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Role of Yes kinase during early zebrafish development

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Abstract

We have identified the Yes kinase in zebrafish eggs and investigated its role in development of the zebrafish embryo. In situ hybridization as well as immunofluorescence techniques demonstrated that Yes kinase is maternally expressed and is localized to the cortical region of the unfertilized egg. Fertilization resulted in concentration of Yes kinase to the blastodisc where it continued to be localized to the blastoderm cells through cleavage, gastrulation, and later development. Yes kinase activity was found to decrease abruptly at fertilization, then increase progressively during epiboly, and was maintained at high levels throughout gastrulation. The role of Yes kinase in development was tested by treating embryos with chemical protein tyrosine kinase (PTK) inhibitors such as 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2) and by injection of antisense morpholinos. Both treatments resulted in the arrest of development at the beginning of the epiboly. Co-immunoprecipitation studies demonstrated that Yes kinase participates in a stable complex with focal adhesion kinase (FAK), which is phosphorylated in vitro. These results demonstrate that Yes kinase plays an important role in epiboly and indicate that Yes kinase participates in signaling by focal adhesion kinase during early development.

Keywords: c-Yes tyrosine kinase; Zebrafish; Egg activation; Fertilization; Epiboly

Introduction

Fertilization and early embryonic development involve a complex series of signal transduction pathways that activate the egg and control cell growth, differentiation, and movement events that together produce a viable embryo. Egg activation appears to require the action of protein tyrosine kinases (PTKs) for events leading up to the sperm-induced calcium transient (Giusti et al., 1999; Shen et al., 1999), for pronuclear migration and fusion (Moore and Kinsey, 1995; Wright and Schatten, 1995), and for reentry into the cell cycle (Kinsey, 1997; Sato et al., 1999). While the role of Src-family PTKs in regulating entry into the cell cycle has been demonstrated in many cell systems (reviewed in Courtneidge, 2002), the developing embryo seems to involve these kinases at several different stages of development. For example, PTK expression studies (Schartl and Barnekow, 1984; Steele et al., 1989a,b, 1990) demonstrated that Src-family kinases are expressed at all stages of development and inhibitor studies (Livingston et al., 1998) demonstrated that PTK activity is required for events critical to gastrulation. Protein tyrosine kinases important in development undoubtedly include growth factor receptors such as epidermal growth factor (EGF) (Boomsma et al., 2001), platelet-derived growth factor (PDGF) (Ataliotis et al., 1995; Ramachandran et al., 1997), and fibroblast growth factor (FGF) family receptor kinases (McCoon et al., 1996; Yamaguchi et al., 1994). These receptors trigger signaling pathways that involve other PTKs such as focal adhesion kinase (FAK) (Furuta et al., 1995;

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FAK, focal adhesion kinase; GST, glutathione S-transferase; HEPES, 2-hydroxyethyl]piperazine-N′-[2-ethane-sulfonic acid; IPTG, isopropylthio-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonylfluoride; PTK, protein tyrosine kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d] pyrimidine; SDS, sodium dodecyl sulfate; SH2 and SH3, Src homology domain 2 and 3, respectively; TCA, trichloroacetic acid.

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Zhang et al., 1995), as well as Src-family PTKs which are essential for mesoderm formation (Hama et al., 2002; Weinstein et al., 1998; Weinstein and Hemmati-Brivanlou, 2001) and for cell movements involved in gastrulation and somitogenesis (Denoyelle et al., 2001; Henry et al., 2001). An understanding of the signaling pathways in which each of these kinases participate will help explain how the complex events that characterize early development are controlled.

In the present study, we have examined the possible contribution of the c-yes encoded kinase (Yes kinase) in fertilization and early development. We have prepared an antibody specific for the zebrafish Yes kinase and measured its activity during fertilization and early development. We report here that Yes kinase activity decreases at fertilization but is strongly activated during epiboly. The Yes kinase is expressed uniformly among the blastula and early gastrula cells, then becomes expressed at higher levels in developing nervous system components. Co-immunoprecipitation studies demonstrated that Yes kinase is associated with FAK and can phosphorylate it in vitro. Functional analysis by PTK inhibitor studies as well as antisense morpholino oligonucleotides indicated that Yes kinase plays an important role in events leading up to or involved with the epiboly process, possibly acting through the FAK signaling pathway.

