

Fig. 5. Quantification of Fyn kinase activity. *Fyn kinase activity was immunoprecipitated from groups of 200 eggs and kinase assays were performed as described in Materials and Methods. The level of autophosphorylation activity was quantified by densitometric scanning of the autoradiographs and the values were normalized to the amount of Fyn protein detected by Western blot analysis of the same sample using the anti-Fyn antibody. The activity obtained in unfertilized (UF) eggs was set equal one, and the activity from the fertilized eggs is expressed relative to that of the unfertilized samples. The activity in eggs activated spontaneously by exposure to aquarium water for five minutes is indicated by the black bar. Values represent the average of three samples +/- S.E.*

Discussion

The role of Src-family protein tyrosine kinases in egg activation has been investigated in several species (Spivack and Maller, 1985; Unger and Steele, 1992; Carroll *et al.*, 1997; Giusti *et al.*, 1999a,b; Abassi *et al.*, 2000; Giusti *et al.*, 2000) and there is evidence that these kinases play a role in events leading up to the

sperm-induced calcium transient (Sato et al., 2000) as well as later steps leading up to cytokinesis. In the sea urchin egg, Fyn kinase is activated shortly after fertilization and remains active through the first cell cycle (Kinsey, 1996). Functional studies using dominant-negative fusion proteins demonstrated a role for Fyn in the sperm-induced calcium transient (Kinsey and Shen 2000). Further analysis of the Fyn activation pathway in the sea urchin egg has been complicated by the low sequence homology of sea urchin proteins to those of vertebrate species. The zebrafish form of Fyn kinase exhibits a high degree of homology to that of mammalian species (Rongish and Kinsey, 2000) and is easily detected by immunoprecipitation and western blot analysis of zebrafish brain. Detection of Fyn in the yolk rich zebrafish egg required that the yolk be separated from the membrane fraction on a sucrose gradient. Fyn kinase activity was highest in the light membrane fraction, which is enriched in marker enzymes for plasma membrane and endoplasmic reticulum. Fyn kinase activity in the unfertilized egg was almost undetectable, but fertilization resulted in stimulation of kinase activity an average of 6-fold, with some groups of eggs exhibiting over 10-fold stimulation.. The fertilization-induced increase in kinase activity was detected as early as 30 seconds after addition of sperm and preceded the elevation of the chorion which is first evident at 45 seconds. Thus Fyn activation occurs prior to or coincident with the cortical reaction (Hart, 1980; Hart and Yu 1990). Spontaneous activation of eggs in response to aquarium water did not trigger Fyn activation, indicating that the fertilizing sperm is required to initiate Fyn activation.

The finding that Fyn kinase is activated at fertilization in the zebrafish egg suggests that this species shares Fyn-regulated enzyme pathway(s) with the fertilized sea urchin egg. The fact that Fyn could be stimulated only by sperm and not by spontaneous parthenogenic activation indicates that these two events occur through different mechanisms. One possibility that would be consistent with observations in *Xenopus* and in marine invertebrate eggs is that the fertilizing sperm triggers events leading to Fyn activation which, in turn stimulates phospholipase C resulting in production of IP3 and the calcium transient, followed by DNA synthesis, and other components of egg activation leading to cytokinesis. Spontaneously activated eggs, however, would bypass Fyn activation and initiate calcium release by a different mechanism possibly involving G-proteins, plasma membrane calcium channels, etc. (see Fig. 1).

In contrast to the above model, a recent study comparing the calcium transient resulting from spontaneous activation with that initiated by fertilization indicated that these events are very similar, although the spontaneous calcium transient does not lead to functional cleavage (Lee *et al.*, 1999). These authors proposed a different model in which the calcium transient in the zebrafish egg does not normally require a signal from the sperm. If this model proves to be the case, it would indicate that sperm-induced Fyn activation is not required for the calcium transient and functions instead in other aspects of egg activation such as cytoskeletal changes or pronuclear movements, etc.

Identification of the egg proteins that Fyn phosphorylates at fertilization and the mechanism by which Fyn itself is regulated remain important questions for which the zebrafish egg will be a useful model system.

Materials and Methods

Eggs were collected from mature *Danio rerio* and maintained in Hanks buffer + 5 mg/ml BSA 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 containing 6.25 μg protein) with the egg suspension, then activating the sperm by addition of 0.5 ml of aquarium water. The success of fertilization was monitored by removing 8-10 eggs from each group and monitoring their development through the early cleavage stages.

Subcellular fractionation

Water was aspirated from a suspension of eggs or zygotes, which were then suspended and homogenized in an equal volume of 70% sucrose in TKM buffer (Tris, pH 7.5, 50 mM; KCl, 25 mM; $MgCl_2$, 5 mM; EGTA, 1 mM, NaN_3, 1 μM; Aprotinin (Sigma-Aldrich, St. Louis, MO) 10 μg/ml). The homogenate was layered on the bottom of a 0.5 ml ultracentrifuge tube, and overlain with 150 μl of 35% sucrose, 150 μl of 23 % sucrose, and 100 μl of 12% sucrose. The samples were centrifuged in an SW50.1 rotor at 100,000 xg for 3 h and the material at each sucrose interface was collected. The bottom 200 μl was collected as the cytosol fraction, and the underlying pellet was also recovered. Fractions were diluted with TKM buffer and centrifuged at 100,000 xg for 1 h. The fractions were resuspended and protein content was determined by Lowry (Lowry *et al.*, 1951) assay in the presence of 1% SDS.

Immunoprecipitation and kinase assay

Membrane fractions were solubilized in immunoprecipitation buffer (NaCl, 150 mM; Tris, 10 mM, EDTA, 1 mM, EGTA, 1 mM; Na_3VO_4, 200 μM; PMSF, 200 μM; NaN_3, 10 μM; NP-40, 1%), and insoluble material was removed by centrifugation at 10,000 xg for 5 min. Detergent extracts were incubated with anti-Fyn antibody (Fyn-3, Santa Cruz Biotechnology, Santa Cruz, CA) at 0.2 μg/ml, or with an equivalent amount of control rabbit IgG (Sigma) for 2 h at 4°C. Immune-complexes were absorbed to protein-A agarose, then washed twice with immunoprecipitation buffer and once with kinase buffer (HEPES, 12.5 mM; $MgCl_2$, 10 mM; β-mercaptoethanol, 2.5 mM; pH 7.2). Kinase reactions were started by addition of 25 μCi of [γ^{32}P]ATP (3000 Ci/mmol) to the immune-complexes which were suspended in 15 μl of kinase buffer, and carried out at 25°C for 1 min. The reaction was stopped by addition of SDS gel sample buffer and samples were analyzed by SDS-PAGE and autoradiography. Autophosphorylation was quantified by digital scanning of the autoradiographs and densitometric analysis using NIH-Image software as modified by Scion Corp (Frederick, MD).

Enzyme assays

The marker enzymes acid phosphatase, succinate dehydrogenase, and NADPH-cytochrome c reductase were assayed as previously described (Ribot *et al.*, 1983). Ouabain-inhibitable Na^+/H^+ ATPase was assayed in 0.1 ml of 50 mM HEPES, 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3.3 mM [γ^{32}P]ATP (0.3 mCi/mmol), pH 7.3 with and without 1 mM ouabain (Sigma Chemical Co. St. Louis, Mo). The reaction was stopped by addition of 0.2 ml of 10% TCA and the unreacted [γ^{32}P]ATP was removed by passing the products through a C-18 SepPak (Waters Assoc. Milford, MA). The $^{32}PO_4$ released was counted in a scintillation counter.

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