Uroplakin III, a novel Src substrate in *Xenopus* egg rafts, is a target for sperm protease essential for fertilization

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Abstract

In a previous study, we identified *Xenopus* egg uroplakin III (xUPIII), a single-transmembrane protein that localized to lipid/membrane rafts and was tyrosine-phosphorylated upon fertilization. An antibody against the xUPIII extracellular domain abolishes fertilization, suggesting that xUPIII acts not only as tyrosine kinase substrate but also as a receptor for sperm. Previously, it has been shown that the protease cathepsin B can promote a transient Ca\(^{2+}\) release and egg activation as seen in fertilized eggs (Mizote, A., Okamoto, S., Iwao, Y., 1999. Activation of *Xenopus* eggs by proteases: possible involvement of a sperm protease in fertilization. Dev. Biol. 208, 79–92). Here, we show that activation of *Xenopus* eggs by cathepsin B is accompanied by tyrosine phosphorylation of egg-raft-associated Src, phospholipase C\(\gamma\) (xPLC\(\gamma\)), and xUPIII. Cathepsin B also promotes a partial digestion of xUPIII both in vitro and in vivo. A synthetic xUPIII-GRR peptide, which contains a potential proteolytic site, inhibits the cathepsin-B-mediated proteolysis and tyrosine phosphorylation of xUPIII and egg activation. Importantly, this peptide also inhibits sperm-induced tyrosine phosphorylation of xUPIII and egg activation. Protease activity that digests xUPIII in an xUPIII-GRR peptide-sensitive manner is present in *Xenopus* sperm. Several protease inhibitors, which have been identified to be inhibitory toward *Xenopus* fertilization, are shown to inhibit sperm-induced tyrosine phosphorylation of xUPIII. Uroplakin Ib, a tetraspanin UP member, is found to be associated with xUPIII in egg rafts. Our results highlight novel mechanisms of fertilization signaling by which xUPIII serves as a potential target for sperm protease essential for fertilization. © 2005 Elsevier Inc. All rights reserved.

Keywords: Fertilization; Egg activation; Membrane microdomains; Src family kinases; Uroplakin III; Sperm proteases

Introduction

During fertilization, contact between egg and sperm initiates a series of numerous and rapid responses in the egg, collectively called ‘egg activation’. Signaling cascade leading to egg activation involves species-specific recognition, interaction, and fusion of egg and sperm and a sperm-induced transient increase(s) in the intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) within the egg cytoplasm (Evans and Florman, 2002; Runft et al., 2002; Santella et al., 2004). In *Xenopus laevis* eggs, fertilization involves a rapid and transient activation of the tyrosine kinase Src, termed xSrc. xSrc phosphorylates and activates phospholipase C\(\gamma\) (xPLC\(\gamma\)) (Sato et al., 1996, 1999, 2000), which in turn promotes hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP\(_3\)), a latter of which is a trigger of calcium release from the endoplasmic reticulum. Thus, it is likely that, in *Xenopus* eggs, sperm-induced transient increase in [Ca\(^{2+}\)]\(_i\) is a result of up-regulation of xSrc/xPLC\(\gamma\)/IP\(_3\) cascade. A similar cascade of events has been shown in other deuterostomes including sea urchin (Abassi et al., 2000; Belton et al., 2001; Giusti et al., 2003; Shearer et al., 1999, 2002; O’Neill et al., 2004; Runft et al., 2004), ascidian (Runft and Jaffe, 2000), and maybe zebrafish (Kinsey et al., 2003; Wu and Kinsey, 2002). On the other hand, a distinct signaling pathway that may involve a sperm-derived PLC\(\xi\) (Saunders et al., 2002), but not Src-dependent phosphorylation (Kurokawa et al., 2004; Mehlmann and Jaffe, 2005), seems to operate for the transient calcium release in the mouse eggs during fertilization.

Abbreviations: [Ca\(^{2+}\)]\(_i\), intracellular calcium concentration; xSrc, *Xenopus* Src; xPLC\(\gamma\), *Xenopus* phospholipase C\(\gamma\); IP\(_3\), inositol trisphosphate; xUPIII, *Xenopus* uroplakin III; UP, uroplakin; xUPIIIb, *Xenopus* uroplakin Ib; APMSF, (p-amidinophenyl)methanesulfonyl fluoride hydrochloride.

