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Evidence for the Involvement of a Src-Related Tyrosine Kinase in *Xenopus* Egg Activation

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Recently, we have purified a Src-related tyrosine kinase, named *Xenopus* tyrosine kinase (Xyk), from oocytes of *Xenopus laevis* and found that the enzyme is activated within 1 min following fertilization [Sato *et al.* (1996) *J. Biol. Chem.* 271, 13250–13257]. A concomitant translocation of a part of the activated enzyme from the membrane fraction to the cytosolic fraction was also observed. In the present study, we show that parthenogenetic egg activation by a synthetic RGDS peptide [Y. Iwao and T. Fujimura, T. (1996) *Dev. Biol.* 177, 558–567], an integrin-interacting peptide, but not by electrical shock or the calcium ionophore A23187 causes the kinase activation, tyrosine phosphorylation, and translocation of Xyk. A synthetic tyrosine kinase-specific inhibitor peptide was employed to analyze the importance of the Xyk activity in egg activation. We found that the peptide inhibits the kinase activity of purified Xyk at IC_{50} of 8 μM. Further, egg activation induced by sperm or RGDS peptide but not by A23187 was inhibited by microinjection of the peptide. In the peptide-microinjected eggs, penetration of the sperm nucleus into the egg cytoplasm and meiotic resumption in the egg were blocked. Indirect immunofluorescence study demonstrates that Xyk is exclusively localized to the cortex of *Xenopus* eggs, indicating that Xyk can function in close proximity to the sperm–egg or RGDS peptide–egg interaction site. Taken together, these data suggest that the tyrosine kinase Xyk plays an important role in the early events of *Xenopus* egg activation in a manner independent or upstream of calcium signaling. © 1999 Academic Press

Key Words: **tyrosine phosphorylation; *Xenopus;* fertilization; egg activation; signal transduction.**

INTRODUCTION

In African clawed frogs, *Xenopus laevis,* as in many other organisms, the union of sperm and egg triggers an egg activation to initiate a developmental program that leads to embryogenesis (Jaffe, 1996; Keller, 1991; Longo, 1997; Snell and White, 1996). Sperm-induced egg activation is accompanied by several specialized events such as a change in membrane potential, which contributes to the block of polyspermy, a transient increase in intracellular calcium which is indispensable for the later developmental events, meiotic resumption of the egg nuclei, and fusion of the male and female pronuclei to form the zygotic nucleus (Longo, 1997). Although there are several possibilities of the mechanism by which the sperm–egg interaction induces the increase of intracellular calcium, it is believed that calcium release from the intracellular store in response to a rise in inositol trisphosphate, a hydrolyzed product of inositol phospholipids, plays an important role (Kline, 1988; Larabell and Nuccitelli, 1992; Longo, 1997; Nuccitelli *et al.*, 1993; Snow *et al.*, 1996; Stewart-Savage and Grey, 1987). However, it has not been established how production of inositol trisphosphate is stimulated upon sperm–egg interaction. Thus, there is a missing link between sperm–egg interaction and intracellular calcium release.

Protein tyrosine phosphorylation and dephosphorylation have been implicated in the regulation of a variety of cellular processes including growth, differentiation, apoptosis, and cell–cell communication (Hunter, 1995, 1997).

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Recent findings indicate that several kinds of cytoplasmic protein tyrosine kinases act in regulating these processes in the vicinity of a large number of cell surface receptor molecules with or without intrinsic catalytic activity (Clark and Brugge, 1995; Gutkind, 1998; Thomas and Brugge, 1997; Weiss, 1993). However, involvement of a protein tyrosine kinase in egg activation which seems to be under the control of egg surface receptor(s) for sperm remains to be understood (Kinsey, 1997). The Src family of protein tyrosine kinases is a prototype of the cytoplasmic tyrosine kinases of which there are at least 10 subspecies (Thomas and Brugge, 1997). In *Xenopus* oocytes/eggs, three members of the Src family, c-Src, c-Yes, and Fyn, have been initially identified at the mRNA level (Steele *et al.,* 1989a,b, 1990). Recently, we have isolated cDNA encoding the *Xenopus* Lyn gene (unpublished result, *Xenopus* Lyn sequence ID No. 2114076), and Weinstein *et al.* have also cloned a cDNA encoding a novel Src family gene named *laloo* (Weinstein *et al.,* 1998). Thus, in *Xenopus* oocytes/eggs, multiple Src family kinases are present at the mRNA level. However, the presence and function of these protein products have not been described.

Recently, we have purified a 57-kDa protein tyrosine kinase, named *Xenopus* tyrosine kinase (Xyk),[2] from oocytes of *Xenopus* (Sato *et al.,* 1996), by using an antibody (anti-pepY antibody; Fukami *et al.,* 1993) raised against a synthetic peptide corresponding to residues 410 to 428 of the chicken c-Src, a region whose amino acid sequence is completely conserved in c-Src, c-Yes, and Fyn. Xyk can be recognized by immunoprecipitation or immunoblotting with the anti-pepY antibody but not by an antibody specific to either c-Src or c-Yes or Fyn, indicating that Xyk is structurally related to but distinct from the Src family members identified in *Xenopus* oocytes (Sato *et al.,* 1996). Importantly, Xyk is localized in the membrane fraction and remains virtually inactive during oocyte maturation but is activated several-fold and tyrosine phosphorylated within 1 min following fertilization and its partial translocation from the membrane fraction to the cytosolic fraction takes place (Sato *et al.,* 1996). More recently, we have found that a number of egg proteins become tyrosine phosphorylated or dephosphorylated within 1 min after fertilization and that tyrosine kinase-specific inhibitors can inhibit fertilization-dependent egg activation (Sato *et al.,* 1998). These results suggest that the tyrosine kinase Xyk is involved in the signal transduction of fertilization, especially in the egg activation process.

In this paper, in order to understand further the mechanism and physiological importance of Xyk action, we examined whether parthenogenetic egg activation causes activation, tyrosine phosphorylation, and translocation of Xyk. We show here that a synthetic RGDS peptide, which acts on the egg surface to induce intracellular calcium release and egg activation (Iwao and Fujimura, 1996), but not electrical shock nor the calcium ionophore A23187, stimulates the kinase activity and induces the translocation of Xyk. Furthermore, results obtained from inhibitor-microinjection experiments and indirect immunofluorescence studies lead us to suggest that Xyk is involved in *Xenopus* egg activation in close proximity to the sperm–egg or RGDS peptide–egg interaction site.

[2] Abbreviations used: Xyk, *Xenopus* tyrosine kinase; APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride; DB, DeBoer's buffer; SB, Steinberg's solution; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; T-TBS, Tris-buffered saline containing Tween 20; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

MATERIALS AND METHODS

Materials

RGDS peptide (Arg-Gly-Asp-Ser) was purchased from Sigma, dissolved in water at 5–20 mM, and stored at −80°C until use. A23187 was purchased from Calbiochem, dissolved in dimethyl sulfoxide at 10 mM, and kept at −80°C until use. Leupeptin was purchased from Peptide Institute (Osaka). (*p*-Amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF) was from Wako Pure Chemicals (Osaka). [γ-^{32}P]ATP (35020) and [^{125}I]protein A (68038) were obtained from ICN. Other reagents were from Wako or Nacalai (Kyoto). Rabbit polyclonal anti-pepY antiserum raised against a synthetic peptide, termed pepY, which corresponds to residues 410–428 of the chicken c-Src, and its preimmune serum were prepared as described previously (Fukami *et al.,* 1993). Anti-pepY IgG was purified by ammonium sulfate precipitation and DEAE–cellulose chromatography as described (Fukami *et al.,* 1993) or by protein A–Sepharose chromatography, and the IgG fraction was used to prepare anti-pepY beads (see below). Purified Xyk was prepared from the membrane fraction of oocytes as described previously (Sato *et al.,* 1996). Specific activity of the enzyme toward Cdc2 peptide under the assay conditions as described below was 0.9 nmol/min/mg. The following synthetic peptides were prepared as described previously (Fukami *et al.,* 1993), dissolved in 20 mM Tris–HCl, pH 7.5, at 3–10 mM, and kept at −80°C until use: Cdc2 peptide (Val-Glu-Lys-Ile-Gly-Glu-Gly-Thr-Tyr-Gly-Val-Val-Tyr-Lys-Ala-Arg-His-Lys-Leu-Ser) which corresponds to residues 7–26 of the fission yeast *cdc2* gene product (Gould and Nurse, 1989) and peptides A7 (Ser-Asp-Ser-Ile-Gln-Ala-Glu-Glu-Trp-Tyr-Phe-Gly-Lys-Ile-Thr-Arg-Arg-Glu) and A9 (Ser-Asp-Ser-Ile-Gln-Ala-Glu-Glu-Trp-Tyr-Phe-Gly-Lys-Ile-Thr), which correspond to residues 140–157 and residues 140–154 of the chicken c-Src, respectively (Fukami *et al.,* 1993).

