

Low density detergent-insoluble membrane of *Xenopus* eggs: subcellular microdomain for tyrosine kinase signaling in fertilization

Ken-ichi Sato^{1,2,*}, Tetsushi Iwasaki¹, Keiko Ogawa³, Masako Konishi³, Alexander A. Tokmakov^{4,†} and Yasuo Fukami^{2,3}

¹Research Center for Environmental Genomics, Kobe University, Nada, Kobe 657-8501 Japan

²Department of Biology, Faculty of Science, Kobe University, Nada, Kobe 657-8501 Japan

³The Graduate School of Science and Technology, Kobe University, Nada, Kobe 657-8501 Japan

⁴Biosignal Research Center, Kobe University, Nada, Kobe 657-8501 Japan

[†]Present address: Genomic Sciences Center, RIKEN Yokohama Institute, Yokohama 230-0045 Japan

*Author for correspondence (e-mail: kksato@kobe-u.ac.jp)

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SUMMARY

Protein-tyrosine phosphorylation plays an important role in egg activation signaling at fertilization. We show that in *Xenopus*, fertilization stimulates a rapid and transient tyrosine phosphorylation of egg proteins, as revealed by immunoblotting with anti-phosphotyrosine antibody. Immunofluorescent microscopic analysis demonstrated that the phosphorylation occurs in cortical area of the egg animal hemisphere. To further characterize subcellular compartment for fertilization-dependent tyrosine kinase signaling, we isolated low density detergent-insoluble membrane (LD-DIM) fraction from *Xenopus* eggs. The egg LD-DIM was enriched in cholesterol and GM1 ganglioside. It also contained signaling molecules such as Xyk (*Xenopus* egg Src), Gq α , Ras, integrin β 1 and CD9. Fertilization stimulated tyrosine phosphorylation of Xyk and some other LD-DIM proteins. Remarkably, sperm stimulated tyrosine

phosphorylation of the LD-DIM proteins in vitro. The sperm-dependent phosphorylation was sensitive to the tyrosine kinase inhibitors PP2 and genistein. We found that pretreatment of eggs with methyl- β -cyclodextrin, a cholesterol-binding substance, led to a decrease in cholesterol, Xyk and sperm-induced tyrosine phosphorylation in LD-DIM. In methyl- β -cyclodextrin-treated eggs, sperm-induced Ca²⁺ transient and first cell division were also inhibited. These findings suggest that the egg LD-DIM might serve as subcellular microdomain for tyrosine kinase signaling in *Xenopus* egg fertilization.

Key words: Fertilization, Egg activation, Protein-tyrosine phosphorylation, Low density detergent-insoluble membrane, Src family kinase, Signal transduction, *Xenopus*

INTRODUCTION

Fertilization produces offspring by virtue of cellular communication between gametes: sperm and egg. At fertilization, eggs of so far examined species, if not all, undergo a transient rise(s) in intracellular Ca²⁺ (Stricker, 1999). Such a sperm-induced Ca²⁺ rise is required for latter biochemical and cell biological processes that lead to the activation of zygotic development. The term, egg activation, compiles a series of events that orchestrate developmental activation of zygote from the time of sperm-egg binding/fusion to the time at which zygote initiates DNA synthesis and cell division cycle. Artificial increase in [Ca²⁺]_i can mimic egg activation. Therefore, it appears that a transient rise in [Ca²⁺]_i is sufficient for egg activation. Now the major problems in the fertilization study are determining how a sperm interacts and fuses with an egg, how a fertilizing sperm elevates [Ca²⁺]_i, and how elevated [Ca²⁺]_i induces egg activation.

In eggs of *Xenopus laevis*, a transient rise in [Ca²⁺]_i takes place at fertilization. The Ca²⁺ response begins near the sperm

entry point within minutes of fertilization and propagates through the entire egg cytoplasm. It is known that inositol trisphosphate [InsPtd(1,4,5)P₃] is necessary and sufficient for the Ca²⁺ response (Nuccitelli et al., 1993; Runft et al., 1999). Accumulating evidence has presented some candidate substances in sperm that may account for the ignition of the InsPtd(1,4,5)P₃-mediated Ca²⁺ response, although none of them are definitive. They include the disintegrin peptide-containing protein (Iwao and Fujimura, 1996; Shilling et al., 1998) and cathepsin B-like endoprotease (Mizote et al., 1999), both of which would interact with egg surface receptor molecules. Sperm cytosolic factor has also been suggested to act as initiator of the Ca²⁺ response in *Xenopus* (Dong et al., 2000; Wu et al., 2001), although its molecular identity has not yet been established.

Egg InsPtd(1,4,5)P₃ is produced by hydrolysis of phosphatidylinositol bisphosphate that is catalyzed by phospholipase C (PLC). Pharmacological and/or ectopic gene expression studies have suggested that two egg components, G-proteins and protein-tyrosine kinases (PTKs), serve as

upstream regulator of egg PLC (Kline et al., 1991; Yim et al., 1994; Sato et al., 1998; Glahn et al., 1999; Runft et al., 1999). They are thought to act under the control of egg membrane receptors or sperm-derived factors. Little is known, however, about the molecular identity and function of egg/sperm endogenous component(s) that activates PLC/InsPtd(1,4,5)P₃/Ca²⁺ pathway.

Previously, we have shown that an egg Src-related protein-tyrosine kinase (PTK), named Xyk, is activated within minutes of fertilization of *Xenopus* eggs (Sato et al., 1996). Xyk is a mixture of *Xenopus* Src1 and Src2 (Steele et al., 1989a), as demonstrated by structural analyses (Iwasaki et al., unpublished). In *Xenopus*, some other Src family PTKs have been identified at the mRNA level: Fyn (Steele et al., 1990) and Yes (Steele et al., 1989b; Weinstein et al., 1998). However, it has been shown that Xyk is a major Src family PTK expressed in eggs (Sato et al., 1999). Xyk localizes to the egg cortex in unfertilized eggs and part of the activated Xyk undergoes translocation to the cytosolic fraction at fertilization (Sato et al., 1996; Sato et al., 1999). Src-specific inhibitors can block Ca²⁺ transient and egg activation induced by sperm, but not by Ca²⁺ ionophore (Sato et al., 1999; Glahn et al., 1999). Ca²⁺ ionophore does not activate Xyk, while an integrin-interacting RGDS peptide and hydrogen peroxide induces a Xyk-dependent Ca²⁺ transient and egg activation (Sato et al., 2000a; Sato et al., 2001). One isoform of PLC (PLC γ) becomes tyrosine-phosphorylated, associated with Xyk and activated in a PTK-dependent manner at fertilization (Sato et al., 2000a). These results suggest that Xyk acts upstream of the PLC/InsPtd(1,4,5)P₃/Ca²⁺ pathway in fertilized *Xenopus* eggs.

In the present study, we further characterized PTK signaling in *Xenopus* egg fertilization in comparison with that at parthenogenetic egg activation. Immunoblotting and indirect immunofluorescent studies were used to analyze spatiotemporal characteristics of protein-tyrosine phosphorylation in eggs. We have isolated low density detergent-insoluble membrane (LD-DIM) fraction from eggs and demonstrated that the sperm-dependent PTK signaling machinery containing Xyk is preorganized in the LD-DIM of unfertilized eggs. LD-DIM from fertilized eggs was shown to contain several tyrosine-phosphorylated proteins. Surprisingly, such fertilization-dependent phosphorylation events could be reproduced at least in part by *in vitro* protein kinase assay using LD-DIM from unfertilized eggs and live sperm. Further pharmacological approaches established the importance of LD-DIM in fertilization signaling. We will discuss the possibility that such an egg subcellular microdomain may constitute a signaling platform regulating sperm-egg interaction, tyrosine kinase activation and Ca²⁺-dependent egg activation.

MATERIALS AND METHODS

Preparation of *Xenopus* eggs and sperm

Xenopus laevis were obtained from the Hamamatsu Seibutsu Kyozai (Hamamatsu, Japan). To obtain eggs, female frogs were injected with 40 IU per animal of pregnant mare serum gonadotropin (PMSG; Seikagaku Kogyo, Tokyo, Japan) 2-3 days before experiment and then injected with 500 IU per animal of human chorionic gonadotropin (hCG; Teikoku Zoki, Tokyo, Japan) half a day before experiment. The hormone-injected frogs were maintained in deionized water (1.5 liter per animal) containing 100 mM NaCl. Ovulation began 6 to 9 hours

after the hCG injection. Ovulated or gently squeezed eggs were immediately washed three times with 1× DeBoer's buffer (DB) containing 110 mM NaCl, 1.3 mM KCl and 0.44 mM CaCl₂ (pH 7.2, by addition of NaHCO₃), kept at ambient temperature (18-22°C), and used within 3 hours of spawning. Before experiments, the jelly coat was removed from eggs by incubation with more than twofold the volume of 1× DB supplemented with 2% cysteine and 0.06 N NaOH for 3 to 8 minutes at ambient temperature. The resulting jelly coat-free eggs were washed five times with 1× DB and subjected to egg activation treatment.