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In unfertilized Xenopus eggs, xSrc localizes to the egg lipid/membrane microdomains, so called rafts (Sato et al., 2002; for review, see Anderson, 1998; Brown and London, 1998; Simons and Ikonen, 1997). On the other hand, xPLCγ localizes mainly to the cytosolic fraction. After fertilization, xPLCγ is rapidly and transiently translocated to rafts, whereby it can be phosphorylated and activated by the raft-associated xSrc (Sato et al., 2003). Therefore, egg rafts may act as platform for sperm contact and subsequent xSrc/xPLCγ activation. In fact, sperm-dependent tyrosine phosphorylation during fertilization occurs predominantly in the egg rafts, and sperm can activate the raft-associated xSrc in vitro (Sato et al., 2002; Sato et al., 2003). These results suggest that the egg rafts contain receptor(s) for sperm, which can connect the sperm signal to xSrc activation.

More recently, we have identified a novel raft-associated protein, uroplakin III (UPIII) that is tyrosine-phosphorylated upon fertilization (Sakakibara et al., 2005). In mammals, UPIII is known as a member of urothelial tissue-specific UP proteins that consist of four members: UPIa, UPIb, UPIII, and UPIV, and that are responsible for permeability control of the apical surface of the urothelial membranes (Liang et al., 2001; Wu et al., 1994; Yu et al., 1994). The mammalian UPs are also known as a host cell receptor for pathogenic bacteria in urinary tract infection (Duncan et al., 2004; Wu et al., 1996; Zhou et al., 2001). Xenopus UPIII (xUPIII) is a single-transmembrane protein that consists of the amino-terminal N-glycosylated extracellular domain, transmembrane domain, and the carboxyl-terminal cytoplasmic domain containing a tyrosine phosphorylation site (Tyr249) (Sakakibara et al., 2005). Physiological importance of xUPIII is suggested by the fact that a specific antibody against the extracellular domain of xUPIII inhibits fertilization (Sakakibara et al., 2005). Here, we address further the functional importance of xUPIII in fertilization signaling. Previously, involvement of sperm-derived protease in Xenopus egg fertilization has been suggested (Iwao et al., 1994, 1995; Mizote et al., 1999). In these studies, the acrosomal serine protease activity has been shown to be essential for sperm-induced egg activation. Interestingly, it has also been shown that exogenously applied protease cathepsin B can promote a transient Ca$^{2+}$ release in Xenopus eggs. However, molecular mechanism by which protease promotes Ca$^{2+}$ release and activation in Xenopus eggs is unknown. Interestingly, a similar protease-dependent egg activation has also been demonstrated in starfish (Carroll and Jaffe, 1995). Therefore, we addressed a specific question as to whether protease-induced Xenopus egg activation involves xUPIII function. Results obtained suggest a novel scheme, in which the extracellular domain of xUPIII serves as a target for sperm protease(s) essential for initiation of egg activation.

Materials and methods

Materials

Antibodies against the recombinant xUPIII extracellular domain (xUPIII ED) or carboxyl-terminus peptide (xUPIII CT) were prepared as described previously (Sakakibara et al., 2005). Antibodies against a part of xSrc amino-terminal region (residues 22–36, EGSHQPPFTSLSASQ) or kinase domain (residues 409–427, LIEDEYNTARQGAKFPKIKW) and an antibody against the carbboxyl-terminus of Xenopus uroplakin Ib (xUPIb CT, residues 241–259, CWTFWLLGSMMHYTTRIEY) were raised in rabbits according to the described method (Harlow and Lane, 1988). Aforementioned peptide was also used as xUPIII-GRR peptide (residues 177–191, SSGTIDTPWPGRSSG) that was obtained from Bex Cooperation (Tokyo, Japan). Anti-phosphotyrosine antibody PY99 was from Santa Cruz (CA, USA). Anti-xPLCγ antibody was from Upstate Biotechnology (NY, USA). Anti-phosphorylated MAP kinase antibody was from BioLabs (MA, USA). Anti-phosphorylated Tyr418 of Src antibody and anti-prohibitin antibody were from Oncogene Research Products (CA, USA). H$_2$O$_2$ was from Santoku Chemical Industries (Tokyo, Japan). A23187 and bovine spleen cathepsin B were from Sigma (MO, USA). Leupeptin and Boc-Gly-Arg-Arg-4-methylcoumarinyl-7-amide (Boc-Gly-Arg-Arg-MCA) were from Peptide Institute (Osaka, Japan). Aprotinin, (p-amidinophenyl)methanesulfonyl fluoride hydrochloride (APMSF), chymostatin, and pepstatin were from Calbiochem (San Diego, CA). Protein A–Sepharose was obtained from Amersham Biosciences (Uppsala, Sweden). Unless otherwise indicated, other chemicals were purchased from Sigma, Wako (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan).

