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Evidence for an Erbstatin-Sensitive Tyrosine Kinase Functioning in Ascidian Egg Activation

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It has been proposed that protein tyrosine phosphorylation plays an important role in sperm-induced egg activation. To obtain evidence for the involvement of tyrosine kinases at an early stage in the egg activation process, we analyzed the effects of tyrosine kinase inhibitors on surface contraction of fertilized eggs of the ascidian *Ciona savignyi* and analyzed their effects on cleavage. We found that only erbstatin analog inhibited surface contraction, which was observed 5 min after insemination. With respect to the cleavage that occurs around 50 min after insemination, tyrophostin A1 and genistein, together with erbstatin analog, showed inhibitory effects. In addition, transient tyrosine phosphorylation of at least five proteins was observed 2-5 min after insemination, followed by tyrosine phosphorylation of one protein 30-40 min after insemination. Among proteins tyrosine-phosphorylated at the former stage, tyrosine phosphorylation of a 75kD protein was inhibited by erbstatin analog. Thus, an erbstatin-sensitive tyrosine kinase functions at an early stage in the ascidian egg activation process. © 1997 Academic Press

Sperm-egg interaction triggers an egg activation process including a transient increase in intracellular free calcium ions, exocytosis of cortical granules, and resumption of the embryonic cell division cycle. The calcium release is observed at an early stage in the egg activation process and is mediated by inositol 1,4,5-triphosphate, which is proposed to be produced through an action of phospholipase C activated by either a G-protein-coupled receptor or a tyrosine kinase receptor (or a tyrosine kinase associated with a receptor) (1-6). Consistent with the latter proposed pathway, i.e., involvement of tyrosine kinases in egg activation, several lines of evidence indicate that fertilization of eggs results in the tyrosine phosphorylation of egg proteins through putative tyrosine kinases (7-12). In addition, molecular interactions between sperm fertilin (an ADAM family protein in sperm plasma membrane) and egg integrin (a cell-adhesion molecule in egg plasma membrane) have been reported to be involved in sperm-egg plasma membrane binding and fusion (13), which suggests that an integrin-mediated signal transduction mechanism including tyrosine phosphorylation works in the egg activation process (13). However, it is still unclear what kind of tyrosine kinase functions in the egg activation process, especially, at its early stage.

In a previous paper (14), we reported that a heparin-blocked inositol 1,4,5-triphosphate receptor-mediated calcium release operates in the sperm-induced activation process of ascidian eggs. Surface contraction, originating from ooplasmic segregation, occurs twice in ascidian eggs (15). The first-phase surface contraction is the earliest recognizable change in egg shape; this change can be observed within 5 min after fertilization at 20°C in eggs of the ascidian *Ciona savignyi*. Here, we investigated the effects of various tyrosine kinase inhibitors on egg activation of *C. savignyi*, based on the above egg surface contraction, to determine what kind of tyrosine kinase functions at an early stage in the ascidian egg activation process. We found that an erbstatin-sensitive tyrosine kinase acts at its early stage.

MATERIALS AND METHODS

Preparation of sperm and eggs. The ascidian *C. savignyi* was collected in Mutsu Bay, Japan. Sperm and eggs were collected by cutting the respective gonaducts. In order to observe egg surface contraction easily, eggs without the chorion (dechorionated eggs) were prepared and used for fertilization experiments. Eggs were incubated in seawater containing 0.05% actinase E (Kaken Seiyaku, Japan) and 1% sodium thioglycolate for 20 min at room temperature and pipetted repeatedly to remove the egg chorion.

Fertilization and egg surface contraction. Dechorionated eggs were suspended in seawater buffered with 20 mM Tris-HCl (pH 8.0) to give a final concentration of 0.5% (vol/vol), and 1 ml of the egg suspension was placed in each well of a 48-well plate that had been previously coated with 0.9% agar. After the eggs were previously incubated for 30 min at 20°C in the presence or absence of tyrosine

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kinase inhibitors at various concentrations, they were fertilized with 10 μl of 5% sperm suspension and allowed to develop over various periods of time. Methanol or dimethylsulfoxide was used as a control. Surface contraction and cleavage of fertilized eggs were microscopically observed at 5 min and 50 min, respectively, after insemination. Tyrosine kinase inhibitors including tyrophostin A1, A23, A51, and B44, erbstatin analog (methyl 2,5-dihydroxycinnamate), and genistein were purchased from LCL Laboratories.