To obtain sperm, male frogs were injected with 20 IU per animal of PMSG half a day before experiment. On the day of experiment, testes were surgically removed from the PMSG-injected frogs, immediately washed three times with 1× DB and kept in siliconized microtube at 4°C until use. Before experiments, a pair of testes was macerated in ice-cold 1× DB. The macerated testes were centrifuged at 100 g for 5 minutes at 4°C. The supernatant was taken and further centrifuged at 1000 g for 5 minutes at 4°C. The resulting pellet was resuspended with 1 ml of egg jelly water (see below) and the suspension was gently rocked for 10 minutes at ambient temperature. After the incubation, the suspension was centrifuged at 1000 g for 5 minutes at 4°C. The sperm pellet was washed once with 1× DB and centrifuged again. The resulting sperm was designated as jelly water-treated sperm and used for egg activation or protein kinase assay. The concentration of sperm in the suspension was determined by counting sperm number by hemacytometer.

Egg jelly water was prepared as described elsewhere (Sato et al., 2000a). Briefly, egg coat-intact eggs (about 4 g) were subjected to gentle rocking with 10 ml of 0.3× modified Ringer's solution containing 33 mM NaCl, 0.6 mM KCl, 0.33 mM MgCl₂, 0.66 mM CaCl₂, 1.67 mM HEPES-NaOH, pH 7.8, for 60 minutes at 18°C. The resulting soluble material (about 3 ml) was collected, mixed with the final concentration of 10% Ficoll (*M_r* 400,000; Sigma, MO, USA), and used as egg jelly water.

Egg activation and solubilization

Jelly coat-free eggs were activated by either insemination with jelly water-treated sperm (about 10⁷ sperm/ml), 0.5 μM A23187 or 10 mM hydrogen peroxide (H₂O₂) as described (Sato et al., 2000a; Sato et al., 2001) for the times as specified in the text. After the activation treatment, eggs were washed twice with ice-cold 1× DB and immediately frozen in liquid nitrogen. Successful sperm-induced egg activation was scored by monitoring cytological markers such as formation of fertilization envelope, cortical contraction of the pigmented animal hemisphere, appearance of sperm entry point (Palecek et al., 1978), cortical rotation and first embryonic cleavage. When analyzing tyrosine phosphorylation profile of total egg proteins, we homogenized eggs with 20 μl per egg of extraction buffer containing 1% SDS, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM β-mercaptoethanol, 10 μg/ml leupeptin, 20 μM (*p*-amidinophenyl)methanesulfonyl fluoride (APMSF), using a Teflon-glass homogenizer (Wheaton, NJ, USA) (20 strokes). Alternatively, eggs were homogenized with 4 μl per egg of the extraction buffer containing 1% Triton X-100, instead of SDS. Homogenized materials were sonicated twice for 1 minute in an ultrasonic bath incubator (VS-100; Iuchi Seieido, Osaka, Japan), incubated on ice for 10 minutes, and centrifuged at 1000 g for 10 minutes to remove yolk platelets and debris. The resulting supernatants were subjected to protein analysis or further fractionated by sucrose-density gradient centrifugation.

Antibodies and other chemicals

Anti-phosphotyrosine mouse monoclonal antibody (PY99) was purchased from Santa Cruz (CA, USA). Horseradish peroxidase (HRPase)-conjugated goat antibody against mouse immunoglobulin G was from Cappel (Belgium). Rabbit polyclonal antibodies against Xyk (*Xenopus* Src1/2; residues 409-427), integrin β1 (*Xenopus*

integrin $\beta 1$; residues 775-798) and CD9 (mouse CD9; residues 217-226) were prepared according to the described methods (Harlow and Lane, 1988). An antibody that recognizes phosphorylated tyrosine-416 of Src (anti-pY416 antibody) was obtained from Oncogene Research Products (MA, USA). Specific antibodies against PLC γ , Shc, actin, β -tubulin, mitogen-activated protein kinase, Ras, Gq α and caveolin 1 were from Transduction Laboratories (KY, USA), Upstate Biotechnology (NY, USA), BioLabs (MA, USA), Santa Cruz, or Sigma (MO, USA). The Ca²⁺ ionophore A23187 was obtained from Sigma. PTK inhibitors PP2 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) and genistein, and their inactive analogs PP3 and daidzein were obtained from Calbiochem (CA, USA), dissolved in dimethyl sulfoxide and kept at -80°C until use. H₂O₂ was from Santoku Chemical Industries (Tokyo, Japan). L-phosphoamino acids, HRPase-conjugated B subunit of cholera toxin, methyl- β -cyclodextrin and fura-2 were purchased from Sigma. [γ -³²P]ATP was from Moravek (CA, USA). A synthetic PTK substrate peptide (Cdc2 peptide; residues 7-26 of the fission yeast Cdc2) was synthesized and purified as described previously (Sato et al., 1999). Other chemicals were analytical grade and purchased from Sigma, Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan).

SDS-PAGE, immunoblotting and immunoprecipitation

Protein samples were separated by SDS-polyacrylamide electrophoresis (SDS-PAGE) (Laemmli, 1970) on acrylamide gels (8 or 12.5 or 16% gels). The separated proteins were visualized by protein silver staining using a Daiichi-kagaku kit (Tokyo, Japan). To examine tyrosine phosphorylation, we performed immunoblotting with anti-phosphotyrosine antibody as described (Sato et al., 2001). Briefly, proteins were transferred from SDS-PAGE gels to polyvinylidene difluoride membranes by using a semi-dry blotting apparatus (BioRad, CA, USA). Membranes were soaked in blocking buffer containing 6 mg/ml bovine serum albumin, 150 mM NaCl, 0.05% Tween 20 and 20 mM Tris-HCl (pH 7.5). Antibody treatment of the membranes was made in the same buffer containing the specified amount of a primary antibody for 2 hours at room temperature. The immune complex was detected by color development catalyzed by alkaline phosphatase labeled to the secondary antibody. In some experiments, protein samples were immunoprecipitated with the specified antibodies for 3 hours at 4°C. The immune complex was absorbed onto Sepharose beads coupled with protein A (Pharmacia, Sweden). The beads were washed three times with RIPA buffer containing 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl and 50 mM Tris-HCl (pH 7.5), and then washed beads were subjected to immunoblotting or protein kinase assay.

Sucrose-density gradient fractionation of eggs

Low density detergent-insoluble membrane (LD-DIM) fraction was prepared by sucrose-density gradient centrifugation. Triton X-100-solubilized egg extract was brought to 42.5% (w/v) sucrose by mixing with equal volume of detergent-free extraction buffer supplemented with 85% (w/v) sucrose. The mixture (about 5 ml) was layered successively with 18 ml of 30% (w/v) sucrose and with 12 ml of 5% (w/v) sucrose in the same buffer, and centrifuged at 144,000 *g* for 24 hours at 4°C (SW28 rotor, Beckman, CA, USA). After the centrifugation, 3 ml aliquots of 12 fractions were collected from the top to the bottom of the centrifuge tube. The fractions were kept on ice and analyzed within 2 days of preparation.

Protein kinase assay

To assess sperm-dependent protein-tyrosine phosphorylation in egg LD-DIM fraction, we performed protein kinase assay. Diluted egg LD-DIM fraction (50 μ l, less than 5% sucrose) was preincubated with or without jelly water-treated sperm (10 μ l of 10⁸ sperm/ml) for 10 minutes at 21°C. The preincubated fraction was then incubated in the

presence of 5 mM MgCl₂, 1 mM ATP, 50 mM Tris-HCl (pH 7.5) and 1 mM DTT, for 10 minutes at 30°C (total volume of 100 μ l). Phosphorylated proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibody. When the effect of inhibitors was examined, phosphorylation incubation was carried out in the presence of 10 μ M PP2, 10 μ M PP3, 50 μ M genistein or 50 μ M daidzein. We also examined overall protein phosphorylation profile and peptide kinase activity in LD-DIM fraction. In this case, protein kinase assay was performed in the presence of 2 μ M [γ -³²P]ATP and 1 mM substrate peptide for 10 minutes at 30°C. Phosphorylated proteins and peptide were separated by SDS-PAGE and analyzed by BAS2000 phosphoimaging analyzer (Fujifilm, Tokyo, Japan). When the immune complexes on the Sepharose beads were assayed for kinase activity, the beads were washed twice with 50 mM Tris-HCl, pH 7.5 and 1 mM DTT and subjected to protein kinase assay.

