Involvement of protein-tyrosine phosphorylation and dephosphorylation in sperm-induced *Xenopus* egg activation

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Received 5 January 1998; revised version received 29 January 1998

Abstract We have analyzed tyrosine-phosphorylated proteins in *Xenopus laevis* eggs before and after fertilization by immunoblotting with anti-phosphotyrosine antibody. A number of egg proteins with different subcellular distribution became tyrosine-phosphorylated or dephosphorylated within 30 min after insemination. Tyrosine kinase-specific inhibitors genistein and herbimycin A were found to inhibit sperm-induced egg activation judged by the egg cortical contraction. Surprisingly, sodium orthovanadate, a tyrosine phosphatase inhibitor, also inhibited the egg activation. Moreover, we found that fertilization-dependent tyrosine dephosphorylation of 42-kDa mitogen-activated protein kinase was inhibited in genistein-treated eggs. These results suggest that both protein-tyrosine phosphorylation and dephosphorylation pathways play an important role in the sperm-induced *Xenopus* egg activation.

Key words: Protein tyrosine phosphorylation/dephosphorylation; *Xenopus laevis* fertilization; Egg activation; Genistein

1. Introduction

Protein phosphorylation/dephosphorylation plays an important role in the regulation of many biological processes such as cell growth, division, differentiation, and cell–cell communication [1]. The proportion between phosphorylated and dephosphorylated forms of proteins in the cell is strictly regulated through 'on/off'-switching of the catalytic activity of specific protein kinases by extracellular signals such as hormones, neurotransmitters, growth factors, and extracellular matrix molecules. By now, however, little is known about the role of protein phosphorylation and dephosphorylation in fertilization, a process leading to egg activation and early embryonic development. An initial step at fertilization in both invertebrates and vertebrates is the binding of sperm to egg in a species-specific manner [2–5]. Upon sperm–egg binding, several biochemical and cell biological events such as membrane depolarization, intracellular Ca^{2+} release, exocytosis of cortical granule, and formation of fertilization envelope are triggered in eggs and the process is generally called 'egg activation'. The egg activation is believed to be indispensable for blocking polyspermy in many organisms and subsequent morphological development. However, molecular events connecting the sperm–egg binding with egg activation is poorly understood.

Recently, we have purified a p57 protein-tyrosine kinase from the crude particulate fraction of oocytes of African clawed frog, *Xenopus laevis*, and found that the enzyme remains inactive during the oocyte maturation but is activated within 1 min following fertilization [6]. Concomitant translocation of a part of the activated enzyme from the particulate fraction to the cytosol fraction was also detected [6]. The p57 *Xenopus* tyrosine kinase (hereafter, Xyk) is the only biochemically and immunochemically characterized member of the Src family proteins in *Xenopus*. The Src family of protein-tyrosine kinases is a kind of plasma membrane-associated non-receptor kinases which function in conjunction with the transmembrane receptor kinases or with other non-kinase receptor/adhesion molecules such as T-cell/B-cell receptor and integrins [7,8]. Thus, we suggested that, in *Xenopus*, Xyk may be involved in fertilization-dependent intracellular signal transduction pathways through the physical and functional interaction with egg surface sperm receptor molecule, which has not yet been identified in *Xenopus*, and that protein tyrosine phosphorylation may play a regulatory role in the fertilization.