Activation of Xenopus Eggs

African clawed frogs, *Xenopus laevis,* were purchased from dealers and maintained in deionized water. All manipulations were carried out at 18–21°C unless otherwise indicated. To obtain unfertilized eggs, adult females were injected with 500–1000 IU of human chorionic gonadtropin (Isei, Yamanashi, Japan) in the dorsal lymph sac and were kept in deionized water for more than 10 h. Laid and squeezed eggs were placed in a 100-mm-diameter plastic dish, immediately washed with 1× DeBoer's buffer (DB) (110 mM NaCl, 1.3 mM KCl, 0.44 mM $CaCl_2$, pH 7.2), and used within 2 h. Sperm suspension was obtained by macerating a piece of testis in 1× DB and stored on ice until use. Sperm at a final concentration of 1–2.5 × 10^6/ml were used for insemination. Unfertilized eggs were activated by either insemination or parthenogenetic activa-

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tion treatment. About 2-ml packed volume of dejellied eggs was used in one activation treatment. For sperm-induced egg activation, excess DB was removed from the dish and 1 ml of the sperm suspension and 8 ml of 0.2× DB were simultaneously added to the monolayer of eggs placed in a 100-mm-diameter plastic dish. For parthenogenetic egg activation, jelly coats of unfertilized eggs were removed by incubation in excess volume of 1× DB supplemented with 2% cysteine–NaOH (pH 8.0) for 3 min, followed by extensive washing with 1× DB. A group of the dejellied eggs were washed twice with 0.2× DB and transferred to an activation chamber filled with 0.2× DB. The bottom of the activation chamber was layered with 2% agarose (0.5 cm depth) in 0.2× DB. Electrical activation (Murray, 1991) was achieved by applying voltage (3.6 V/cm for 5 s, one pulse) across the chamber. For activation by A23187 (Capco and Bement, 1991) or RGDS peptide (Iwao and Fujimura, 1996), dejellied eggs were further washed twice with 0.5× Steinberg's solution (SB) (29 mM NaCl, 0.34 mM KCl, 0.17 mM $CaCl_2$, 0.43 mM Mg_2SO_4, and 2.3 mM Tris–HCl, pH 7.4) and transferred to a 100-mm-diameter plastic dish filled with 0.5× SB. The bottom of the dish was covered with 2% agarose in 0.2× SB. After removing excess solution, 5 ml of 0.5× SB containing A23187 or RGDS peptide, at a final concentration of 2 or 500 μM, was added. In every activation treatment, the reactions were stopped as follows when more than 90% of the eggs showed cortical contraction. To stop the activation procedure, inseminated eggs were subjected to dejelly treatment at 10 min postinsemination by incubation with excess volume of 1× DB supplemented with 2% cysteine–NaOH (pH 8.0) for 2 min and then washed with 1× DB for 1 min. We usually had one- or two-time exchange with fresh and prewarmed cysteine-containing buffer during the first 2 min of dejelly treatment of eggs. In addition, we performed continuous and gentle shaking of the specimens. This method allowed us to observe direct contact between eggs within 3 min of treatment, indicating that the dejelly process was working well, although it may not have been complete. Eggs activated by electrical shock, A23187, and RGDS peptide were subjected to wash treatment with 1× DB for 1 min at 4, 5, and 8 min postactivation treatment. After washing and removal of excess buffer, eggs were immediately frozen by liquid nitrogen and kept at −80°C until use.

Subcellular Fractionation of Eggs and Partial Purification of Xyk

Subcellular fractionation was done according to the procedure as described (Sato *et al.,* 1996, 1998) with some modifications. All procedures were carried out at 0–4°C. Frozen dejellied eggs prepared as above (2 ml in a packed volume) were mixed with 10 ml of buffer A [20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 20 μM APMSF] and sonicated five times with a TOMY UD-201 ultrasonic disrupter (Tomy Seiko, Tokyo). The sonicated sample was centrifuged at 100*g* for 10 min to remove yolk platelets. The supernatant was collected and centrifuged at 150,000*g* for 1 h. After centrifugation, the clear supernatant was set aside as the cytosolic fraction. The pellet and fluffy part of the pellet were then carefully taken, diluted with buffer A, and recentrifuged at 150,000*g* for 30 min. The pellet fraction obtained was suspended in 5 ml of buffer A containing 1% Triton X-100 and sonicated for 5 min. The solubilized material was recovered by centrifugation at 10,000*g* for 10 min and used as the membrane fraction. By this procedure, 2 ml of packed volume of eggs yielded proteins of 24–28 mg of cytosolic fraction and 10–12 mg of membrane fraction. Partial purification of Xyk was carried out according to the method as described previously (Sato *et al.,* 1996) with some modifications. Cytosolic (24–28 mg protein in 5 ml) and membrane (10–12 mg protein in 5 ml) fractions from 2 ml of packed volume of eggs prepared as above were separately applied onto a DEAE–cellulose (Whatman, DE52) column (1 × 6 cm) equilibrated with buffer A containing 1% Triton X-100. Unbound materials were washed with 10 column volumes of the same buffer, and proteins bound to the column were eluted with a 10-ml linear gradient of NaCl (0–0.2 M) in the same buffer. Fractions of 1 ml (10 fractions) were collected as eluates. Fractions 4 to 7 (total 4 ml), which contained 0.05–0.10 M NaCl, as judged by measuring the refractive index of the fractions, were combined and used as the DEAE fraction. By this procedure, proteins of 3–3.6 and 1–1.4 mg were recovered as the cytosolic and membrane DEAE fractions, respectively. To all DEAE fractions, glycerol was added to a final concentration of 10% and kept frozen at −80°C until use. Protein was determined spectrophotometrically by protein assay mixture (Bio-Rad) with bovine serum albumin as standard.

Immunoprecipitation, Immunoblotting, and Cdc2 Peptide Kinase Assay

DEAE fractions prepared as above were immunoprecipitated with anti-pepY IgG immobilized onto protein A–Sepharose which had been prepared according to the manual of Harlow and Lane (1988). One-milliliter portions of cytosolic DEAE fraction (0.75–0.9 mg protein) and membrane DEAE fraction (0.25–0.3 mg protein) were separately incubated with 20 μl of anti-pepY beads containing about 10 μg IgG for 2–4 h at 4°C, and the beads were washed three times with RIPA buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 20 μM APMSF]. Washed beads were divided into aliquots and analyzed by immunoblotting. For immunoblotting, immunoprecipitated samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) on 8% gels and transferred to polyvinylidene difluoride membranes using a semidry blotting apparatus (Bio-Rad). Membranes were blocked with buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 (T-TBS) supplemented with 3 mg/ml bovine serum albumin for 1 h at room temperature and incubated for 2 h at 37°C with 100-fold diluted anti-pepY antiserum or 10 μg/ml mouse monoclonal anti-phosphotyrosine antibody, a mixture of 4G10 (Upstate Biotechnology) and PY20 (ICN). When mouse monoclonal antibody was used, rabbit polyclonal anti-mouse IgG antiserum (Cappel) at a 500-fold dilution was added as the secondary antibody. To detect immunoreactive proteins, antibody-treated membranes were incubated with T-TBS containing $[^{125}I]$protein A (50 kBq/ml) and analyzed by BAS2000 bioimaging analyzer (Fuji film, Tokyo). Alternatively, antibody-treated membranes were further treated with alkaline phosphatase-conjugated goat polyclonal anti-rabbit IgG antibody (Cappel). In this case, the immune complex was detected by incubating the membranes with buffer containing 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and nitroblue tetrazolium. Cdc2 peptide kinase assay was carried out as described previously (Sato *et al.,* 1996). The un-immunoprecipitated DEAE fractions (1.25–2 μg protein in 5 μl per assay) were incubated for 10 min at 30°C with kinase assay reaction mixture (15 μl) containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 μM $[\gamma\text{-}^{32}P]$ATP (3.7 kBq/pmol), 1 mM dithiothreitol, and 1 mM Cdc2 peptide. The kinase reaction was terminated by the addition of SDS sample buffer (Laemmli, 1970) and boiling for 3 min. The sample was subjected to SDS–PAGE