Protein, cholesterol and GM1 assays

The sucrose-density gradient fractions prepared as above were analyzed spectrophotometrically for total protein, cholesterol and GM1-ganglioside. Protein was determined by the dye-binding method (Bradford, 1976) using a BioRad assay kit. Measurement of cholesterol was made at 505 nm, according to a previously described method (Chang et al., 1992) using a Wako assay kit. To determine GM1-ganglioside in the fractions, samples were absorbed onto non-coated plastic wells for 12 hours at 4°C. After washing with phosphate-buffered saline (PBS), the sample wells were treated with HRPase-conjugated B subunit of cholera toxin (~140 ng/ml protein) for 1 hour at 21°C. The extent of the specific binding of HRPase activity was monitored at 600 nm after 10 minutes of addition of PBS supplemented with 0.5 mg/ml diaminobenzidine and 5 mM H₂O₂.

Methyl- β -cyclodextrin treatment

Unfertilized eggs were pretreated with the specified concentrations of methyl- β -cyclodextrin (MCD) in 1 \times DB for 60 minutes at room temperature. After the treatment, eggs were washed three times with 1 \times DB and subjected to egg activation treatment and/or sucrose density gradient centrifugation. To assess the reversibility of the MCD-induced cholesterol removal in *Xenopus* eggs, we performed repletion of cholesterol according to the method described previously (Racchi et al., 1997). Briefly, cholesterol dissolved in chloroform (100 mM, 500 μ l) was completely dried under nitrogen. Then, MCD solution at 25 mM (500 μ l) was added to the dried material. We clarified the mixture by bath-sonication and vigorous mixing at room temperature. After 12 hours of incubation at 37°C, soluble material was used as reconstituted cholesterol/MCD mixture. When the repletion experiments were conducted, MCD-treated eggs were further treated with excess volume of the cholesterol/MCD mixture for 60 minutes at room temperature.

Other methods

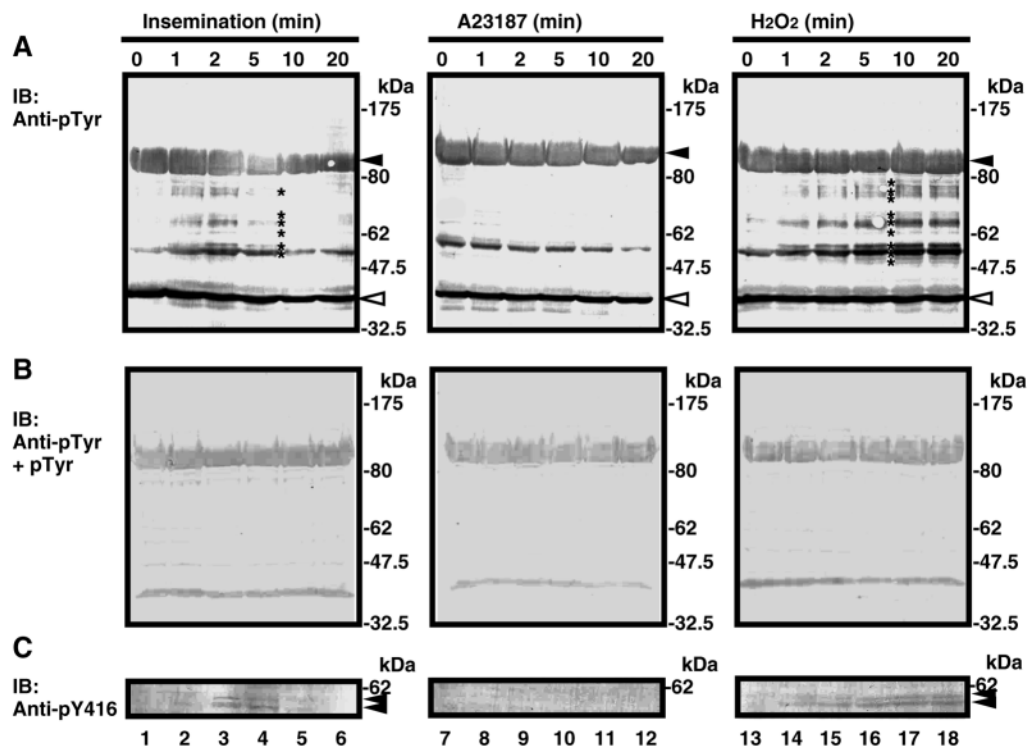
Indirect immunofluorescent microscopic analysis of egg sections, microinjection, monitoring of egg activation and Ca²⁺ measurement of fura-2-injected eggs, were carried out as described previously (Sato et al., 1999; Sato et al., 2000a; Sato et al., 2001).

RESULTS

Transient PTK signaling in the cortical area of fertilized *Xenopus* eggs

Time course of egg activation-associated tyrosine phosphorylation was analyzed by immunoblotting of SDS-solubilized egg extracts with anti-phosphotyrosine antibody. Fertilization causes a transient rise in tyrosine phosphorylation of a subset of proteins: their molecular sizes are 55, 57, 60, 65,

Fig. 1. Tyrosine phosphorylation of *Xenopus* egg proteins in response to fertilization, Ca^{2+} ionophore and H_2O_2 . SDS-solubilized extract was prepared from *Xenopus* eggs that had been treated with either sperm (insemination, lanes 1-6), A23187 (0.5 μM , lanes 7-12) or H_2O_2 (10 mM, lanes 13-18) for the indicated times. Egg proteins (each lane contains 50 μg protein that corresponds to one egg) were separated by SDS-PAGE and analyzed by immunoblotting (IB) with either (A) anti-phosphotyrosine antibody (PY99, 1 $\mu\text{g}/\text{ml}$), (B) anti-phosphotyrosine antibody plus 10 mM L-phosphotyrosine or (C) anti-pY416 antibody (10 $\mu\text{g}/\text{ml}$). Asterisks in A indicate the positions of tyrosine-phosphorylated proteins. Black and white arrowheads in A indicate the positions of a 90 kDa protein and a 42 kDa MAP kinase, respectively. Black arrowheads in C indicate the position of Xyk (57 kDa and partially degraded 55 kDa bands). Prestained molecular size markers are maltose binding protein (MBP)-fusion β -galactosidase (175 kDa), MBP-fusion paramyosin (80 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa) and triosephosphate isomerase (32.5 kDa).



67 and 72 kDa. It peaked 2 minutes after insemination and declined thereafter. During the first 20 minutes after insemination, 42 kDa MAP kinase is stably and highly tyrosine-phosphorylated (Fig. 1A, left). The specificity of anti-phosphotyrosine immunoblotting was verified by the fact that fertilization-dependent tyrosine phosphorylation bands as well as the p42 band could be efficiently eliminated in the presence of L-phosphotyrosine. It appears that a prominent band at 90 kDa is not due to tyrosine phosphorylation (Fig. 1A,B, left). Treatment of eggs with the Ca^{2+} ionophore A23187 did not cause a transient tyrosine phosphorylation of egg proteins (Fig. 1A, middle), indicating that the increase in $[\text{Ca}^{2+}]_i$ does not lead to upregulation of egg tyrosine kinases. However, H_2O_2 treatment of eggs induced a sustained increase in tyrosine phosphorylation (Fig. 1A, right). It showed a similar phosphorylation profile to that observed at fertilization.

We employed an antibody that recognizes the autophosphorylated Src family PTKs (anti-pY416 antibody) to assess the level of Xyk autophosphorylation. The anti-pY416 immunoblotting revealed that Xyk was transiently phosphorylated in fertilized eggs (Fig. 1C, left) and permanently phosphorylated in H_2O_2 -treated eggs (Fig. 1C, right). By contrast, A23187 did not show such an effect (Fig. 1C, middle).

We next examined whether egg activation-associated tyrosine phosphorylation is localized to the specific area in the egg. Such spatial characterization of tyrosine phosphorylation was carried out by indirect immunofluorescent methods. Thin sections of the egg samples were treated with anti-phosphotyrosine antibody and analyzed by confocal laser-scanning microscopy. Unfertilized eggs gave very faint but significant signal in the entire cortex and the cytoplasm (Fig.

2A,G). We found that in fertilized eggs (5 minutes after insemination), the signal apparently increased in the cortical area of the animal hemisphere (Fig. 2C,I). The signal could be eliminated by the addition of L-phosphotyrosine (Fig. 2E). Such localized increase of the anti-phosphotyrosine-specific signal was more evident in H_2O_2 -treated eggs (Fig. 2M, 5 minutes after 10 mM H_2O_2 treatment). The immunostained image obtained with ionophore-treated eggs (5 minutes after 0.5 μM A23187 treatment) was indistinguishable from that obtained with unfertilized eggs (Fig. 2K).

Isolation of egg low density detergent-insoluble membrane (LD-DIM)

Recently, compartmentalization of the signaling molecules in low density detergent-insoluble membrane (LD-DIM) has been shown in many cell systems. LD-DIM, also designated as DRM (detergent-resistant membrane) or GEM (glycosphingolipid-enriched membrane) or raft, is enriched in specific subsets of lipid and protein and has been implicated in receptor-mediated signal transduction (Anderson, 1998; Brown and London, 1998). Src PTKs have been identified in LD-DIM of several cell types. By analogy to this model, we expected that Xyk is present in LD-DIM and involved in fertilization-dependent signal transduction of *Xenopus* eggs.