In this report, we present the results of biochemical and cell biological studies exploring a possible involvement of protein tyrosine phosphorylation in *Xenopus* fertilization. Results obtained indicate that a number of egg proteins with different subcellular localization were tyrosine phosphorylated or dephosphorylated in a fertilization-dependent manner. We also examined the effect of a tyrosine kinase-specific inhibitor, genistein [9], on the activity of Xyk and on fertilization-dependent cellular processes, such as egg activation and early development. The results obtained suggest that protein tyrosine phosphorylation plays an important role in the early stage of the fertilization process in *Xenopus*.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP (35020) and [^{125}I]protein A (68038) were obtained from ICN. Leupeptin was purchased from Peptide Institute (Osaka). (*p*-Amidinophenyl)methanesulfonyl fluoride hydrochloride was from Wako Pure Chemicals (Osaka). Genistein and daidzein were purchased from Biomol Research Laboratories. Herbimycin A was from Calbiochem. H-89 was from Seikagaku Kogyo (Tokyo). Sodium orthovanadate (Na_3VO_4) and okadaic acid were from Wako. All the compounds but not Na_3VO_4 were dissolved in dimethylsulfoxide (DMSO). Other reagents were from Wako or Nacalai (Kyoto). A synthetic peptide, termed Cdc2 peptide, which corresponds to residues 7–26 (Val-Glu-Lys-Ile-Gly-Glu-Gly-Thr-Tyr-Gly-Val-Val-Tyr-Lys-

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Abbreviations: Xyk, *Xenopus* tyrosine kinase; Na_3VO_4, sodium orthovanadate; DMSO, dimethylsulfoxide; DB, DeBoer's solution; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; MAP kinase, mitogen-activated protein kinase

PII S0014-5793(98)00123-9

Ala-Arg-His-Lys-Leu-Ser) of the fission yeast *cdc2* gene product was synthesized and purified as described [10].

2.2. Oocytes, unfertilized eggs, and fertilized eggs

Adult frogs, *Xenopus laevis*, were purchased from dealers and maintained in deionized water at 21°C. Preparation of oocytes, unfertilized eggs, and fertilized eggs was done according to the described methods with some minor modifications [6]. Briefly, oocytes were surgically removed from females and defolliculated by incubation in a modified Barth's solution [6] containing 0.5 mg/ml collagenase (Wako) for 2 h. The defolliculated oocytes were washed extensively in the modified Barth's solution. Full grown oocytes (stage VI) were sorted by size under a dissecting microscope and used within 2 h or frozen in liquid nitrogen and stored at −80°C until use. Ovulation was induced by administration of 1000 units of human chorionic gonadotropin (Isei, Yamanashi, Japan). The ovulated eggs were immediately washed with 1×DeBoer's solution (DB) (110 mM NaCl, 1.3 mM KCl, 0.44 mM $CaCl_2$, pH 7.2) and used within 2 h. Fertilized eggs were obtained by adding sperm suspension to monolayers of unfertilized eggs in plastic dishes followed by the addition of excess 0.05×DB. Sperm at a final concentration of at least 5×10^6/ml were used to fertilize 250–500 eggs per dish. Insemination was stopped at a specified time by washing eggs with ice-cold 1×DB. Eggs were dejellied in 2% cysteine-NaOH (pH 8.0), and kept frozen until use. Under these conditions, 0, 55, 80, 95, 95, and 95% eggs underwent cortical contraction/rotation after 0, 1, 5, 10, and 30 min of insemination, respectively.

2.3. Pretreatment of eggs with inhibitors

Unfertilized eggs were preincubated in 1×DB containing specified concentrations of each inhibitor for 1 h, washed with 1×DB for 3 min, and inseminated in the inhibitor-free medium. Concentrated stock solutions of each inhibitor were prepared and diluted 100-fold with DB so that the final DMSO concentration is to be 1% in DB. Thus, control eggs were pretreated with 1% DMSO and inseminated.