Western blot analysis. Aliquots (300 μl each) of the above egg suspensions before and after insemination were pelleted in a hand centrifuge for 5 sec. After seawater was removed, the pellets were homogenized in 60 μl of ice-cold homogenization buffer containing 5 mM Tris-HCl (pH 7.6), 20 mM NaCl, 5 mM KCl, 50 mM NaF, 20 mM EDTA, 1 mM Na_3VO_4, 3 mM phenylmethanesulfonyl fluoride (Sigma), and 0.4 mM leupeptin (Peptide Institute, Japan), and were then centrifuged at 10,000 $\times g$ for 10 min. The resulting pellets were washed with the same buffer, mixed with sample buffer for SDS-polyacrylamide gel electrophoresis that included 1 mM Na_3VO_4, 3 mM phenylmethanesulfonyl fluoride, and 0.4 mM leupeptin, and were then heated at 90°C for 3 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel (16), and subjected to Western blotting (17) using anti-phosphotyrosine antibody PY20 (Leinco Technologies) as a primary antibody and peroxidase-conjugated anti-mouse IgG (TAGO) as a secondary antibody. The phosphotyrosine content of egg proteins was determined by detection using the enhanced chemiluminescence system (ECL) (Amersham), and the bands were visualized with X-ray film. High molecular mass standards for SDS-polyacrylamide gel electrophoresis were obtained from Sigma.

Immunoprecipitation experiments. Egg pellets obtained from the egg homogenate were solubilized in lysis buffer (1 ml/tube) containing 5 mM Tris-HCl (pH 7.4), 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 50 mM NaF, 20 mM EDTA, 1 mM Na_3VO_4, 3 mM phenylmethanesulfonyl fluoride, and 0.4 mM leupeptin. The egg lysate was centrifuged at 15,000 $\times g$ for 10 min, and tyrosine-phosphorylated proteins were immunoprecipitated from the resulting supernatant with PY20 antibody (1.0 μg/ml) and protein A-Sepharose CL-4B (Sigma). The pellets were washed three times with the lysis buffer, suspended in the sample buffer for SDS-polyacrylamide gel electrophoresis, heated at 95°C, and were then subjected to Western blotting using PY20 antibody.

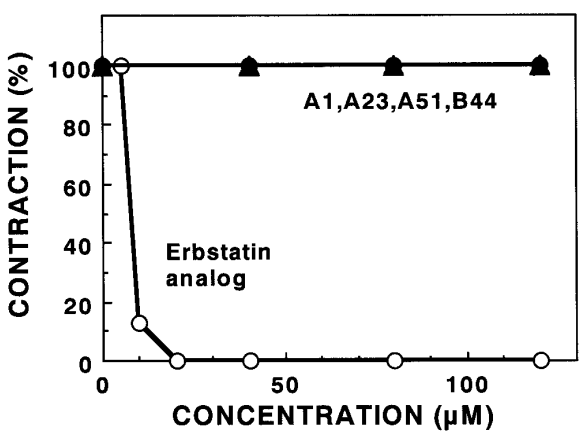

FIG. 1. Effects of tyrosine kinase inhibitors on egg surface contraction in *C. savignyi*. Dechorionated eggs were previously incubated for 30 min at 20°C in the presence or absence of erbstatin analog (○), tyrophostin A1 (●), A23 (△), A51 (▲), and B44 (□), and were then fertilized. Surface contraction of fertilized eggs was microscopically observed 5 min after insemination. Its extent in solvent (methanol or dimethylsulfoxide) was defined as 100%.