Triton X-100-solubilized extract of unfertilized eggs was subjected to sucrose density-gradient centrifugation and fractionated (Fig. 3A). A distinctive light-scattering band was obtained in the low density region (interface between 5% and 30% sucrose, Fig. 3B). Protein staining of the fractions by silver (Fig. 3C) and protein assay (Fig. 3D) demonstrated that LD-DIM fractions (fractions 3-6) contained a limited amount

Fig. 2. Immunofluorescent microscopic visualization of localized tyrosine phosphorylation in fertilized and H₂O₂-treated *Xenopus* eggs. Sections (10 μ m) of *Xenopus* unfertilized eggs (A,B,G,H), fertilized eggs (C-F,I,J; 5 minutes after insemination), A23187-treated eggs (K,L; 0.5 μ M, 5 minutes), or H₂O₂-treated eggs (M,N; 10 mM, 5 minutes) were immunostained with anti-phosphotyrosine antibody (PY99, 1 μ g/ml). The samples were analyzed by confocal laser-scanning microscopy (model LSM410, Zeiss, Germany). Indirect immunostained images obtained with fluorescein isothiocyanate (FITC) and the corresponding Nomarski cell images are shown. White arrowheads in C indicate the area showing fertilization-dependent tyrosine phosphorylation. Scale bars: 250 μ m in A-F,K-N; 62.5 μ m in G-J.

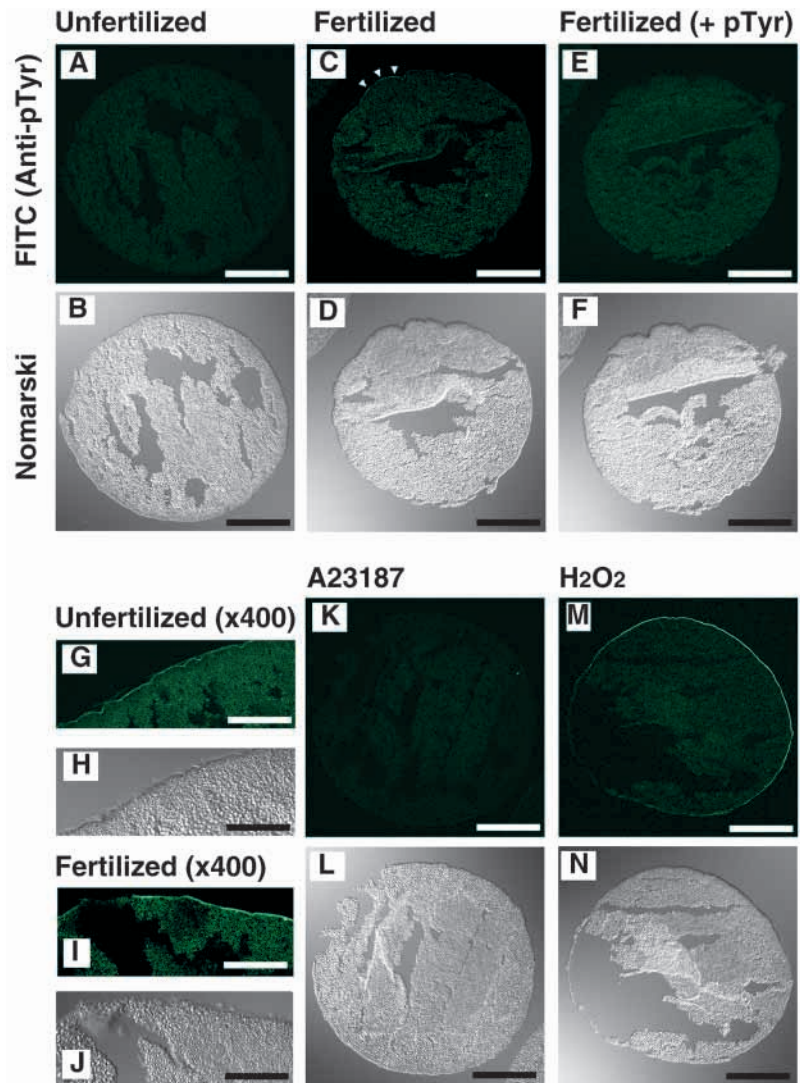
of proteins of less than 0.1% (ca. 50 ng protein/egg) of total proteins (ca. 50 μ g/egg). Most proteins were recovered in high-density detergent-soluble fractions (fractions 10-12, Fig. 3C,D). Cholesterol and GM1-ganglioside were found to be significantly enriched in LD-DIM fractions (Fig. 3E,F), indicating that LD-DIM fractions of *Xenopus* eggs have characteristics of so-called 'lipid/membrane raft'. Immunoblotting analysis demonstrated that Xyk was present in LD-DIM fractions (Fig. 3G). Several other molecules were also present in LD-DIM fractions (Fig. 3G). They include actin, β -tubulin, MAP kinase, Ras, Gq α , CD9, caveolin 1, and integrin β 1. However, neither phospholipase C γ nor Shc could be detected in LD-DIM fractions (Fig. 3G).

Egg LD-DIM contains sperm-dependent Src PTK signaling machinery

To analyze whether fertilization induces protein-tyrosine phosphorylation and activation of Xyk in the egg LD-DIM, we performed anti-phosphotyrosine immunoblotting. We found that tyrosine phosphorylation of proteins of 34, 55, 57, 60, 74 and 95 kDa became apparent in LD-DIM fractions after fertilization (Fig. 4A,B; pTyr, fractions 4 and 5). The 57 kDa band might be related to the phosphorylation of Xyk because anti-pY416 immunoblotting gave the same band in fractions 4 and 5 (Fig. 4B; pY416). The anti-pY416-specific signal could not be detected in detergent-solubilized proteins (Fig. 4A,B; pY416, fractions 10 to 12). In addition, total protein-tyrosine phosphorylation pattern in the heavy fractions was not affected by fertilization (Fig. 4A,B; pTyr, fractions 10 to 12).

We also analyzed relative efficiency of tyrosine phosphorylation using 10 \times concentrated LD-DIM fraction (fraction 4) and 100-fold diluted non-LD-DIM fractions (fraction 12), in which about 1 μ g protein each was analyzed. We found that, even after dilution, fertilization-dependent tyrosine phosphorylation was hardly detectable in the non LD-DIM fraction (data not shown). So, it seems that egg LD-DIM might serve initially as a major site for fertilization-induced tyrosine phosphorylation. In H₂O₂-treated eggs, however, both LD-DIM and non LD-DIM fractions showed augmented protein-tyrosine phosphorylation (Fig. 4C; pTyr) and tyrosine phosphorylation of Xyk (Fig. 4C; pY416).

Results obtained in Fig. 4 strongly suggest that functional PTK machinery is present in egg LD-DIM. To address this



issue more directly, we performed in vitro protein kinase assay by using LD-DIM fraction of unfertilized eggs. Phosphorylated proteins were analyzed by immunoblotting with anti-phosphotyrosine antibody in the absence or the presence of L-phosphotyrosine. Egg LD-DIM was found to contain tyrosine kinase activity in the presence of Mg/ATP (Fig. 5A; lanes 1 and 2). Surprisingly, addition of fertilization-competent, i.e. jelly water-treated sperm to the kinase reaction mixture augmented the phosphorylation (lanes 3 and 4). The phosphorylation was observed in proteins of 25, 34, 50, 55 and 57 kDa. It should be noted that a subset of the phosphorylated proteins (34, 55 and 57 kDa) was also detected in LD-DIM fractions of fertilized eggs (Fig. 4B). A phosphoprotein of 18 kDa was detected in the presence of sperm alone (lanes 3-6, an open asterisk), indicating that it was derived from sperm. We also performed kinase assay using non LD-DIM fraction from unfertilized eggs (fraction 12). As shown in Fig. 5, however, sperm-dependent tyrosine phosphorylation was hardly detectable: only an 18 kDa tyrosine-phosphorylated sperm protein could be detected in the presence of sperm (lanes 7 and 8).