2.4. Subcellular fractionation of oocytes/eggs

Subcellular fractionation was done according to the procedure described [6] with some modifications. All procedures were carried out at 0–4°C. Defolliculated oocytes and dejellied eggs were mixed with 10-fold volume 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 (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride) and homogenized in a Teflon-glass homogenizer (10 strokes). The homogenate was centrifuged at $1000\times g$ for 10 min. The supernatant was decanted and centrifuged at $10\,000\times g$ for 20 min. The resulting supernatant was further centrifuged at $150\,000\times g$ 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 $150\,000\times g$ for 30 min. The pellet fraction obtained was solubilized with buffer A containing 1% Triton X-100 with the aid of 5-min sonication. The resultant solubilized material was used as the particulate fraction containing microsomal proteins. The $10\,000\times g$ pellet (plasma membrane-enriched fraction) was suspended in buffer A containing Triton X-100 and sonicated for 5 min. The solubilized material was recovered by centrifugation at $10\,000\times g$ for 15 min and used as the plasma membrane fraction. To all preparations, glycerol was added to a final concentration of 10% and kept frozen until use. By this procedure, 1-ml packed volume of oocytes/eggs (ca. 750 oocytes/eggs) yielded proteins of 12.5 mg of cytosol, 2.0 mg of particulate, and 2.5 mg of plasma membrane. Protein was determined spectrophotometrically by Bio-Rad protein assay mixture (Bio-Rad) with bovine serum albumin (BSA) as a standard.

2.5. Antibodies and immunoblotting

Mouse monoclonal anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology. For immunoblotting proteins of egg subcellular fractions were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [11] on 3–18% linear gradient polyacrylamide gels and transferred to polyvinylidene difluoride membranes using a semi-dry blotting apparatus (Bio-Rad). Membranes were blocked with T-TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20) containing 3 mg/ml BSA and incubated for 2 h at 37°C with 10 μg/ml 4G10 in T-TBS containing 3 mg/ml BSA. Membranes were then incubated with rabbit anti-mouse IgG (Cappel). To detect immunoreactive proteins, antibody-treated membranes were incubated with T-TBS containing [^{125}I]protein A (50 kBq/ml), washed extensively with T-TBS, and analyzed by BAS2000 Bioimaging Analyzer (Fuji film, Tokyo).

2.6. Purified enzyme and kinase assay

Purification of p57 *Xenopus* tyrosine kinase from oocytes is described previously [6]. Specific activity of the purified enzyme toward an exogenous substrate, Cdc2 peptide, in the standard assay conditions described below was 1.4 nmol/min/mg and 6.5 nmol/min/mg in the absence and presence of kinase-activating anti-pepY IgG [10], respectively. Rabbit anti-pepY antibody was raised against a synthetic peptide, termed pepY, which corresponds to residues 410–428 of the chicken c-Src [12]. Protein kinase assay was carried out for 20 min at 30°C. The standard assay mixture (25 μl) contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 μM [γ-^{32}P]ATP (3.7 kBq/pmol), 1 mM dithiothreitol, 1 mM Cdc2 peptide, and 5 μl of the enzyme fraction (200 ng protein) which had been preincubated with or without anti-pepY IgG for 1 h at 4°C. The final concentration of anti-pepY antibody in the assay mixture was 40 μg/ml. Phosphorylation reaction was started by the addition of the enzyme fraction and terminated by the addition of SDS-sample buffer [11] and boiling for 3 min. The samples were subjected to SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue. The radioactivity in the gel was visualized and quantified by the Bioimaging Analyzer.