Materials and methods

Egg collection

Wild-type, random bred Zebrafish (*Danio rerio*) were maintained on a 14:10-h light–dark cycle at 28°C (Westerfield, 1995). Fertilization was accomplished by mixing sperm (5 μl containing 7 μg protein) with the egg suspension, then activating the sperm by addition of ten volumes of aquarium water. The fertilized eggs were then washed and suspended in aquarium water and incubated in petri dishes maintained at 28°C.

Culture and treatment of embryos

To evaluate the requirement for Src-family tyrosine kinase activity during early development, eggs were fertilized and then incubated with the Src-family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine (PP2) or the inactive analog 4-amino-7-phenylpyrazolo[3,4-d] pyrimidine (PP3; Calbiochem-Novabiochem, San Diego, CA). The inhibitors were solubilized in dimethyl sulfoxide (DMSO) and suspended in aquarium water just before use. The final concentration of DMSO in the culture water was less than 0.1% vol/vol. Embryos were cultured in the presence of inhibitors for 5 h at 28.5°C after which the inhibitor was washed out and the progress of the embryos was monitored with a dissecting microscope so that the embryos could be rotated manually to allow estimation of the progress through epiboly.

Antibody production

The U + SH3 domains of c-Yes (AA 13–136) were amplified from a cDNA library constructed from 24 h zebrafish embryos (Stratagene, La Jolla, CA) by polymerase chain reaction (PCR) using the following primers: sense, 5′-TAGAATTCAACACAGAAGTACCGACCAGA-3′; antisense, 5′-ATGCTCGAGCGAGCCTCACACCAGTCAC-3′). The PCR product was gel purified and cloned into pGEM-T-easy vector (Promega, Madison, WI) for sequencing. The insert containing the zebrafish Yes sequence was excised by digestion with *Eco*RI and *Xho*I, and ligated into the *Eco*RI and *Xho*I sites of pGEX-4T3 (Pharmacia Biotech Inc., Piscataway, NJ). The ligation product was transformed into DH5α bacteria (GIBCO BRL Life Technologies, Grand Island, NY), and the DNA sequence was again confirmed (GenBank accession no. AY157872). The zebrafish Yes-U + SH3-GST fusion protein was induced and purified as previously described (Kinsey and Shen, 2000) then further purified by SDS-polyacrylamide gel electrophoresis (PAGE). The 39-kDa band was excised and used to inoculate rabbits. The anti-Yes antibodies were partially purified by absorption with glutathione S-transferase (GST) protein bound to glutathione–agarose to remove antibodies to GST. Antibodies directed against the phosphorylated tyrosine 861 in FAK were obtained from Biosource International, Camarillo, CA.

Immunoprecipitation and kinase assay

Membrane fractions were prepared as previously described (Wu and Kinsey, 2000), solubilized in immunoprecipitation buffer [TritonX-100, 1%; SDS, 0.1%; Na_3VO_4, 200 μM; phenylmethylsulfonylfluoride (PMSF), 200 μM; ethylenediaminetetraacetic acid (EDTA), 1 mM in PBS buffer pH 7.2], and insoluble material was removed by centrifugation. Detergent extracts were incubated with anti-Yes antibody, or with an equal amount of preimmune antiserum for 2 h at 4°C. Immune complexes were absorbed to protein A-Sepharose, then washed twice with immunoprecipitation buffer and once with kinase buffer [12.5 mM 2-hydroxyethyl]piperazine-N′-[2-ethane-sulfonic acid (HEPES), pH 7.2, 10 mM $MgCl_2$, 2.5 mM β-mercaptoethanol].

PTK activity in the immunoprecipitates was detected by an autophosphorylation reaction performed in kinase buffer containing 25 μCi [γ-^{32}P]ATP (3000 Ci/mmol). Reactions were carried out at 25°C for 1 min, then terminated by addition of SDS gel sample buffer. The products were resolved by SDS-PAGE and the gels were stained with Coomassie blue, destained, and treated with 1 M KOH at 50°C for 30 min prior to detection of radiolabeled proteins by autoradiography. Tyrosine protein kinase activity was quantitated by phosphorylation of a synthetic peptide substrate (GNQHIYQPIGK), which was quantitated by reverse phase HPLC as previously described (Moore and Kinsey, 1994).