The Src-specific inhibitor PP2 and the universal PTK inhibitor genistein could block the Mg/ATP/sperm-dependent

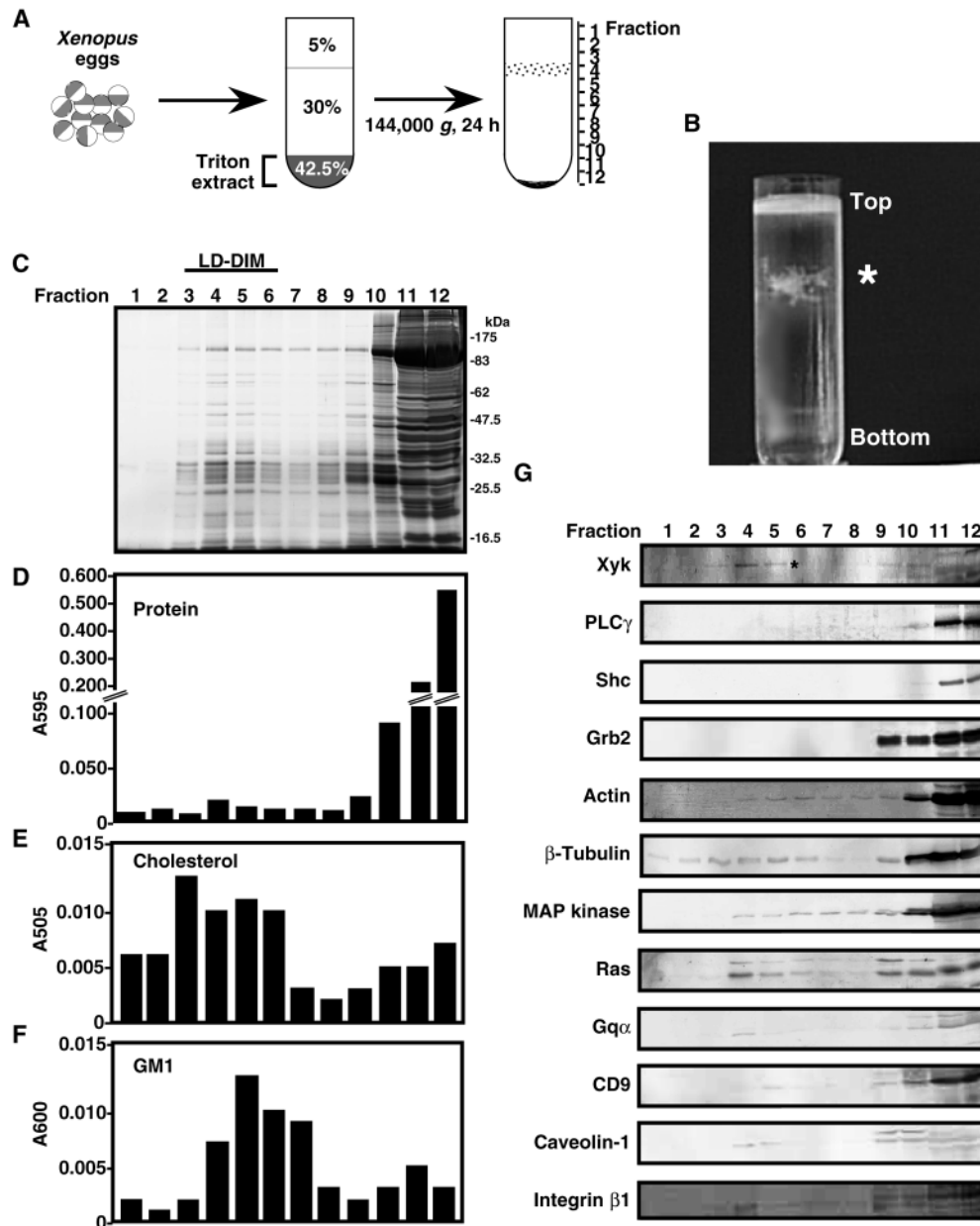


Fig. 3. Isolation and characterization of low density detergent-insoluble membrane (LD-DIM) fraction from *Xenopus* unfertilized eggs. (A) Schematic diagram for preparation of LD-DIM fraction from *Xenopus* eggs. (B) Egg LD-DIM fraction, a light-scattering band, is indicated by an asterisk. (C) Protein silver staining of sucrose gradient fractions. Proteins (20 μ l of each fraction) were separated by SDS-PAGE on a 12.5% gel. Prestained molecular size markers are as in Fig. 1 plus β -lactoglobulin A (25.5 kDa) and lysozyme (16.5 kDa). Each sucrose density gradient fraction was assayed for the content of (D) protein, (E) cholesterol and (F) GM1-ganglioside. (G) Fractions were separated by SDS-PAGE on 8%, 12.5% or 16% gels, and analyzed by immunoblotting with the indicated specific antibodies. An asterisk indicates the position of Xyk in the LD-DIM fractions.

tyrosine phosphorylation in LD-DIM fraction while their inactive analogs PP3 and daidzein had no effect (Fig. 6A, top). We next analyzed overall protein phosphorylation in LD-DIM fraction by kinase assay in the presence of [γ - 32 P]ATP. Results shown in Fig. 6B demonstrate that protein phosphorylation was augmented by sperm (lanes 1 and 2). The phosphorylation was partly sensitive to PP2 or genistein (Fig. 6B, lanes 3 and 5). We also observed that an exogenous PTK substrate peptide, Cdc2 peptide, was phosphorylated by LD-DIM fraction in a sperm-dependent manner (Fig. 6C, lanes 1 and 2). In addition, it was sensitive to the PTK inhibitors (Fig. 6C, lanes 3 and 5).

An antibody specific to Xyk immunoprecipitated a 57 kDa protein, and the immunoprecipitated protein was recognized by anti-pY416 antibody (Fig. 6D, top panel). It was also found that the anti-Xyk immunoprecipitates contained tyrosine kinase activity that underwent autophosphorylation at a position of 57 kDa (Fig. 6D, middle panel) and phosphorylation

of Cdc2 peptide (Fig. 6D, bottom panel). From these results, we conclude that the egg LD-DIM contains Xyk that can phosphorylate substrate proteins and peptides in response to sperm *in vitro*.

Methyl- β -cyclodextrin inhibits PTK signaling in LD-DIM, Ca $^{2+}$ release and egg activation in fertilized *Xenopus* eggs

If specific localization of Xyk and other molecules to egg LD-DIM is important for the fertilization signaling, then disruption of the LD-DIM should abolish the

ability of sperm to cause successful egg activation upon fertilization. We employed the cholesterol-binding substance methyl- β -cyclodextrin (MCD) to address this issue. *Xenopus* unfertilized eggs were incubated in the medium supplemented with the different concentrations of MCD (0-25 mM), and then inseminated. Sperm-induced activation of the inseminated eggs was monitored by the formation of the first cleavage furrow. We found that MCD at 25 mM inhibited the furrow formation by about 50% (27 of 59 eggs were inhibited; Fig. 7A). The MCD inhibition was dose-dependent (Fig. 7A). Concomitant decrease in the LD-DIM PTK activity was also observed in MCD-treated eggs (Fig. 7B). MCD pretreatment of eggs also caused a significant decrease in tyrosine phosphorylation of LD-DIM proteins after fertilization and H $_2$ O $_2$ treatment (Fig. 7D). Protein profile of LD-DIM as assessed by silver staining, however, was not affected by MCD (Fig. 7C).

To ascertain whether the Ca $^{2+}$ transient, a crucial step for

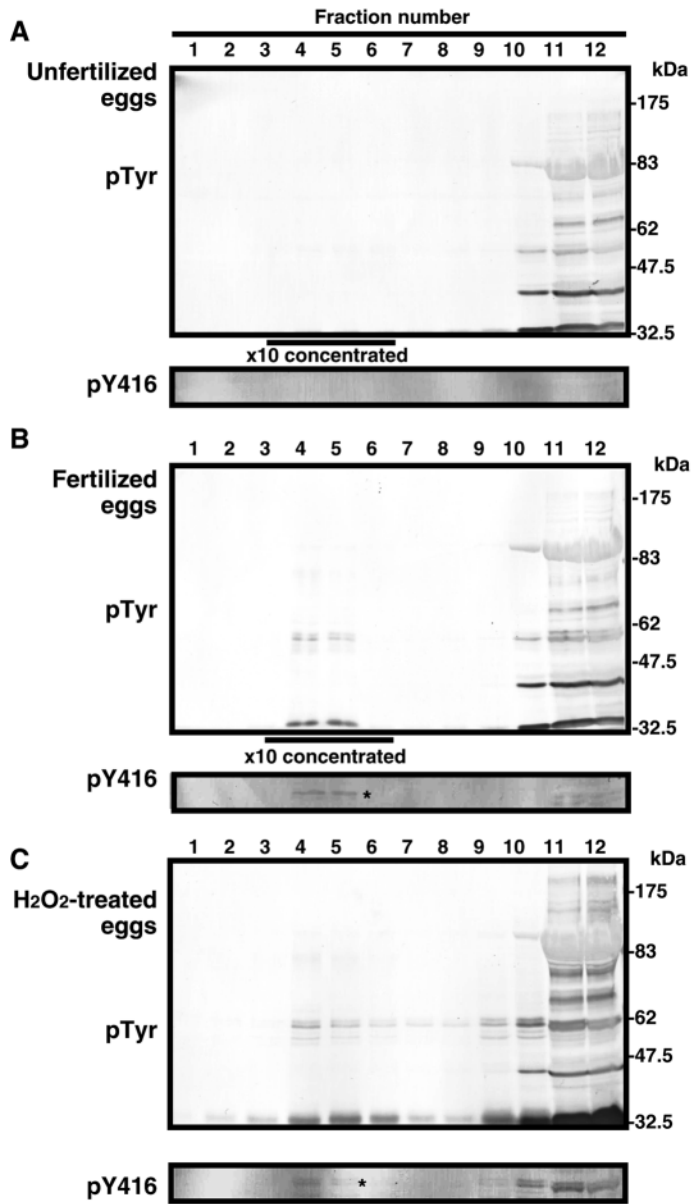


Fig. 4. Fertilization stimulates protein-tyrosine phosphorylation in LD-DIM fractions. Sucrose density gradient fractions (20 μ l of each fraction per lane) were prepared from (A) unfertilized, (B) fertilized (5 minutes after insemination) and (C) H₂O₂-treated (10 mM, 5 minutes) eggs and analyzed by immunoblotting with anti-phosphotyrosine antibody (pTyr, 1 μ g/ml of PY99) or anti-pY416 antibody (pY416, 5 μ g/ml). Fractions 3-6 of unfertilized and fertilized eggs were concentrated ten times before the experiment. Asterisks indicate the positions of tyrosine-phosphorylated Xyk.