3. Results

Xenopus immature oocytes, unfertilized eggs, and fertilized eggs were fractionated into cytosol, particulate, and plasma membrane fractions, and phosphotyrosine-containing proteins were analyzed by immunoblotting with anti-phosphotyrosine antibody, 4G10. As shown in Fig. 1, a number of tyrosine-phosphorylated proteins were detected. The detected bands were phosphotyrosine-specific, because addition of 10 mM phosphotyrosine in the immunoblotting reaction resulted in a competitive disappearance of the signals (data not shown). In addition, oocyte maturation or fertilization caused no dramatic alterations of protein distribution or content (data not shown). A 42-kDa protein (p42) was found to be a major phosphotyrosine-containing protein in the cytosolic fraction (lanes 2–6) of unfertilized eggs. It is clearly shown that fertilization triggered a dramatic decrease in the intensity of p42 band during the time periods analyzed. The intensity of p42 band started to decrease at 5 min after insemination and disappeared almost completely by 30 min. The p42 band is supposed to be the tyrosine-phosphorylated form of mitogen-activated protein kinase (MAP kinase) [13,14], since anti-MAP kinase antibody stained the p42 protein (data not shown). The phosphorylation of p42 was not detected in oocytes (Fig. 1, lane 1), which is consistent with the fact that p42 MAP kinase is not tyrosine-phosphorylated and inactive in immature oocytes [13]. Other proteins in the cytosol, for example a 58-kDa band, were also observed to be phosphorylated upon fertilization (Fig. 1, lanes 1–6). In the particulate fraction (lanes 7–12), oocyte maturation caused a dramatic increase in immunoreactivity of some proteins. In the course of fertilization, these bands remained stable and no other proteins appeared to be tyrosine-phosphorylated. On the other hand, some plasma membrane-associated proteins were found to be tyrosine-phosphorylated in a fertilization-dependent manner (lanes 13–18). In particular, the 57-kDa band, which is supposed to be Xyk, was prominent. These results indicate that at an early time period of fertilization, a number of egg proteins in different subcellular localization are subjected to tyrosine phosphorylation or dephosphorylation.

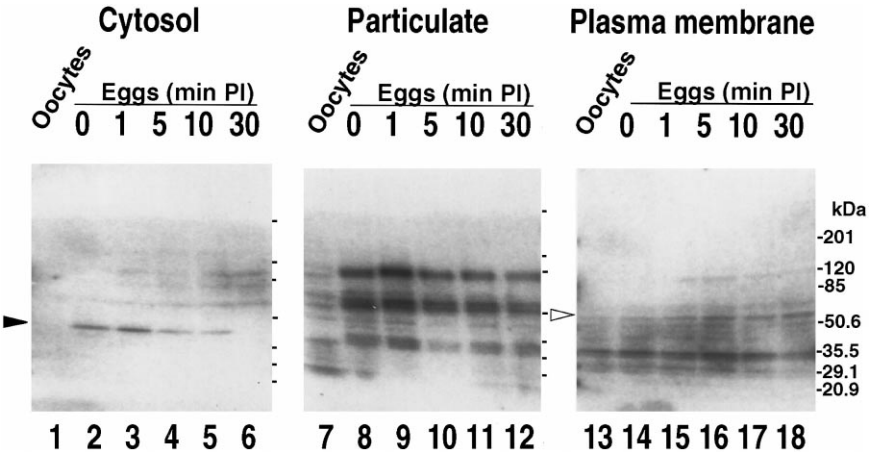

Fig. 1. Tyrosine-phosphorylated proteins in *Xenopus* oocytes/eggs subcellular fractions before and after fertilization. Cytosol (lanes 1–6), particulate (lanes 7–12), and plasma membrane (lanes 13–18) fractions were prepared from *Xenopus* oocytes (lanes 1, 7, and 13) and eggs at various times after insemination (PI, post insemination) as described in Section 2.4. Protein samples were subjected to SDS-PAGE on linear gradient polyacrylamide gels and analyzed by anti-phosphotyrosine immunoblotting. Each lane contains 20–35 μg protein, which corresponds to 0.03 ml of oocytes/eggs in case of cytosol and 0.1 ml of oocytes/eggs in case of other fractions. The positions of p42 MAP kinase in the cytosol fraction and p57 Xyk in the plasma membrane fraction are indicated by closed and opened arrowheads, respectively. Result is a representative of three independent experiments. Prestained molecular size markers (Bio-Rad) were myosin (201 kDa), β-galactosidase (120 kDa), bovine serum albumin (85 kDa), ovalbumin (50.6 kDa), carbonic anhydrase (35.5 kDa), soybean trypsin inhibitor (29.1 kDa), and lysozyme (20.9 kDa).