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using an 18% polyacrylamide gel, and the gel was stained with Coomassie brilliant blue. Radioactivity in the gel was visualized by the bioimaging analyzer and phosphorylation of the Cdc2 peptide was analyzed. When the effect of synthetic tyrosine kinase inhibitor peptide was examined, Cdc2 peptide kinase assay was carried out with the purified Xyk (Sato *et al.,* 1996). The enzyme (200 ng protein in 5 μl per assay) was preincubated with 10 μl of 20 mM Tris–HCl, pH 7.5, containing various amounts of synthetic peptides for 10 min at 30°C and then mixed with 10 μl of a concentrated kinase reaction mixture to give a final concentration of reagents as described above for 10 min at 30°C. Final concentrations of synthetic peptides in the kinase assay are indicated below.

Microinjection

Synthetic peptides were dissolved at concentrations of 0.005, 0.05, 0.5, and 1 mM in a buffer containing 20 mM Tris–HCl, pH 7.5, and 110 mM NaCl and centrifuged at 15,000*g* for 10 min to remove insoluble materials. Jelly-intact unfertilized eggs were prepared as described above and placed in 1× DB. The following manipulations were carried out at 18–21°C. To avoid artificial activation by insertion of the micropipet for microinjection, eggs were preincubated for 15 min in a buffer containing 110 mM NaCl, 1.3 mM KCl, 20 mM $MgSO_4$, 0.1 mM EGTA, and 10 mM chlorobutanol (1,1,1-trichloro-2-methyl-2-propanol; Wako Pure Chemicals). Then, eggs were injected using glass micropipets with an oil-filled constriction. The tip diameter of micropipets was 15–30 μm. Quantitative injection was made by manual handling using precalibrated micropipets or by use of micropipets equipped with a pulse-directed microinjector (Drummond, Nanoject). Injected volumes (40 to 50 nl) were about 5% of the average total volume of the egg cytoplasm, which had been calculated to be 935 ± 17 nl including 495 ± 29 nl of pigment and yolk layers (Iwao *et al.,* 1997). After injection, the eggs were immediately incubated in 1× DB for 15 min and then in 0.5× SB for 15 min. During these processes, prick-activated or dead eggs, which show cortical contraction or abnormal outlook, were discarded. After the incubation, eggs were inseminated or activated by either A23187 or RGDS peptide as described above. The eggs which showed cortical contraction within 30 min after the treatments were scored as activated. Sections of inseminated eggs were prepared to examine the localization and structure of sperm nuclei and egg chromosomes. Eggs of 40 min postinsemination were fixed in Smith's solution (20 mM potassium dichromate, 3.7% formalin, 2.5% acetic acid) for 24 h followed by more than 24 h incubation in water and embedded in paraffin. Serial sections of 10-μm thickness were prepared and stained with Feulgen's reaction and Fast Green. Photographs of the samples were taken under microscopic observation using a Nikon X2UW microscope with object lens magnifications of 10, 40, and 100× and an ocular lens magnification of 10×.

Immunocytochemistry

To observe Xyk in *Xenopus* eggs, the unfertilized eggs were dejellied as described above and fixed and processed for immunocytochemistry. Dejellied eggs were fixed in a buffer containing 10 mM NaH_2PO_4/Na_2HPO_4 and 145 mM NaCl, pH 7.2 (PBS), supplemented with 4% paraformaldehyde for 3 h at 18°C. Fixed eggs were further treated with absolute methanol for 12–18 h at −30°C. Then the eggs were rehydrated with PBS supplemented with 20% sucrose, embedded in a medium containing polyvinyl alcohol and polyethylene glycol (O.C.T. compound, Tissue-Tek), and frozen at −80°C. Serial sections of 10-μm thickness were made in the plane of the animal–vegetal axis and placed on poly-L-lysine-coated slide glasses at −20°C and washed with PBS for 30 min at room temperature. All manipulations were carried out at room temperature unless otherwise indicated. The specimens were treated for 15 min with PBS supplemented with 3% hydrogen peroxide to quench endogenous peroxidase activity. After washing with PBS (six times), the specimens were blocked for 1 h with a buffer supplied by a kit (TSA-Direct, Daiichikagaku, Tokyo, Japan). After treatment, the specimens were treated with anti-pepY antiserum or preimmune serum at 4°C for 12–18 h. The specimens were washed with PBS and blocked again for 30 min and then treated with horseradish peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (Cappel) for 1 h. After washing with PBS, the specimens were treated for 30 min with fluorescein isothiocyanate (FITC)-conjugated tyramide supplied by the kit. After washing with PBS (5 min, six times), the specimens were mounted with 50 mg/ml 1,4-diazabicyclo[2,2,2]octane and 90% glycerol in PBS and kept under dark conditions. Fluorescence was observed with a confocal laser-scanning microscope (Model LSM410 Invert, Carl Zeiss, Germany) at 488-nm argon excitation using a 515-nm long-pass barrier filter. The same sample was also observed using a microscope equipped with Nomarski interference.

RESULTS

Translocation and Tyrosine Phosphorylation of Xyk upon Egg Activation

To investigate whether the subcellular localization, tyrosine phosphorylation, and kinase activity of Xyk are affected by parthenogenetic egg activation, partially purified Xyk was prepared from both cytosolic and membrane fractions of unfertilized and activated eggs. Parthenogenetic activation was done by either electrical shock, calcium ionophore A23187, or a synthetic RGDS peptide and compared with that done by sperm. In each egg activation treatment, the reactions were stopped when more than 90% of the eggs showed cortical contraction. Accordingly, egg activations by electrical shock, A23187, and RGDS peptide were stopped by washing at 4, 5, and 8 min postactivation treatment, respectively. Fertilization, i.e., sperm-induced egg activation, was stopped by adding an excess volume of cysteine-containing buffer to remove the jelly coat at 10 min postinsemination. The dejelly treatment takes 2–3 min. Each of the activated eggs was collected and fractionated into cytosolic and membrane fractions, and DEAE fractions were prepared.

In Figs. 1A and 1B, the DEAE fractions were immunoprecipitated with anti-pepY antibody and the immunoprecipitates were analyzed by immunoblotting with either anti-pepY antibody (Fig. 1A) or anti-phosphotyrosine antibody (Fig. 1B). In this experiment, it can be not only Xyk but also c-Src, Fyn, and/or c-Yes proteins that are present in the anti-pepY immunoprecipitates since the amino acid sequence of the pepY region is completely conserved among these Src family proteins. However, as we describe later, the DEAE fractions used in this experiment contained only one anti-pepY immunoreactive protein of 57 kDa, Xyk, that had not been recognized by specific antibodies to c-Src, Fyn, and