egg activation, is abolished in MCD-treated eggs, we performed measurement of intracellular Ca²⁺ by using the Ca²⁺ indicator fura-2. We found that eight out of 13 albino eggs treated with MCD (25 mM) did not show sperm-induced Ca²⁺ response (Fig. 7E, MCD + sperm) while the remaining five MCD-treated eggs showed the normal response (data not shown) as control eggs did (Fig. 7E, control). The extent of the inhibition of Ca²⁺ release (about 60%) was comparable with the extent of inhibition of the formation of cleavage furrow

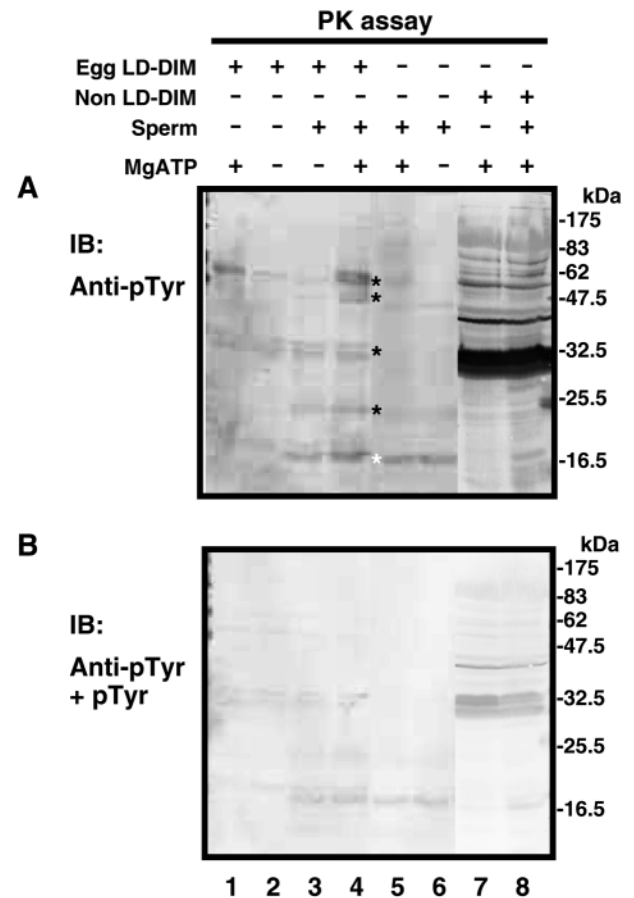


Fig. 5. Sperm stimulates protein-tyrosine phosphorylation in LD-DIM fractions in vitro. Aliquots (50 μ l) of LD-DIM fraction (lanes 1-4) or non LD-DIM fraction (lanes 7 and 8) from unfertilized eggs were preincubated in the absence or the presence of jelly water-treated sperm (10⁸ sperm/ml) and subjected to protein kinase assay with or without 5 mM MgCl₂ and 1 mM ATP as described in Materials and Methods. As a control, sperm alone was also examined for its kinase activity (lanes 5 and 6). Phosphorylated proteins were separated by SDS-PAGE and analyzed by immunoblotting with anti-phosphotyrosine antibody (PY99, 1 μ g/ml) without (A) or with (B) 10 mM L-phosphotyrosine. Black asterisks indicate the positions of sperm-dependent phosphorylation bands. A white asterisk indicates the position of a band that can be seen in the presence of sperm alone.

(about 50%) (Fig. 7A). All the results obtained with the use of MCD, however, could be due to toxicity of the compound. So, we analyzed artificial activation of the eggs treated with MCD. As shown in Fig. 7E (MCD + sperm + A23187), MCD-treated eggs that failed to activate by fertilization could still undergo transient Ca²⁺ rise in response to A23187 treatment (three out of three MCD-treated eggs). Consistently, all these artificially activated eggs showed elevation of fertilization envelope and cortical contraction (data not shown). So, we conclude that the fertilization defect in MCD-treated eggs is due to the absence of sperm-induced Ca²⁺ release within the eggs. This is consistent with previous results showing that sperm-induced tyrosine kinase signaling, which may act upstream of Ca²⁺ release, was abolished in MCD-treated eggs (Fig. 7B,D).

Then we asked why tyrosine kinase signaling was defected in MCD-treated eggs. We found that eggs treated with 25 mM

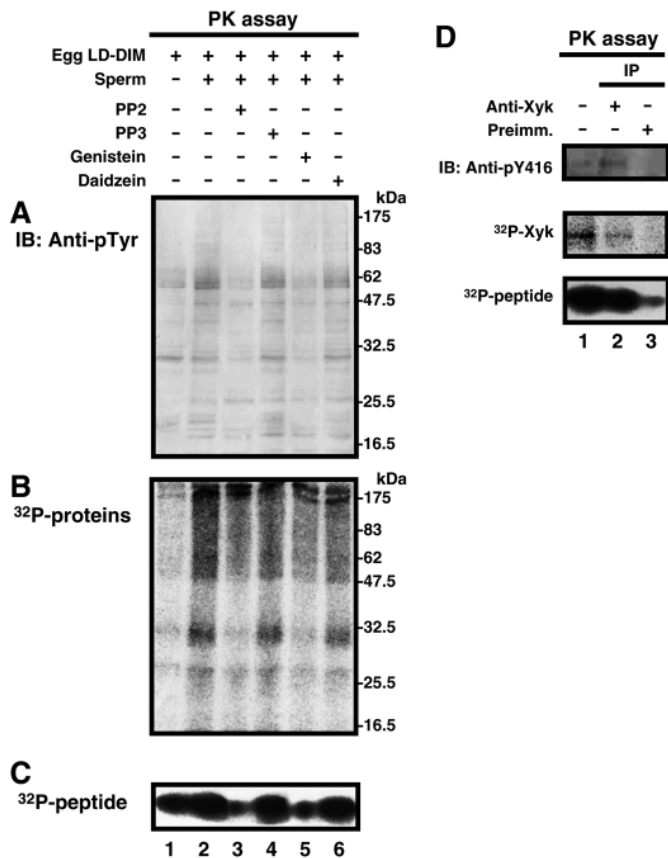


Fig. 6. Characterization of sperm-dependent PTK activity in egg LD-DIM fraction. (A) LD-DIM fraction (50 μ l) was preincubated in the absence or the presence of jelly water-treated sperm (10^8 sperm/ml). The samples were subjected to protein kinase assay in the presence of either DMSO alone (0.2%, lanes 1 and 2), 10 μ M PP2 (lane 3), 10 μ M PP3 (lane 4), 50 μ M genistein (lane 5) or 50 μ M daidzein (lane 6). (A) Phosphorylation was carried out with non-labeled ATP and analyzed by anti-phosphotyrosine immunoblotting as in Fig. 5. (B) Phosphorylation was carried out with [γ - 32 P]ATP and analyzed by BAS2000 phosphoimaging analyzer. (C) Phosphorylation was performed as in B in the presence of Cdc2 peptide. A phosphoimage for 32 P-labeled peptide is shown. (D) LD-DIM fraction was solubilized with extraction buffer containing 2% (v/v) n-octyl- β -D-glucoside (Wako). The samples (500 μ l) were immunoprecipitated with either anti-Xyk antibody (lane 2, 1 μ l of rabbit serum) or preimmune antibody (lane 3, 1 μ l of rabbit serum). The immunoprecipitates were subjected to protein kinase assay and analyzed by immunoblotting with anti-pY416 antibody (top panel). We also performed protein kinase assay of the immunoprecipitates in the presence of [γ - 32 P]ATP and Cdc2 peptide. Autophosphorylated Xyk (middle panel) and phosphorylated peptide (bottom panel) were visualized as in B. Intact LD-DIM fraction was used as a control in each assay (lane 1).