Genistein, an isoflavone tyrosine kinase-specific inhibitor [9], was employed to examine the role of protein-tyrosine phosphorylation in fertilization. We first examined the in vitro effect of genistein on the kinase activity of purified Xyk. Xyk is partially active when purified from unfertilized eggs but can be activated several-fold by an antibody, termed anti-pepY antibody, raised against the inter-DFG-APE region of c-Src [10]. Thus, we examined the effect of genistein in the presence or the absence of anti-pepY antibody. As a control, we used daidzein, a structurally similar compound to genistein, which is non-inhibitory toward protein-tyrosine kinases. As shown in Fig. 2, phosphorylation of exogenous substrate by both partially active and antibody-activated Xyk was significantly but incompletely inhibited by 30 μM of genistein and almost completely inhibited by 100 μM, while no effect was observed with the same concentrations of daidzein.

We next examined the effect of genistein on *Xenopus* egg fertilization. Unfertilized eggs were preincubated in 1×DB solution supplemented with 1% DMSO and various concentrations of genistein or daidzein for 1 h. The treated eggs were washed and then inseminated. Fertilization-dependent cellular process, i.e. egg activation, first cell division, and later cell morphogenesis toward tadpole (formation of stage-25 embryo), was monitored at specified time after insemination. Egg activation was judged by occurrence of cortical contraction by 30 min after insemination. Pretreatment of eggs with genistein or daidzein itself was not toxic and had no ability to cause egg activation (data not shown). As shown in Fig. 3, 100 μM genistein was found to abolish the cortical contraction and later embryogenesis (top panels). On the other hand, 100 μM daidzein did not show such effect and allowed eggs to develop almost normally (bottom panels). The results for the effects of other concentrations of compounds are summarized in Table 1. It is found that egg activation and first cell division were little affected by 30 μM genistein. Even at this concentration, however, genistein completely inhibited the formation of stage-25 embryos (Table 1). On the other hand, daidzein had no effect on egg activation at 30 and 100 μM.

We also examined the effect of another tyrosine kinase inhibitor, herbimycin A [15], along with cAMP-dependent protein kinase-specific inhibitor, H-89 [16]. It was found that egg activation was significantly inhibited by 100 μM herbimycin A (Table 1), the concentration which could inhibit the kinase activity of Xyk efficiently in vitro (data not shown). On the other hand, H-89 showed only a limited inhibitory action on the egg activation at 30 μM and 100 μM (Table 1). At these higher concentrations, however, H-89 may inhibit not only cAMP-dependent protein kinase but also other serine/threonine kinases and tyrosine kinases. Thus, it is difficult to know which kind of protein kinase is affected in this case. Since the fertilization-dependent protein-tyrosine dephosphorylation was observed in Fig. 1, we tried to examine the effect of a

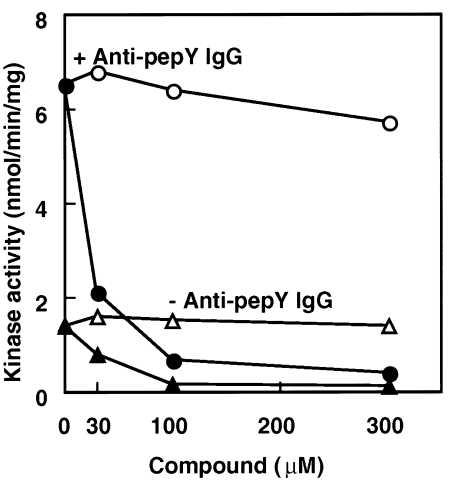

Fig. 2. Effect of genistein and daidzein on p57 *Xenopus* tyrosine kinase (Xyk) activity in vitro. Purified Xyk (200 ng protein) was preincubated in the presence (●,○; fully active Xyk) or absence (▲,△; partially active Xyk) of kinase-activating anti-pepY IgG (1 μg/assay), and then subjected to kinase assay with various concentrations of genistein (●,▲) or daidzein (○,△) as described in Section 2.6. The results are representative of four independent experiments.