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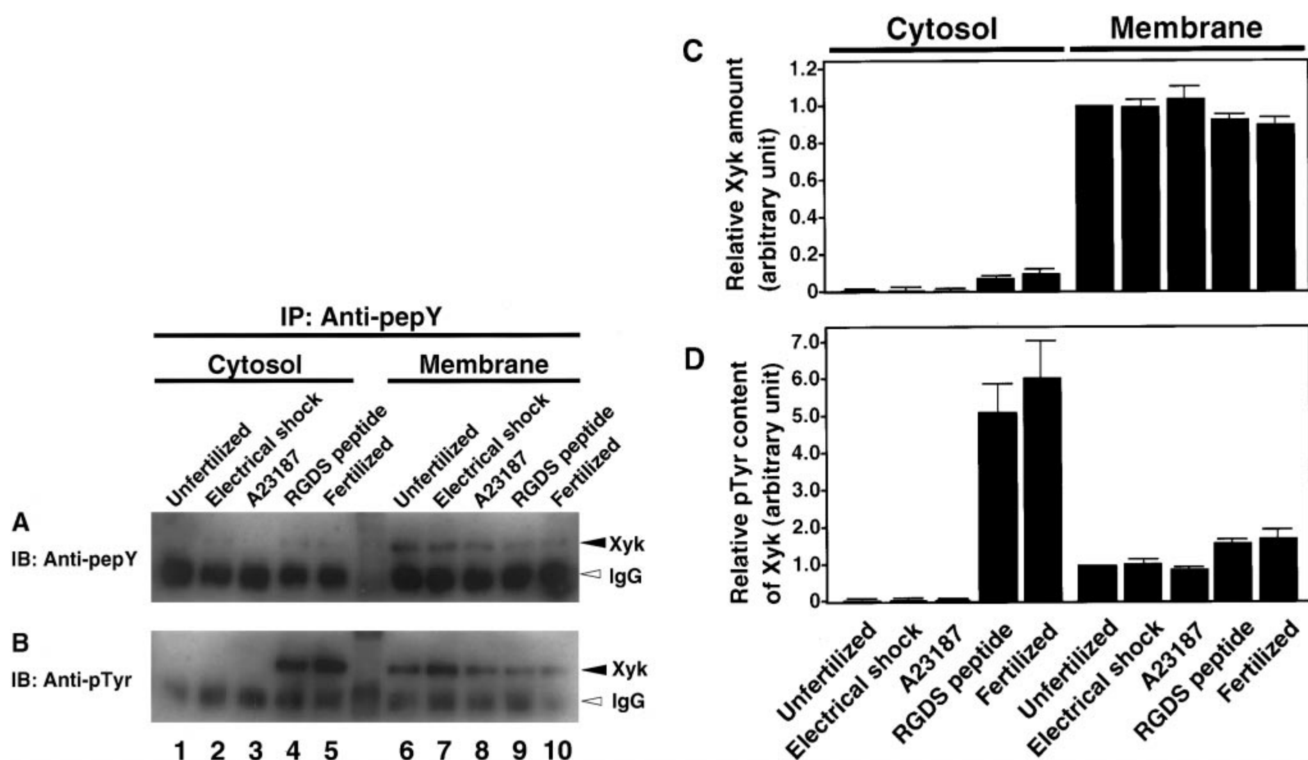

FIG. 1. Translocation and tyrosine phosphorylation of Xyk upon egg activation. Unfertilized eggs and activated eggs by treatment with electrical shock (4 min after activation), A23187 (5 min after activation), RGDS peptide (8 min after activation), or sperm (10 min after insemination) were prepared and fractionated as described under Materials and Methods. DEAE–cellulose fractions were prepared from the cytosolic (lanes 1–5) and membrane fractions (lanes 6–10) and immunoprecipitated with anti-pepY beads as described under Materials and Methods. Three milligrams of proteins from the cytosolic DEAE fractions and 0.5 mg of proteins from the membrane DEAE fractions, which correspond to ca. 2 ml packed volume of eggs, were used for immunoprecipitation. Immunoprecipitates were subjected to immunoblotting with either anti-pepY antibody (A) or anti-phosphotyrosine antibody (B) as described under Materials and Methods. Immune complexes treated with [^{125}I]protein A were visualized by BAS2000 bioimaging analyzer (Fujifilm). The positions of Xyk and immunoglobulin heavy chain are indicated. This result is a representative of three independent experiments. In C and D, the Xyk bands shown in A and B were quantified and relative Xyk amount (C) and tyrosine phosphorylation level (D) were calculated. In each graph, the value obtained with the membrane sample of unfertilized eggs was taken as 1.0. The graphs show means ± SE of three independent experiments.

c-Yes (Sato *et al.,* 1996), indicating that Xyk is the major Src family kinase in the DEAE fractions used in this experiment. It should be noted that a difficulty in detecting the presence of c-Src, Fyn, and c-Yes may be also due to the use of antibodies made to mammalian proteins because Tsai *et al.* (1998) have reported that all three Src family members are present in *Xenopus* eggs. As shown in Fig. 1A, Xyk was localized exclusively in the membrane fraction of unfertilized eggs (Fig. 1A, lanes 1 and 6) and also appeared in the cytosolic fraction upon fertilization (lanes 5 and 10) as we reported previously (Sato *et al.,* 1996). Relative Xyk content in the cytosolic fraction of unfertilized eggs was less than 0.01 (n = 3), taking the content of the membrane Xyk activity in unfertilized eggs as 1.0 (Fig. 1C). Cytosolic Xyk content was shown to elevate several-fold in eggs activated by RGDS peptide and by sperm (Figs. 1A and 1C).

An Xyk-related band was also detected in the cytosolic fraction of electrical shock-treated eggs (Fig. 1A, lane 2). However, we believe this is not significant because (1) the appearance of the band was not reproducible (n = 3) and (2) no signal was detected in the same fraction in both the anti-phosphotyrosine immunoblot (see below, Fig. 1B, lane 2) and the immune complex Cdc2 peptide kinase assay (data not shown). A23187 (lane 3) was also shown to have no effect on subcellular localization and tyrosine phosphorylation of Xyk (Figs. 1A and 1B, lanes 3 and 8). Relative Xyk contents in the cytosolic fraction of RGDS peptide-activated eggs and fertilized eggs were 0.07 ± 0.1 (n = 3) and 0.09 ± 0.3 (n = 3), respectively, taking the content of the membrane Xyk activity in unfertilized eggs as 1.0 (Fig. 1C). Correspondingly, a slight but significant decrease in the membrane Xyk activity is detected in eggs activated by RGDS peptide and by fertilization, the relative values of 0.90 ± 0.05 and 0.86 ± 0.07, respectively (n = 3) (Fig. 1C).

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The results are consistent with the idea that fertilization and RGDS peptide specifically cause partial translocation of Xyk. It is possible that we may miss a crucial change in electrical shock- and A23187-treated eggs by preparing samples too early. However, we confirmed that the longer activation treatment of eggs (10 min) did not alter subcellular localization of Xyk (data not shown).

Anti-phosphotyrosine immunoblotting of the same sample demonstrated that both the cytosolic and membrane Xyk in fertilized eggs are tyrosine phosphorylated (Fig. 1B, lanes 5 and 10). The phosphorylation of the cytosolic Xyk was much higher than that of the membrane Xyk. Relative phosphotyrosine content of the cytosolic Xyk in the fertilized eggs was 6.0 ± 1.1 ($n = 3$), taking that of the membrane Xyk in unfertilized eggs as 1.0 (Fig. 1D). Augmented tyrosine phosphorylation was observed with eggs activated by RGDS peptide (Fig. 1B, lane 4). Relative phosphotyrosine content of the cytosolic Xyk in RGDS peptide-activated eggs was 5.1 ± 0.7 ($n = 3$) (Fig. 1D). These results indicate that sperm and RGDS peptide specifically induce the translocation and augmented tyrosine phosphorylation of Xyk.

It was difficult to detect a dramatic change in tyrosine phosphorylation of the membrane Xyk before and after egg activation. This is because the membrane Xyk in unfertilized eggs is already tyrosine phosphorylated to some extent (Fig. 1B, lane 6). As shown in Fig. 1D, however, it is found that relative tyrosine phosphorylation of the membrane Xyk increases significantly in eggs activated by RGDS peptide (1.45 ± 0.12, $n = 3$) and by fertilization (1.55 ± 0.25, $n = 3$) but not by electrical shock (1.05 ± 0.15) and A23187 (0.91 ± 0.11).