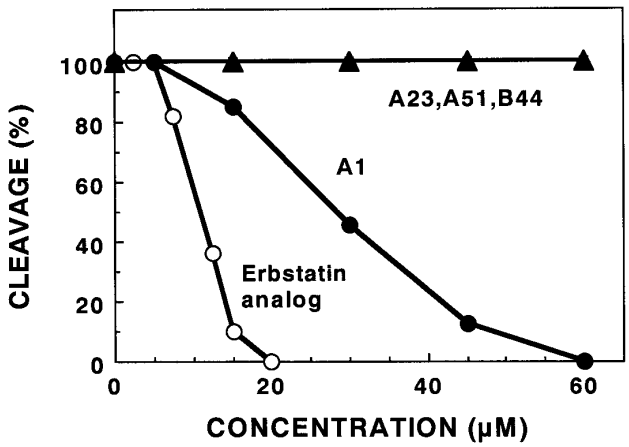

FIG. 2. Effects of tyrosine kinase inhibitors on egg cleavage in *C. savignyi*. Dechorionated eggs were previously incubated for 30 min at 20°C in the presence or absence of erbstatin analog (○), tyrophostin A1 (●), A23 (△), A51 (▲), and B44 (□), and were then fertilized. Cleavage of fertilized eggs was microscopically observed 50 min after insemination. Its extent in solvent (methanol or dimethylsulfoxide) was defined as 100%.

RESULTS AND DISCUSSION

Eggs of the ascidian *C. savignyi* undergo a first-phase surface contraction, which is observable 5 min after insemination. We investigated the effects of tyrosine kinase inhibitors on the surface contraction of dechorionated eggs to determine whether tyrosine kinases function at an early stage in the egg activation process. Among the inhibitors used, only erbstatin analog inhibited the egg surface contraction; genistein and tyrophostin A1, A23, A51, and B44 scarcely inhibited it (Fig. 1). Based on cleavage of the dechorionated eggs, which is observable 50 min after insemination, the former inhibitor also inhibited it (see Fig. 2). Taken together, these results strongly suggest that an erbstatin-sensitive tyrosine kinase functions at an early stage in the ascidian egg activation process.

In addition to erbstatin analog, tyrophostin A1 inhibited the cleavage, although to a lesser extent, whereas other tyrophostin analogs did not (Fig. 2). Inhibition by genistein was detected when microscopically observed 50 min after insemination but not when observed 60 min after insemination, which indicates that this inhibitor delays cleavage (Fig. 3). Thus, at least two different kinds of tyrosine kinases function in the egg activation process: One is a erbstatin-sensitive kinase, functioning at its early stage, while the other is a kinase susceptible to tyrophostin A1 and/or genistein, functioning at its later stage. It should be noted that the effect of genistein in ascidian eggs is similar to that observed in sea urchin eggs, while the effects of erbstatin analog in these two eggs are different. In sea urchins, both inhibitors inhibit the later events of egg activation but not the early events (10). In addition,

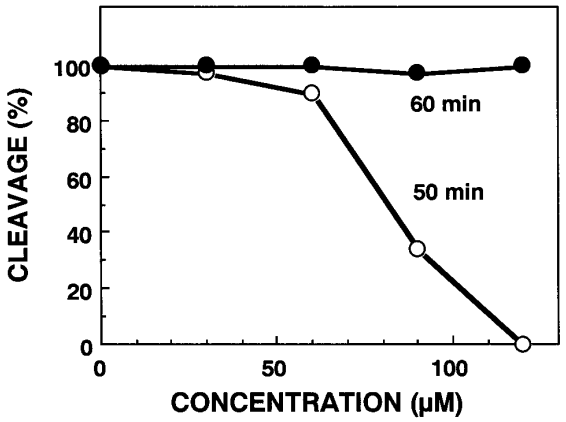

FIG. 3. Effects of genistein on egg cleavage in *C. savignyi*. Dechorionated eggs were previously incubated for 30 min at 20°C in the presence (○, ●) or absence of genistein, and were then fertilized. Cleavage of fertilized eggs in the presence of genistein was microscopically observed 50 (○) or 60 min (●) after insemination. Its extent in solvent (dimethylsulfoxide) was defined as 100%.