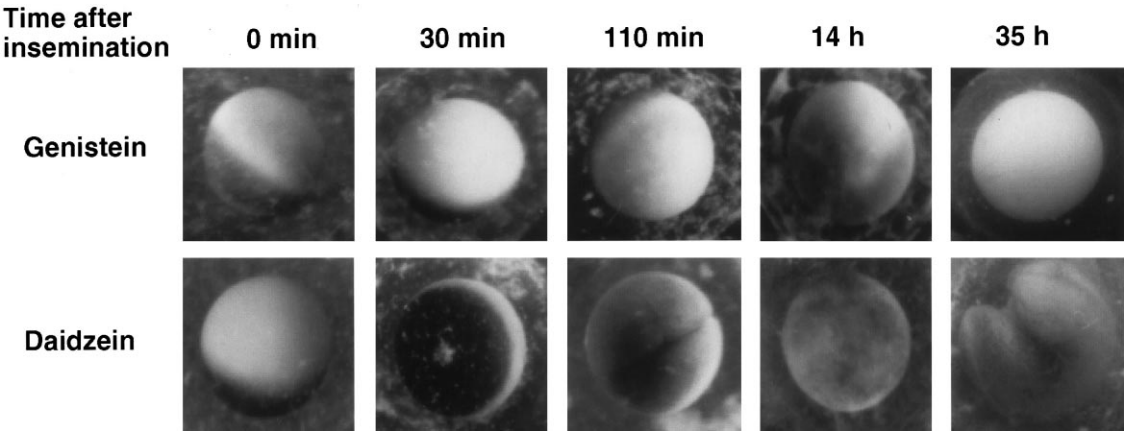

Fig. 3. Effect of genistein and daidzein on egg activation and early development of *Xenopus*. Unfertilized eggs were pretreated with 1×DB solution supplemented with 1% DMSO and 100 μM of genistein (top panels) or 100 μM of daidzein (bottom panels) for 1 h, washed extensively with 1×DB, and inseminated. Photographs were taken at the indicated time. Shown are eggs prior to insemination (0 min), cortical contraction/rotation (30 min), first cell division (110 min), initial gastrula (14 h), and stage-25 embryo (35 h).

tyrosine phosphatase inhibitor, Na_3VO_4, and a serine/threonine phosphatase inhibitor, okadaic acid. Inhibition of egg activation was observed with Na_3VO_4 but not with okadaic acid (Table 1) although a high concentration (1 μM) of okadaic acid inhibited the first cell division of about 40% of the eggs (Table 1). Taken together, it is indicated that a network of multiple protein phosphorylation and dephosphorylation events participates in the sequential process of *Xenopus* fertilization.

Further evidence for involvement of protein tyrosine phosphatase in egg activation was obtained by the following experiments. In Fig. 4, tyrosine phosphorylation of the cytosolic p42 MAP kinase in genistein-treated eggs before and after 10- and 30-min insemination are examined by anti-phosphotyrosine immunoblotting. It is shown that dephosphorylation of MAP kinase was inhibited in genistein-treated eggs. The result suggests that genistein-pretreatment alters the phosphotyrosine content of egg proteins not only through direct action on tyrosine kinases but also through indirect action toward tyrosine phosphatases.