MCD showed a decrease of Xyk and integrin β 1, but not Ras nor Gq α , in LD-DIM (Fig. 7F). The cholesterol content in LD-DIM of MCD-treated eggs also showed about 25% decrease when compared with the control eggs (Fig. 7G). These results demonstrate that MCD cause a fertilization defect in eggs by affecting the composition of specific subset of lipid/protein that may account for the inhibition of tyrosine kinase activity in LD-DIM and subsequent egg activation.

All the defects in sperm-induced egg activation events might

be due to primarily an inability of sperm to interact with the MCD-treated eggs. So, we examined the effect of MCD treatment on the successful rate of sperm-egg binding/fusion, as judged by the appearance of sperm entry point in pigmented wild type *Xenopus* eggs (Palecek et al., 1978). We found that a significant number of MCD-treated eggs (seven out of 30 eggs) did not show sperm entry point and subsequent egg activation process, as judged by the occurrence of cortical contraction (Fig. 8). The result indicates that in these eggs, sperm-egg binding/fusion process is the primary target of MCD-mediated egg activation defect. The remaining 70% of MCD-treated eggs (23 of 30 eggs tested) showed sperm entry point (Fig. 8). However, these eggs showed only a limited rate of egg activation (14 of 23 eggs, Fig. 8). Total inhibitory rate of egg activation in MCD-treated eggs was 53% (16 of 30 eggs). The rate is comparable with that obtained for Ca $^{2+}$ release (ca. 60%, Fig. 7E, see above). Thus, the results suggest that MCD-mediated defect in sperm-induced egg activation is due to at least two mechanisms: one involves a defect in sperm-egg binding/fusion, and the other involves defect(s) in post sperm-egg binding/fusion process.

Finally, we addressed to the effect of repletion of cholesterol on the recovery of egg activation ability of MCD-treated eggs. We found that the rate of sperm entry and egg activation was significantly recovered in eggs added back with cholesterol (Fig. 8). The results show that MCD-induced removal of cholesterol is reversible event, and confirm again that MCD treatment under our experimental condition is not toxic for eggs.

DISCUSSION

In the present study, we have studied spatial and temporal characteristics of fertilization-dependent tyrosine phosphorylation of egg proteins in *Xenopus*. A concomitant activation of Xyk was also evaluated. Further analysis was performed with egg LD-DIM fractions prepared from unfertilized, fertilized or H $_2$ O $_2$ -treated eggs. The results obtained support an idea that LD-DIM is a subcellular microdomain for sperm-dependent PTK signaling in *Xenopus* egg fertilization. The signaling microdomain may have impact on the sperm-induced Ca $^{2+}$ transient and egg activation.

Fertilization-dependent PTK signaling has been reported in several model organisms. They include sea urchin (Ciapa and Epel, 1991; Wright and Schatten, 1995; Shearer et al., 1999; Kinsey and Shen, 2000; Abassi et al., 2000), ascidian (Ueki and Yokosawa, 1997; Runft and Jaffe, 2000), starfish (Giusti et al., 1999; Giusti et al., 2000), fish (Wu and Kinsey, 2000), frog (Sato et al., 1998), rat (Ben-Yosef et al., 1998), pig (Kim et al., 1999) and mouse (Dupont et al., 1996). However, none of these studies has uncovered the spatial characteristics of tyrosine phosphorylation of egg proteins. We analyzed rapid and transient tyrosine phosphorylation by immunoblotting and indirect immunofluorescent methods (Figs 1 and 2). The fertilization-induced tyrosine phosphorylation was evident in a part of the animal hemisphere of the egg cortex. It might be related to the fact that in *Xenopus*, fertilizing sperm prefers the animal half of the egg cortex than the vegetal half (Grey et al., 1982; Stewart-Savage and Grey, 1987). So, it is suggested that *Xenopus* egg cortex contains a specific subcellular domain for

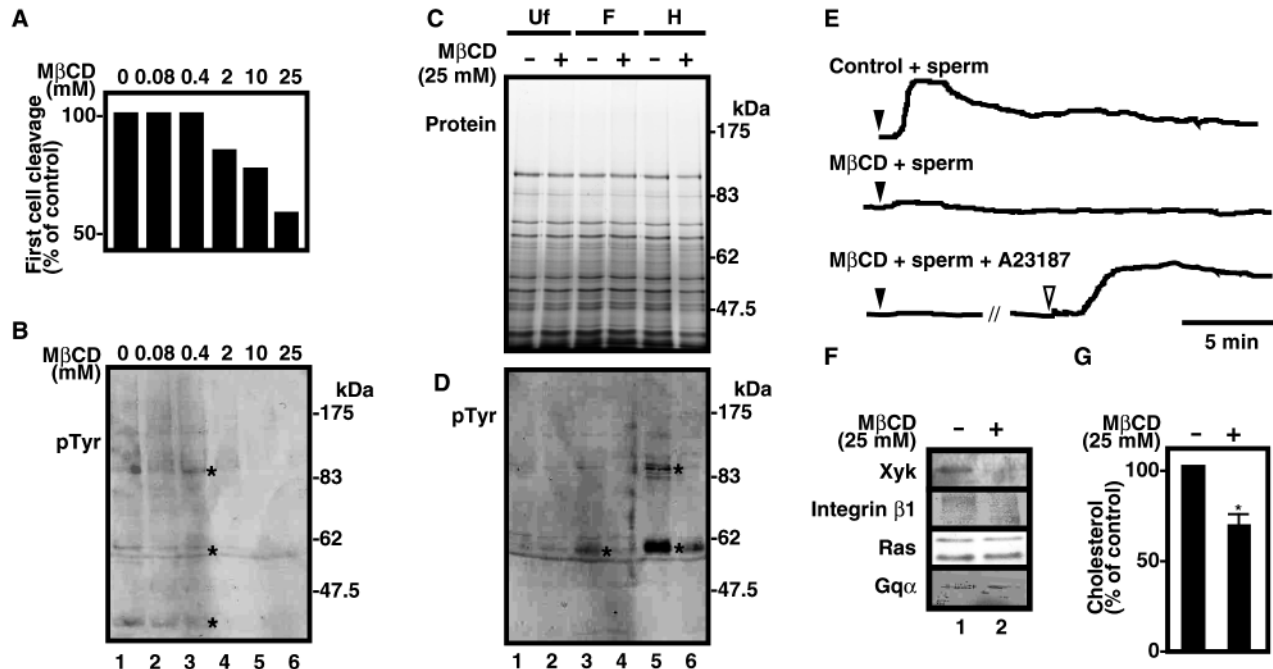


Fig. 7. Methyl- β -cyclodextrin (MCD) inhibits tyrosine kinase signaling and egg activation in fertilized *Xenopus* eggs. (A) *Xenopus* unfertilized eggs were pretreated with $1\times$ DB containing the indicated concentrations of MCD for 60 minutes, washed and inseminated. Appearance of the first cleavage furrow in the eggs was monitored for 100 minutes after insemination. More than 50 eggs were examined in each MCD concentration. (B) LD-DIM fraction was prepared from unfertilized eggs that had been treated with or without MCD as in A. The samples (50 μ l each) were subjected to protein kinase assay and analyzed by immunoblotting with anti-phosphotyrosine antibody (PY99, 1 μ g/ml). Asterisks indicate the positions of bands that disappear by high dose of MCD. (C) *Xenopus* unfertilized eggs were pretreated with or without 25 mM MCD for 60 minutes and then left untreated (Uf), or activated by either fertilization (F, 5 minutes of insemination) or H_2O_2 (H, 10 mM, 5 minutes). LD-DIM fractions (20 μ l per lane) were obtained from the egg samples and analyzed for their protein profile by silver staining. (D) LD-DIM fractions (20 μ l each) prepared as in C were analyzed by immunoblotting with anti-phosphotyrosine antibody. Asterisks indicate the positions of bands that disappear in the presence of MCD. (E) Eggs were injected with fura-2 and then pretreated with or without 25 mM MCD for 60 minutes. After the pretreatment, eggs were inseminated while recording the fluorescent ratio signal using a high-frame digital CCD imaging ARGUS/HISCA system (Hamamatsu Photonics, Japan). Shown are traces of the fura-2 signal as a function of time. Black arrowheads indicate the time at which insemination was started. In some experiments, A23187 (0.5 μ M) was applied after 30 minutes of insemination of MCD-treated eggs (white arrowhead). (F) LD-DIM fractions were prepared from untreated and MCD (25 mM)-treated unfertilized eggs and analyzed by immunoblotting with either anti-Xyk (100 \times dilution of antiserum), anti-integrin β 1 (100 \times dilution of antiserum), anti-Ras (0.5 μ g/ml) or anti-Gq α (2 μ g/ml) antibody. (G) LD-DIM fractions of untreated and MCD-treated eggs prepared as in F were assayed for their relative cholesterol content. The cholesterol content in the untreated egg LD-DIM was taken as 100%. Data represent mean \pm standard deviations of three independent trials. A bar with an asterisk is significantly different from the control. $P < 0.01$.

sperm binding and subsequent PTK activation. We used H_2O_2 -treated egg sample as a tyrosine phosphorylation-positive control because H_2O_2 has been shown to cause sustained tyrosine phosphorylation of egg proteins accompanied by egg activation-related processes (Sato et al., 2001). We also used eggs activated by Ca^{2+} ionophore, in which upregulation of egg PTK would not occur, as a negative control. Comparative analysis of these artificially activated eggs further characterized the localized nature of PTK signaling in eggs.