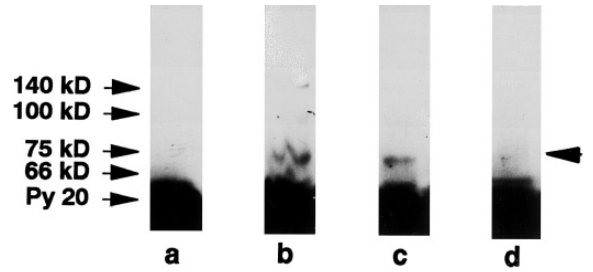

FIG. 5. Effects of tyrosine kinase inhibitors on tyrosine phosphorylation at the early stage in the egg activation process of *C. savignyi*. Dechorionated eggs were previously incubated for 30 min at 20°C in the presence or absence of erbstatin analog and tyrophostin A1, and were then fertilized. Eggs collected 4 min after insemination were homogenized and centrifuged, and the pellets were solubilized and centrifuged, as described in MATERIALS AND METHODS. The supernatant was subjected to immunoprecipitation with PY20 antibody and protein A-Sepharose CL-4B, and the immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blotting using PY20 antibody. (a) PY20 antibody only. (b) Solvent (methanol). (c) Tyrophostin A1 (20 μM). (d) Erbstatin analog (20 μM).

it has been reported that erbstatin-sensitive tyrosine kinases control the microtubule assembly in the sea urchin egg activation process (11). In our case, egg surface contraction is thought to be mediated by microfilaments, not by microtubules (15). This discrepancy between ascidian and sea urchin eggs may be due to species difference, but its precise reason remains unknown.

Next, we analyzed tyrosine phosphorylation during ascidian egg activation by Western blotting using anti-phosphotyrosine antibody to determine what kinds of proteins are tyrosine-phosphorylated (Fig. 4). Transient tyrosine phosphorylation of at least five proteins of 190 kD, 140 kD, 100 kD, 75 kD and 66 kD was observed 2-5 min after insemination, followed by tyrosine phosphorylation of 75 kD protein, which indicates that there is different timing of tyrosine phosphorylation in the process of egg activation. These results are consistent with the above results showing the involvement of different tyrosine kinases at two stages in the egg activation process, i.e., one stage between sperm-egg interaction and egg surface contraction and another between surface contraction and cleavage.

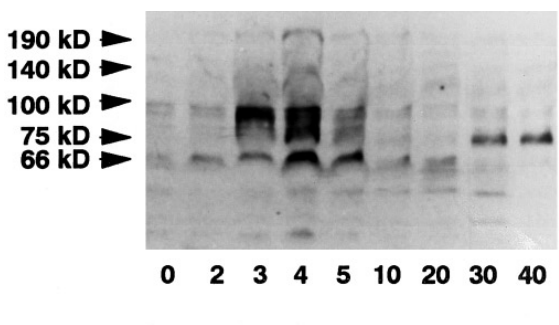

FIG. 4. Tyrosine phosphorylation during egg activation in *C. savignyi*. Eggs collected in the process of egg activation were homogenized and centrifuged at 10,000 ×*g* for 10 min. The resulting pellets were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blotting using anti-phosphotyrosine antibody PY20.

To obtain definitive evidence for the involvement of the erbstatin-sensitive tyrosine kinase at the early stage in the ascidian egg activation process, proteins tyrosine-phosphorylated in the presence and absence of tyrosine kinase inhibitors were immunoprecipitated by using anti-phosphotyrosine antibody to enrich tyrosine-phosphorylated proteins, and then the proteins were subjected to Western blotting using anti-phosphotyrosine antibody (Fig. 5). Erbstatin analog inhibited tyrosine phosphorylation of 75 kD protein during surface contraction, while tyrophostin A1 did not. This result strongly suggests that the erbstatin-sensitive tyrosine kinase catalyzes the tyrosine phosphorylation of at least 75 kD protein at the early stage in the egg activation process.

In conclusion, we propose that the erbstatin-sensitive tyrosine kinase plays an important role in ascidian egg activation. Studies on the purification and characterization of the tyrosine kinase are now in progress in our laboratories.

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