Table 1
Effect of various inhibitors on sperm-induced activation and early development of *Xenopus* eggs

Sample	Activated eggs/eggs treated (%)	Two-cell embryos/eggs treated (%)	Stage-25 embryos/eggs treated (%)
Control			
(1% DMSO)	268/288 (93.0)	259/288 (89.9)	251/288 (87.2)
Genistein			
30 μM	206/263 (78.3)	147/263 (55.9)	0/263 (0.0)
100 μM	15/192 (7.8)	0/192 (0.0)	0/192 (0.0)
300 μM	6/69 (8.7)	0/69 (0.0)	0/69 (0.0)
Daidzein			
30 μM	102/104 (98.1)	102/104 (98.1)	99/104 (95.2)
100 μM	184/191 (96.3)	175/191 (91.6)	175/191 (91.6)
300 μM	32/52 (61.5)	32/52 (61.5)	28/52 (53.8)
Herbimycin A			
30 μM	16/21 (76.2)	4/21 (19.0)	4/21 (19.0)
100 μM	2/23 (8.7)	2/23 (8.7)	2/23 (8.7)
H-89			
30 μM	18/28 (64.3)	9/28 (32.1)	9/28 (32.1)
100 μM	8/21 (38.1)	4/21 (19.0)	4/21 (19.0)
Na_3VO_4			
300 μM	22/22 (100)	16/22 (72.7)	11/22 (50.0)
1 mM	0/23 (0)	0/23 (0)	0/23 (0)
Okadaic acid			
0.3 μM	19/19 (100)	18/19 (94.7)	18/19 (94.7)
1 μM	20/21 (95.2)	12/21 (57.1)	10/21 (47.6)

Unfertilized eggs were preincubated with the indicated concentrations of inhibitors, washed, and inseminated as described in Sections 2.2 and 2.3. Activation was scored by the appearance of cortical contraction and cortical rotation by 30 min after insemination. Two-cell (stage-2) and stage-25 embryos were scored at 110 min and 35 h, respectively. Eggs used were obtained from 12 animals.

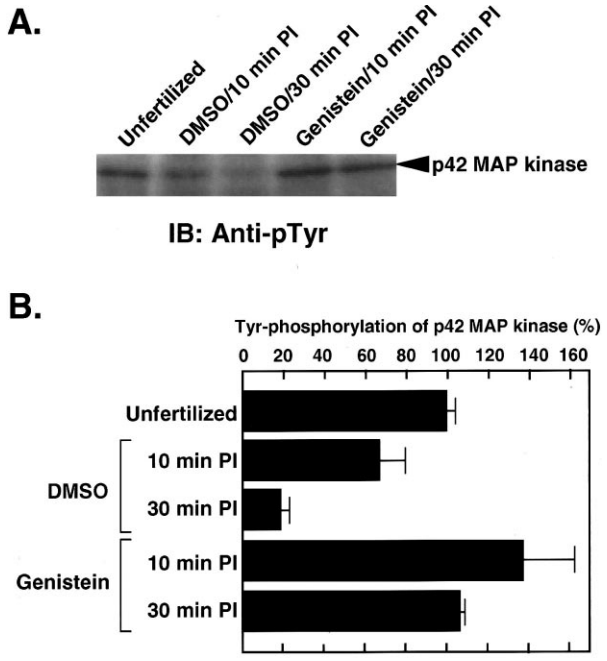

Fig. 4. Genistein inhibits sperm-induced tyrosine dephosphorylation of p42 MAP kinase. Unfertilized eggs were pretreated with 1% DMSO alone or 100 μM genistein in 1% DMSO for 1 h, and inseminated. Samples were taken at 10 min and 30 min post insemination (PI). A: Tyrosine phosphorylation of the cytosolic p42 MAP kinase was visualized by immunoblotting with anti-phosphotyrosine antibody as in Fig. 1. B: The band intensity was analyzed by Bioimaging Analyzer (Fuji Film BAS2000). The data are presented as a percent of the unfertilized eggs and represent mean ± S.D. of four independent experiments.