Stimulation of Specific Kinase Activity of Egg Fraction Enriched in Xyk upon Egg Activation

We then analyzed whether the specific kinase activity of Xyk is stimulated by parthenogenetic egg activation. In Fig. 2, the membrane DEAE fractions, which contained Xyk as demonstrated in Fig. 1, were directly subjected to the Cdc2 peptide kinase assay without immunoprecipitation. This method allowed us to determine the specific activity of Xyk in each membrane fraction. It should be noted that a similar assay could not be conducted in the cytosol fractions because of the contamination of a large amount of unrelated serine/threonine kinase activity (data not shown). It is shown that the Cdc2 peptide kinase activity in the eggs activated by RGDS peptide and by sperm showed 2.1 ± 0.5- ($n = 3$) and 2.9 ± 0.2- ($n = 3$) fold stimulation, respectively, over that obtained with the unfertilized eggs. On the other hand, no stimulation of kinase activity was observed with the eggs activated by electrical shock or by A23187. The total amount of Xyk present in each membrane DEAE fraction was normalized as demonstrated in Figs. 1A and 1C. Therefore, the increased kinase activity in the membrane DEAE fractions of RGDS peptide- and sperm-activated eggs is likely due to an increase in the specific activity of Xyk.

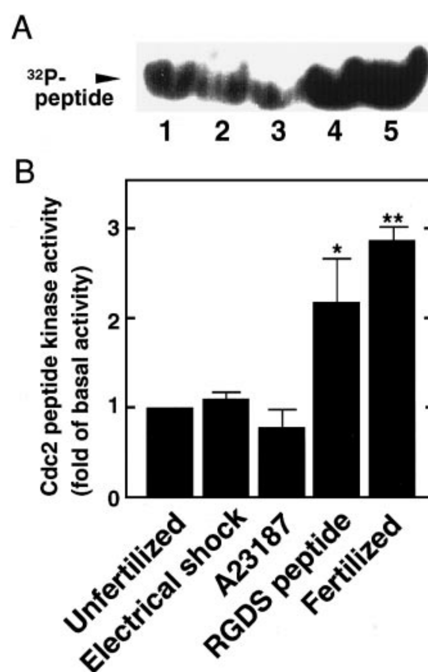

FIG. 2. Elevation of specific kinase activity of egg fraction enriched in Xyk upon egg activation by sperm and RGDS peptide. The membrane DEAE fractions used in Fig. 1 were subjected to Cdc2 peptide kinase assay as described under Materials and Methods. Twenty-five micrograms of proteins, which corresponds to 0.1 ml packed volume of eggs, was used. In A, phosphorylation of Cdc2 peptide (indicated as ^{32}P-peptide) was visualized by the bioimaging analyzer. Lane 1, unfertilized eggs; lane 2, electrical shock-treated eggs; lane 3, A23187-treated eggs; lane 4, RGDS peptide-treated eggs; lane 5, fertilized eggs. In B, phosphorylation was analyzed and presented as percentage of unfertilized eggs. The data represent means ± standard deviations of at least three independent experiments. Bars marked with asterisks are significantly different from the control. $*P < 0.05$, $**P < 0.01$.

Inhibition of Purified Xyk by a Src Family-Specific Tyrosine Kinase Inhibitor Peptide A7

A synthetic 18-amino-acid tyrosine kinase-specific inhibitor peptide was used to examine the role of the tyrosine kinase Xyk in *Xenopus* egg activation. The peptide used was peptide A7 that corresponds to residues 140 to 157 of the chicken c-Src and has been shown to inhibit the kinase activity of v-Src with a half-inhibition concentration of 3 μM (Fukami *et al.,* 1993). The peptide has been shown to inhibit Src activity through its binding to the Src autophosphorylation site, a well-conserved and specific region of the Src family of protein tyrosine kinases. Therefore, we believe that the peptide can be used as an inhibitor specific to the Src family kinases, although it can inhibit other types of

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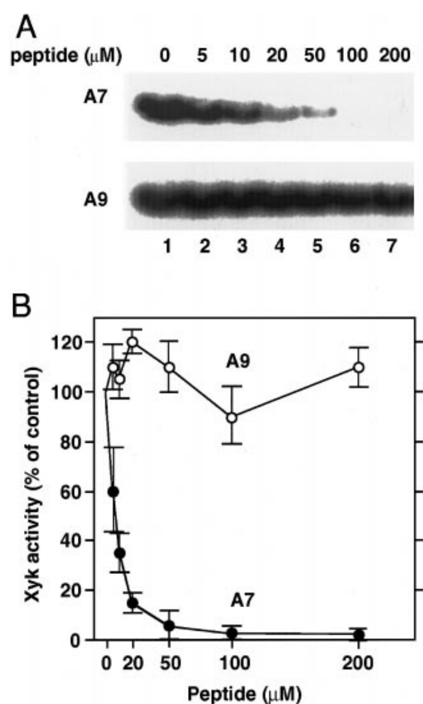

FIG. 3. Inhibition of tyrosine kinase activity of Xyk by peptide A7. Purified Xyk (200 ng protein) was subjected to Cdc2 peptide kinase assay in the absence or presence of various amounts of either peptide A7 or A9 as described under Materials and Methods. In A, phosphorylation of Cdc2 peptide was visualized as in Fig. 2A. In B, phosphorylation was analyzed and presented as percentage of control without peptide. The data represent means ± standard deviations of three independent experiments. ●, peptide A7; ○, peptide A9.

tyrosine kinases such as epidermal growth factor receptor/kinase at much higher concentrations (Sato *et al.,* 1990). We first examined the *in vitro* effect of peptide A7 on the kinase activity of an Xyk preparation which had been purified to near homogeneity by successive chromatographies (Sato *et al.,* 1996). As a control, we employed another synthetic peptide with no inhibitory effect, named peptide A9, which is a 15-amino-acid-long peptide with deletion of the 3 amino acid residues from the carboxyl-terminus of peptide A7. As shown in Fig. 3, phosphorylation of an exogenous substrate Cdc2 peptide by purified Xyk was effectively inhibited by peptide A7 in a dose-dependent manner, but not by peptide A9. The half-inhibition concentration was 8.0 ± 1.8 μM ($n = 3$).

Inhibition of Sperm- and RGDS Peptide-Induced Egg Activation by Peptide A7

To inhibit Xyk during egg activation, we injected the inhibitor peptide A7 into jelly-intact *Xenopus* unfertilized eggs and examined its effect on the subsequent sperm-induced or parthenogenetic egg activation. In eggs injected with peptide A7 at a concentration of 50 μM to 1 mM, but not 5 μM, sperm-induced egg cortical contraction was inhibited in more than 80% of the eggs challenged (Table 1). We confirmed by cytological sections of eggs injected with 1 mM peptide A7 and fixed 40 min after insemination that penetration of sperm nuclei into the egg cytoplasm and meiotic resumption of egg nuclei were inhibited (Figs. 4A and 4B). We have done this experiment with 10-μm-thick sections of the entire egg of more than five different batches. However, with this resolution, we could not determine whether or not sperm–egg fusion occurred in eggs injected with peptide A7. The control peptide, peptide A9, did not show such an effect at 1 mM (Table 1) and allowed monospermic penetration and the formation of a sperm pronucleus with a growing sperm aster by 40 min after insemination (Fig. 4C).

The total volume of the egg cytoplasmic components has been calculated to be 935 ± 17 nl including 495 ± 29 nl of pigment and yolk layers (Iwao *et al.,* 1997); thus, the injection of 40 to 50 nl of peptide will result in about 10-fold dilution of the peptide in the egg. Therefore, the result indicates that peptide A7 at a final concentration of more than 5 μM was effective in inhibiting sperm-induced egg activation. Injection of peptide A7 at 1 mM also exerted an inhibitory effect on egg activation by RGDS peptide. On the other hand, egg activation induced by A23187 was not affected by injection of the same amount of peptide A7 (Table 1). Neither 1 mM peptide A9 nor buffer alone showed an inhibitory effect toward parthenogenetic egg activation (Table 1). It should be noted that injection of the peptide at any concentration did not cause egg activation by itself. These results strongly indicate that egg activation by sperm and RGDS peptide but not by A23187 involves the activity of protein tyrosine kinase sensitive to peptide A7.