As a candidate of the egg subcellular domain for sperm-induced signaling in *Xenopus*, we isolated egg LD-DIM. As it has been reported for LD-DIM of other cell systems, the egg LD-DIM was enriched in cholesterol and a ganglioside GM1 (Fig. 3). The egg LD-DIM contained Xyk as well as other signaling proteins. The localization of Xyk to the egg LD-DIM fraction is consistent with the previous findings that Src family PTKs are identified in LD-DIM or caveolae fractions of the cells (Arni et al., 1996; Draberova and Draber, 1993; Field et al., 1997; Katagiri et al., 1999; Yamamura et al., 1997). It has

been reported that myristoylation and/or palmitoylation is important for the recruitment of Src family PTKs to LD-DIM (Kabouridis et al., 1997; Robbins et al., 1995; Shenoy-Scaria et al., 1994). Consistently, the myristoylation of Xyk has been demonstrated by mass spectroscopic analysis (Iwasaki et al., unpublished). However, some fertilization-related but unacylated proteins such as PLC γ (Sato et al., 2000) and Shc (Aoto et al., 1999) were not detected in LD-DIM. So, *Xenopus* egg LD-DIM involves a specific subset of proteins, although it contains very small amount of proteins (less than 0.1% of the total).

We show that fertilization-dependent protein-tyrosine phosphorylation could be detected in LD-DIM fraction. It involves tyrosine autophosphorylation of Xyk, as judged by immunoblotting with anti-phospho-specific (anti-pY416) antibody (Fig. 4). The results suggest that Xyk in the egg LD-DIM can be activated soon after sperm-egg interaction and contributes to the phosphorylation of other LD-DIM proteins. Similar results were obtained with the H_2O_2 -treated eggs

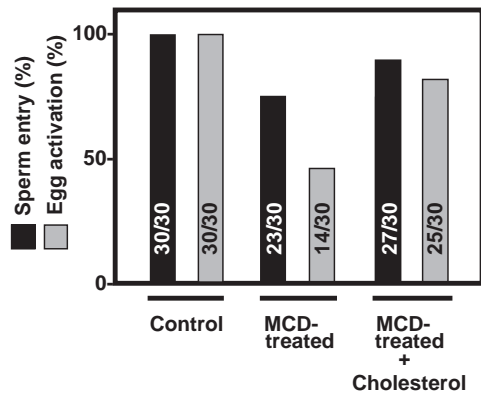


Fig. 8. Effect of MCD-mediated removal of cholesterol and its repletion on *Xenopus* egg fertilization. A group of *Xenopus* unfertilized eggs (30 eggs) were untreated (control), pretreated with 25 mM MCD (middle) or pretreated with 25 mM MCD then treated with cholesterol (right), and subjected to insemination as in Fig. 7A. The appearance of sperm entry point and cortical contraction (a marker of egg activation) were scored after microscopic observation. Number of positive eggs/total number of eggs tested is indicated.

(Fig. 4) where the sustained activation of Xyk has been demonstrated (Sato et al., 2001). The fertilization-dependent response was not so evident in the heavy sucrose fractions that contained detergent-soluble egg proteins. The LD-DIM-specific tyrosine phosphorylation may reflect the fact that fertilization-dependent tyrosine phosphorylation is limited to a part of the egg cortex (discussed above). In contrast, H_2O_2 -treated eggs contained tyrosine-phosphorylated proteins even in the heavy fractions. This might be related to the fact that tyrosine phosphorylation in H_2O_2 -treated eggs involves not only the egg cortex but also the cytoplasmic area (Sato et al., 2001).

The presence of functional PTK activity in the egg LD-DIM was shown by *in vitro* protein kinase assay (Fig. 5). To our surprise, the kinase activity was stimulated in the presence of sperm (Fig. 6). The results suggest that the egg LD-DIM contains a sperm-interacting molecule(s) that can lead to upregulation of PTK activity. The sperm-dependent PTK activity was effectively inhibited by genistein, a universal PTK inhibitor, and by PP2, a Src-specific inhibitor (Fig. 6). In addition, PTK activity in the egg LD-DIM could be immunoprecipitated with anti-Xyk antibody (Fig. 6). These data strongly suggest that Src PTK, i.e. Xyk, is the main sperm-dependent PTK in the egg LD-DIM. Ligand-induced activation of PTKs as well as phosphorylation of substrate proteins have been shown in LD-DIM *in vitro* (Stefanova et al., 1991; Thomas and Samelson, 1992; Morgan et al., 1993; Shenoy-Scaria et al., 1992; Liu et al., 1997). Our present findings highlight the possibility that sperm and egg LD-DIM can reconstitute at least in part signaling events in egg fertilization.

We used MCD to clarify further the functional and structural importance of the egg LD-DIM. This substance has been used for efficient and selective removal of cholesterol from several types of cells. It enables us to analyze the importance of LD-DIM in signal transduction (for a review, see Simons and Ikonen, 2000). MCD pretreatment of eggs was shown to inhibit fertilization (Fig. 7). The inhibitory effect was dependent on

the concentration of MCD. However, the effect was limited to about 50% inhibition even in the presence of 25 mM MCD. We used 25°C in MCD treatment because higher temperature will damage eggs (data not shown). Such low temperature treatment might affect the efficiency of cholesterol depletion. Under these conditions, however, we could detect some significant changes in the LD-DIM of MCD-treated eggs. A significant loss of cholesterol was demonstrated (Fig. 7). As indicated by *in vitro* kinase assay and/or immunoblotting analysis, MCD caused a reduction of PTK activity and protein-tyrosine phosphorylation in the LD-DIM fraction, although there was no detectable change in the protein profile. Such selective loss of PTK activity in the LD-DIM was found to involve a decrease of the LD-DIM Xyk. Several groups have reported MCD-mediated selective loss of PTK activity in cells (Huby et al., 1999; Sheets et al., 1999; Xavier et al., 1998; Moran and Miceli, 1998). In addition, more than half of the MCD-treated eggs tested did not show sperm-induced Ca^{2+} transient. At present, however, it is difficult to conclude that tyrosine kinase-dependent Ca^{2+} signaling pathway is a specific target of the MCD treatment, because we have observed that sperm-egg fusion, as judged by the appearance of sperm entry point, was also abrogated in some but not all MCD-treated and fertilization-defected eggs. Nevertheless, our studies using MCD suggest that the egg LD-DIM may play essential role in fertilization signaling of *Xenopus* eggs. Further study will be necessary to clarify a role of egg LD-DIM in sperm-egg binding and subsequent fusion processes.

Recent progress in molecular and cellular biology has allowed identifying multiple signaling pathways that involve complex array of information network created by signaling molecules. In the studies on fertilization, however, only a limited part of the signaling network has been established. Now, the involvement of egg PTKs, phospholipases and intracellular Ca^{2+} in egg activation is clear in several organisms (Sato et al., 2000b; Stricker, 1999). Very little is known, however, about the molecular identity of the egg plasma membrane receptor for sperm. It seems that each organism has common and species-specific mechanism of sperm-egg interaction and egg activation (for reviews, see Ciapa and Chiri, 2000; Iwao, 2000; Jaffe et al., 2001; Swann and Parrington 1999). In mammals, several egg molecules have been identified as sperm binding/fusion-related molecules: integrin $\alpha 6/\beta 1$ (Almeida et al., 1995), glycoposphatidylinositol-anchored proteins (Coonrod et al., 1999), CD9 (Kaji et al., 2000; Miyado et al., 2000; Le Naour et al., 2000), etc. Their homologs (integrin and CD9) have been identified in the *Xenopus* egg LD-DIM, so it will be interesting to analyze their function in egg fertilization. Alternatively, it is possible to conduct the comprehensive analysis of sperm interacting and/or egg activating molecules using the egg LD-DIM, where molecular machinery for sperm-induced PTK activation is preorganized. Similar approach has recently been initiated in the studies on the LD-DIM of sea urchin sperm (Ohta et al., 2000) and egg (Belton et al., 2001). Such comprehensive analysis will not require any knowledge about the unidentified molecules. Therefore, isolated egg LD-DIM in combination with cell-free experimental systems for analyzing egg activation and cell cycle regulation (Tokmakov et al., 2001) will be a powerful instrument for identifying fertilization-signaling molecules and for *in vitro* reconstitution of the fertilization signaling.

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