4. Discussion

The results presented in this study are consistent with the idea that protein tyrosine phosphorylation plays an important role in the process of fertilization and early development in *Xenopus*. By anti-phosphotyrosine immunoblotting, a number of egg proteins were found to be phosphorylated on or dephosphorylated from tyrosine residues upon fertilization. p42 MAP kinase was identified as a major tyrosine-phosphorylated protein in the cytosol fraction of unfertilized eggs and was shown to be dephosphorylated completely by 30 min after insemination. By that time completion of meiosis II and egg cortical rotation take place. Dephosphorylation of p42 MAP kinase implies that fertilization causes the rapid inactivation of the responsible kinase, MAP kinase kinase, or activation of the tyrosine phosphatase specific to MAP kinase [14,17]. Some other cytosolic or plasma membrane-associated proteins were newly identified as fertilization-dependent tyrosine phosphorylated proteins. In particular, p57 in the plasma membrane fraction is supposed to be Xyk, since (i) Xyk has already been identified as a fertilization-dependent tyrosine-phosphorylated protein [6]; and (ii) immunoprecipitation kinase assay of the subcellular fractions with anti-pepY antibody revealed that Xyk is detected predominantly in the plasma membrane (unpublished results). In the particulate fraction, some proteins were found to be tyrosine phosphorylated upon oocyte maturation, i.e. in unfertilized eggs, but their phosphotyrosine content was constant during fertilization. Involvement of tyrosine phosphorylation in oocyte maturation has been established [18]. Thus, these particulate proteins may contribute to oocyte maturation rather than fertilization.

Egg activation and later developmental processes were found to be inhibited by genistein in a dose dependent manner (Fig. 3 and Table 1). Genistein at 30 μM showed only a limited effect on early events like egg activation and first cell division. A dramatic inhibitory effect on egg activation was observed at 100 μM. At any concentrations tested, later embryogenesis was severely impaired. These results suggest that the multiple tyrosine kinase-dependent signal transduction pathways with different sensitivity to genistein may function in the different time periods of fertilization. In this context, it is attractive to surmise that Xyk, that required 100 μM genistein for the efficient inhibition in vitro (Fig. 2), may be a primary target of genistein at 100 μM in vivo and a responsible kinase for egg activation. This idea is supported by the finding that herbimycin A, a distinct tyrosine kinase inhibitor that can inhibit the kinase activity of Xyk in vitro, also inhibited the egg activation (Table 1). Na_3VO_4 inhibited egg activation (Table 1) and tyrosine dephosphorylation of p42 MAP kinase was blocked in genistein-treated eggs (Fig. 4). These results suggest that a tyrosine phosphatase activity, which seemed to be under the control of tyrosine kinase activity, is also required for egg activation.

Some previous studies on sea invertebrates are concerned with the effect of tyrosine kinase inhibitors on the fertilization process [19]. There is a growing body of evidence implying the role of protein tyrosine phosphorylation in the early stage of sea urchin fertilization. However, the effects of tyrosine kinase inhibitors, such as genistein, erbstatin and tyrphostin B42, were limited to later events such as pronuclear migration, DNA synthesis, and gastrulation, and not prominent on earlier events like elevation of fertilization envelope when 100 μM compounds were used [19–21]. It should be noted that we used 1% DMSO as solvent while others used 0.1% DMSO. Solubility of the tyrosine kinase inhibitors may affect their working concentrations in the medium. Another study by Wright and Schatten [21] used erbstatin in 1% DMSO and demonstrated that it caused defects in fertilization-dependent mitotic spindle formation, although its effect on egg activation was not described [21]. Differences between organisms should also be regarded. Involvement of tyrosine kinase in egg activation is also suggested in the study with another sea invertebrate, starfish [22,23] and ascidian [24]. To our knowledge, however, this is the first report to demonstrate that the inhibition of the tyrosine kinase pathway leads to the disruption of sperm-induced egg activation of *Xenopus*.

Acknowledgements: We are grateful to Dr. Yasutomi Nishizuka for his support and encouragement. We are also grateful to Dr. Yasuhiro Iwao for helpful discussion and critical reading of the manuscript. This work was supported in part by research grants from the Ministry of Education, Science, Sports, and Culture of Japan.

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