TABLE 1
Inhibition of *Xenopus* Egg Activation by a Src Family-Specific Tyrosine Kinase Inhibitor Peptide

		Activated eggs/eggs treated with		
Peptide	mM	Sperm (%)	A23187 (%)	RGDS peptide (%)
A7	1.0	3/26 (11.5)	10/10 (100.0)	2/13 (15.4)
	0.5	2/11 (18.2)	ND	ND
	0.05	2/10 (20.0)	ND	ND
	0.005	8/11 (72.7)	ND	ND
A9	1.0	9/10 (90.0)	4/4 (100.0)	4/5 (80.0)
None	—	27/32 (84.4)	11/11 (100.0)	11/11 (100.0)

Note. Unfertilized eggs were microinjected with 40–50 nl of buffer (none) or synthetic peptides of the indicated concentrations and then inseminated or treated with either A23187 or RGDS peptide as described under Materials and Methods. Activation was scored by the occurrence of cortical contraction by 30 min after activation treatment. Each data point was obtained with eggs from two to six animals. ND, not done.

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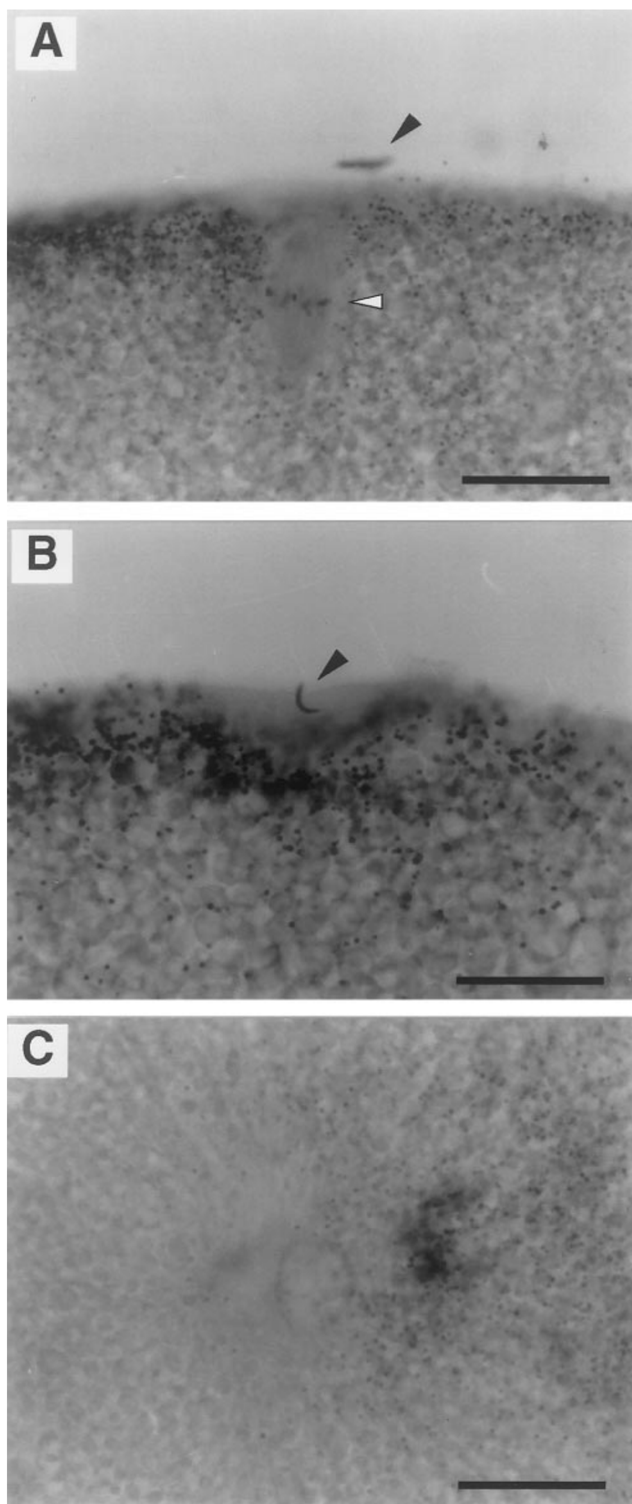

FIG. 4. Inhibition of sperm penetration and resumption of meiosis II by peptide A7. Shown are light microscope images of sections of *Xenopus* eggs. Unfertilized eggs were microinjected with 1 mM peptide A7 or A9 and then inseminated as described in the note to Table 1. Forty minutes after insemination, eggs were fixed, embedded, sectioned into 10-μm thickness, and stained to observe nuclei as described under Materials and Methods. (A and B) Sperm nucleus near the surface of two different eggs microinjected with 1 mM peptide A7, showing inhibition of penetration of sperm into the egg cytoplasm. Meiosis II metaphase-arrested egg chromosomes are also shown in A. The positions of the sperm nucleus and egg chromosomes are indicated by closed and open arrowheads, respectively. (C) A sperm pronucleus with a developing aster in an egg microinjected with 1 mM peptide A9. The results shown are representatives of more than three independent experiments. Bars are 20 μm.

Localization of Xyk in the Cortex of Xenopus Eggs

To determine subcellular localization of Xyk in *Xenopus* eggs, we performed immunofluorescence confocal microscopy. Dejellied unfertilized eggs were fixed, sectioned, and treated with anti-pepY antiserum or the preimmune serum followed by further treatment with the horseradish peroxidase-conjugated secondary antibody and FITC-conjugated tyramide as described under Materials and Methods. In Figs. 5A to 5D, it is demonstrated that the anti-pepY antiserum-specific signals are highly concentrated in the cortex. Cytoplasmic signals obtained with anti-pepY antiserum were very weak but more intense than those with the preimmune serum (Figs. 5B and 5D). The anti-pepY-specific signals on the cortex were distributed over the animal hemisphere (Figs. 5E and 5F), the junction of the animal and vegetal hemispheres (Figs. 5G and 5H), and the vegetal hemisphere (Figs. 5I and 5J) with similar intensity. Direct immunoblotting of the DEAE fractions of unfertilized eggs used in Figs. 1 and 2 demonstrated that only p57 Xyk is specifically recognized by anti-pepY antibody (Fig. 5K, lanes 2 and 4). No clear band is seen in the total egg extract (lanes 1 and 3), indicating that abundance of p57 Xyk is quite low. These results indicate that anti-pepY antibody-immunoreactive protein Xyk localizes to the egg cortex in close proximity to where the sperm–egg or RGDS peptide–egg interaction takes place.

DISCUSSION

Our present findings demonstrate that Xyk is activated and tyrosine phosphorylated and undergoes translocation upon *Xenopus* egg activation induced by either RGDS peptide or sperm and that neither the calcium ionophore A23187- nor electrical shock-induced egg activation can mimic these events (Figs. 1 and 2). There are at least two possibilities as to the mechanism by which the egg cortex tyrosine kinase Xyk is activated at sperm-induced egg activation. The first one is the introduction of kinase itself and/or kinase-regulating factor(s) from sperm into egg. Sperm-derived factors, which can be introduced into the egg upon sperm–egg fusion, have been suggested to account for egg activation (Dale *et al.,* 1985; Stricker *et al.,* 1997; Swann, 1990). In hamster eggs, the

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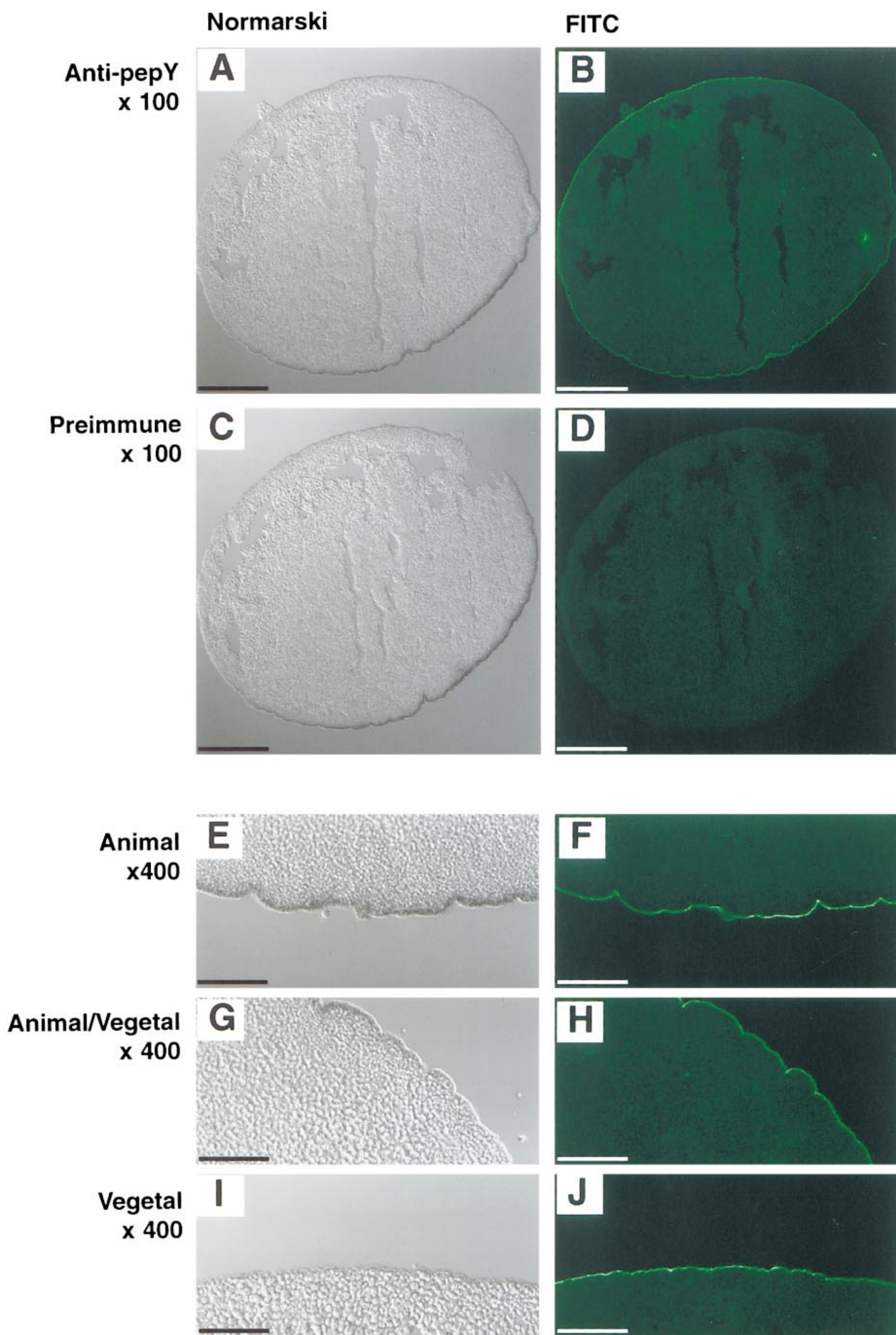
Normarski
FITC
Anti-pepY
x 100
A
B
Preimmune
x 100
C
D
Animal
x400
E
F
Animal/Vegetal
x 400
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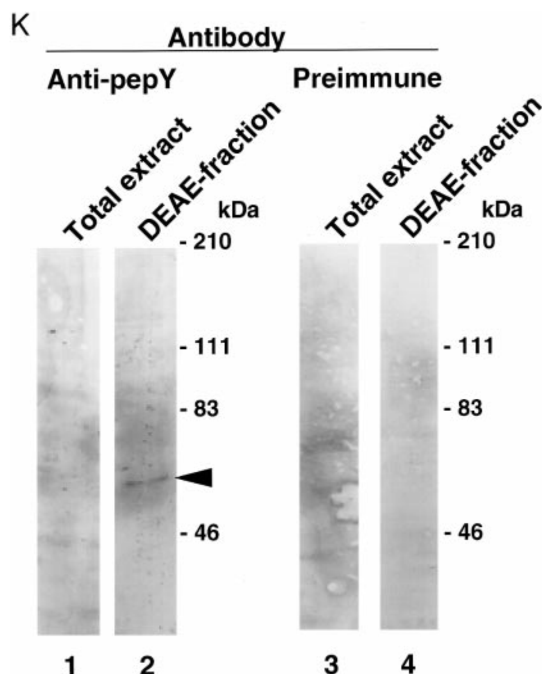

FIG. 5. Localization of Xyk in egg cortex. Indirect immunofluorescence staining of 10-μm sections of *Xenopus* unfertilized eggs was performed as described under Materials and Methods and analyzed by a confocal laser-scanning microscope (Zeiss, LSM410). (A, C, E, G, and I) Views are taken at 100× magnification in A and C (serial sections of an egg) or at 400× magnification in E, G, and I (the same egg shown in A) under Nomarski interference. (B, D, F, H, and J) The same samples are observed for immunostained images with either anti-pepY antiserum in B, F, H, and J or the preimmune serum in D. Note that the anti-pepY antiserum-specific signals are localized in the cortex of both animal and vegetal hemispheres. Bars in A–D are 250 μm. Bars in E–J are 62.5 μm. In K, total unfertilized egg extract (30 μg protein, lanes 1 and 3) and the DEAE fraction (30 μg protein, lanes 2 and 4) used in Figs. 1 and 2 were subjected to immunoblotting with anti-pepY antibody (lanes 1 and 2) or the preimmune antibody (lanes 3 and 4). The position of Xyk is indicated by an arrowhead. Prestained molecular size markers (Bio-Rad) were myosin (210 kDa), β-galactosidase (111 kDa), bovine serum albumin (83 kDa), and ovalbumin (46 kDa).

sperm-derived protein oscillin has been shown to cause intracellular calcium release when microinjected (Parrington *et al.,* 1996), although Wolosker *et al.* (1998) have reported that oscillin is the mammalian form of glucosamine-6-phosphate deaminase and that purified recombinant glucosamine-6-phosphate deaminase fails to elevate intracellular calcium when injected into mouse eggs. Another candidate for the sperm-derived factor has been proposed by Sette *et al.* (1997) who have shown that in mouse eggs, a fragment of the tyrosine kinase c-kit that does not contain the catalytic domain causes egg activation when microinjected. Thus, it is possible that introduction of a cytosolic kinase related to Xyk and/or a kinase-activating factor from the sperm into the egg cytoplasm contributes to the sperm-induced activation of Xyk, although we present evidence showing that Xyk is an egg cortex protein (Fig. 5). It is very difficult to rule out this possibility; however, we believe it is unlikely because (1) we could not detect Xyk-related protein in extracts prepared from *Xenopus* sperm (our unpublished results) and (2) as presented in this study, Xyk is activated by treatment of eggs with RGDS peptide alone that has been suggested to act through its binding to egg plasma membrane (Iwao and Fujimura, 1996). More recently, Shilling *et al.* (1998) reported that synthetic peptides derived from *Xenopus* sperm surface disintegrin cause the voltage-dependent activation of *Xenopus* eggs. It is of course uncertain that such peptide-induced egg activation is relevant to physiological egg activation. Thus, it should be examined whether introduction of *Xenopus* sperm-derived factors can cause activation of both Xyk and eggs.

Alternatively, an egg plasma membrane-associated receptor(s) for sperm may initiate the sperm-dependent intracellular signaling events. It is possible that a sperm receptor molecule(s) itself is a tyrosine kinase which is initially activated by a sperm ligand and, in turn, activates the downstream cytoplasmic tyrosine kinase like Xyk as in the case of receptor/kinase-Src-mediated pathways in mammalian cells (Thomas and Brugge, 1997). It is more likely that direct or indirect noncatalytic interaction between the sperm receptor molecule(s) and Xyk causes the activation of Xyk as in the case of nonkinase receptors that include T-cell/B-cell receptors, seven transmembrane G-protein-coupled receptors, and integrins (Thomas and Brugge, 1997). In *Xenopus,* however, plasma membrane-associated receptor molecules for sperm have not yet been identified.

The fact that Xyk can be activated upon RGDS peptide-induced egg activation implies the involvement of an integrin or integrin-related molecule in physiological Xyk activation and egg activation. In mouse eggs, integrin $\alpha6/\beta1$ has been found to function as the sperm receptor (Almeida *et al.,* 1995; Snell and White, 1996). However, it should be noted that integrin $\alpha6/\beta1$ is not a receptor for RGDS peptide and that it is not clear whether integrin $\alpha6/\beta1$ is involved in the egg activation process. As to the sperm ligand, Evans *et al.* (1997a,b) and Yuan *et al.* (1997) demonstrated that both sperm protein fertilin α and β subunits and cyritestin have an ability to bind to egg surface integrin molecules. More recently, Cho *et al.* (1998) have reported the fertilization defects in sperm from mice lacking one of the fertilin subunits. In that study, the sperm lacking fertilin β showed a significantly reduced rate of successful sperm–egg membrane binding and sperm–egg fusion when compared to normal sperm. However, it has also been shown that eggs fused with sperm lacking fertilin β undergo normal egg activation (Cho *et al.,* 1998), suggesting that fertilin β is not required for egg activation. In mammalian cell systems, it is well known that integrin-dependent intracellular signaling involves the activation and altered subcellular distribution of c-Src and other Src family tyrosine kinases (Clark and Brugge, 1995; Schwartz *et al.,* 1995; Thomas and Brugge, 1997). As for *Xenopus* integrin, expression of mRNAs

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and/or protein products for various integrin genes and possible roles in early development have already been well documented (Alfandari *et al.,* 1995; Gawantka *et al.,* 1992; Ransom *et al.,* 1993; Whittaker and DeSimone, 1993). Moreover, Shilling *et al.* (1997, 1998) have demonstrated that metalloprotease/disintegrins are present in *Xenopus* testis and a part of the disintegrin molecules is capable of activating *Xenopus* eggs. Thus, it is possible that such identified or unidentified integrin and/or integrin-related molecules may function in the *Xenopus* egg activation system. Therefore, it is important to identify a molecule which is responsible for the binding to sperm and RGDS peptide. It is also important to identify the upstream (kinase-regulating protein) and downstream components (tyrosine phosphorylation substrate) of Xyk.

Since microinjection of a synthetic tyrosine kinase-specific inhibitor peptide, which can inhibit Xyk activity *in vitro* (Fig. 3), abolishes the sperm- and RGDS peptide-induced but not A23187-induced egg activation (Table 1), it appears that Xyk is involved in the sperm ligand– or RGDS peptide–egg surface receptor interaction-mediated egg activation. It is possible, however, that the peptide also affected other Src family kinases which might be present in eggs. In our previous study (Fukami *et al.,* 1993), a region containing the autophosphorylation site of c-Src, pepY region, has been identified as the target site of the tyrosine kinase inhibitor peptide used in this study. Amino acid sequence of the pepY region is completely conserved among three *Xenopus* Src family members: c-Src, Fyn, and c-Yes. Thus, it can be c-Src, Fyn, and/or c-Yes proteins that are inhibited by microinjection of the inhibitor peptide and it is very important to clarify whether eggs contain multiple Src family kinases. In this connection, Tsai *et al.* (1998) have reported that these Src family members are present in *Xenopus* eggs and become tyrosine phosphorylated upon sperm-induced egg activation. However, we have shown that p57 Xyk is the major anti-pepY immunoreactive protein in egg extract used in this study (Fig. 5K) and that antibodies specific to c-Src, Fyn, or c-Yes do not recognize p57 Xyk (Sato *et al.,* 1996). In addition, we have detected c-Src, Fyn, and c-Yes proteins at later developmental stages of embryos or organs (Sato *et al.,* unpublished results), indicating that these Src family proteins are less abundant than Xyk in eggs. Thus, we believe that it may be Xyk that is mainly affected by the inhibitor peptide in our present study. The result that the inhibitor peptide blocked sperm penetration (Fig. 4) suggests a requirement of tyrosine kinase activity at a very early stage of egg activation, e.g., post sperm–egg adhesion/binding events like intracellular calcium release and endocytosis of sperm into the egg. This is consistent with our previous study showing that eggs pretreated with tyrosine kinase inhibitors genistein and herbimycin A are not activated by insemination (Sato *et al.,* 1998). Entry of sperm into the egg fertilization body/cone structure has been shown to require reorganization of polymerized actin (Chow and Elinson, 1993), suggesting a role of tyrosine kinase activity in this process. Recently, Carroll *et al.* (1997) have reported that starfish eggs injected with a fragment of phospholipase Cγ (PLCγ) containing Src homology 2 domains do not undergo sperm-induced activation. In that study, when insemination was carried out with high concentrations of sperm, the injected eggs showed delayed intracellular calcium release, no fertilization envelope elevation, and multiple incorporations of sperm nuclei, indicating the loss of the slow block to polyspermy (Carroll *et al.,* 1997). The authors suggested that the fragment of PLCγ may act as an inhibitor of PLCγ-mediated inositol trisphosphate production which might be under the control of an upstream tyrosine kinase (Carroll *et al.,* 1997). Polyspermic fertilization also has been observed with sea urchin eggs treated with genistein (Moore and Kinsey, 1995).

Together with the observations discussed above, the question arises as to whether a tyrosine kinase is the upstream component of intracellular calcium release at egg activation. In this connection, it has been demonstrated that reconstituted epidermal growth factor receptor/kinase can cause *Xenopus* egg activation accompanied by intracellular calcium release in an epidermal growth factor-dependent manner (Yim *et al.,* 1994). On the other hand, it seems that a tyrosine kinase may not be the sole upstream component of intracellular calcium release at egg activation because Kline *et al.* (1991) have shown that injection of the nonhydrolyzable GTP analogue, GTPγS, alone can cause *Xenopus* egg activation, indicating a possible involvement of a G-protein in this process. Involvement of heterotrimeric and/or monomeric G-proteins in physiological egg activation has also been documented in mouse (Moore *et al.,* 1994) and starfish eggs (Shilling *et al.,* 1994). Furthermore, the activity of multiple phosphatidylinositol lipid kinases is regulated to control the transient increase of intracellular inositol trisphosphate in *Xenopus* eggs (Snow *et al.,* 1997). Therefore, there might be a complex signaling network involving at least tyrosine kinases, lipid kinases, and G-proteins, although their molecular identity remains to be determined, to transmit an egg activation signal evoked from egg surface to the calcium-dependent pathway. Further study will be directed to establish this point.

In the present study, an indirect immunofluorescence method was used to demonstrate that Xyk is localized mainly to the cortex of *Xenopus* eggs (Fig. 5). Similar subcellular distribution of a sea urchin egg tyrosine kinase has been described for a 57-kDa tyrosine kinase (Peaucellier *et al.,* 1993) and a 220-kDa Abl-related kinase (Moore and Kinsey, 1994), although their role in egg activation remains to be determined. More recently, Talmor *et al.* (1998) demonstrated that in rat eggs Fyn, a Src family protein tyrosine kinase, is localized in the cortex of unfertilized eggs and becomes enriched in the polar body and fertilizing cone structure after sperm-induced egg activation. The group has also demonstrated that tyrosine phosphorylation and dephosphorylation in a number of egg proteins are regulated during egg activation (Ben-Yosef *et al.,* 1998). Our present biochemical studies demonstrate that a fraction of the Xyk protein undergoes cytoplasmic translocation upon

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egg activation. Thus, it is of interest to determine whether such an event is confirmed by the indirect immunofluorescence method. In addition, it is important to analyze the localization of tyrosine-phosphorylated proteins including Xyk before and after egg activation, especially at the point where the sperm–egg interaction takes place.

In summary, our present study investigates the molecular mechanism by which the *Xenopus* egg is activated physiologically or parthenogenetically. The identification of Src-related tyrosine kinase Xyk as a possible important component of egg activation is presented and discussed. Present data are consistent with the idea that tyrosine kinase Xyk plays an important role in the early events of egg activation in a manner independent or upstream of calcium signaling. More detailed study with biochemical and immunocytochemical approaches will contribute to better understanding about the spatial and temporal sequence of biological events of egg activation not only in *Xenopus* but also in other organisms.

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