Animals, gametes, and embryos

Maintenance of frogs, collection of eggs and sperm, removal of jelly layer from eggs, and jelly water treatment of sperm were carried out as described previously (Sato et al., 2000, 2002). Activation of jelly-layer-free eggs was done by insemination with jelly-water-treated sperm (10$^6$–10$^7$ sperm/ml) or by parthenogenetic activation with the calcium ionophore A23187 (0.5 μM). H$_2$O$_2$ (10 mM), or cathepsin B (5 U/ml) for the times as specified in the text. After the activation treatments, egg samples were washed with ice-cold DeBoer’s solution containing 110 mM NaCl, 1.3 mM KCl, and 0.44 mM CaCl$_2$, pH 7.2, immediately frozen in liquid nitrogen, and kept at −80°C. Activation was monitored by the occurrence of cortical contraction and first cell cleavage in a group of control eggs, and those batches showing more than 90% activation were used for experiments. In some experiments, egg activation treatment was done in the presence of synthetic peptide, purified IgG, or protease inhibitors/substrate. When the effect of protease inhibitors/substrate was examined, the jelly-water-treated sperm were pretreated with these materials for 15 min at room temperature and subjected to insemination. The mammalian UPs are also known as a host cell receptor for pathogenic bacteria in urinary tract infection (Duncan et al., 2004; Wu et al., 1996; Zhou et al., 2001). Xenopus UPIII (xUPIII) is a single-transmembrane protein that consists of the amino-terminal N-glycosylated extracellular domain, transmembrane domain, and the carboxyl-terminal cytoplasmic domain containing a tyrosine phosphorylation site (Tyr249) (Sakakibara et al., 2005). Physiological importance of xUPIII is suggested by the fact that a specific antibody against the extracellular domain of xUPIII inhibits fertilization (Sakakibara et al., 2005). Here, we address further the functional importance of xUPIII in fertilization signaling. Previously, involvement of sperm-derived protease in Xenopus egg fertilization has been suggested (Iwao et al., 1994, 1995; Mizote et al., 1999). In these studies, the acrosomal serine protease activity has been shown to be essential for sperm-induced egg activation. Interestingly, it has also been shown that exogenously applied protease cathepsin B can promote a transient Ca$^{2+}$ release in Xenopus eggs. However, molecular mechanism by which protease promotes Ca$^{2+}$ release and activation in Xenopus eggs is unknown. Interestingly, a similar protease-dependent egg activation has also been demonstrated in starfish (Carroll and Jaffe, 1995). Therefore, we addressed a specific question as to whether protease-induced Xenopus egg activation involves xUPIII function. Results obtained suggest a novel scheme, in which the extracellular domain of xUPIII serves as a target for sperm protease(s) essential for initiation of egg activation.

Subcellular fractionation

Eggs were extracted and subjected to raft preparation as described previously (Sato et al., 2002). Briefly, eggs were homogenized with 5-fold volume of ice-cold extraction buffer containing 20 mM Tris–HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM Na$_2$VO$_4$, 10 μg/ml leupeptin, and 20 μM APMSF. The homogenates devoid of debris were brought to be 42.5% (w/v) sucrose and centrifuged at 200,000 × g for 24 h in an SW55 rotor (Beckman). After the centrifugation, aliquots of 12 fractions were collected from the top to the bottom of the tubes. Fractions 3–6 were pooled as rafts, whereas fractions 9–12 were pooled as non-rafts. Protein concentrations were determined by the dye-binding assay (Bio-Rad, CA, USA).

Immunoprecipitation, SDS-PAGE, and immunoblotting

Protein samples (50–500 μg, 1 mg/ml) were immunoprecipitated with antibodies as specified in the text for 3–6 h at 4°C. The immune complexes were adsorbed onto 10 μl of protein A–Sepharose beads by gentle agitation for 30 min at 4°C. The beads were washed three times with 500 μl of buffer containing 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 50 mM Tris–HCl, pH 7.5, 1 mM Na$_2$VO$_4$, 10 μg/ml leupeptin, and 20 μM APMSF. The washed beads were treated with Laemmli’s SDS sample
buffer (Laemmli, 1970) at 98°C for 5 min. SDS-denatured proteins were separated by SDS-PAGE and analyzed by immunoblotting or silver stain as described previously (Sato et al., 2002).

Proteolytic digestion of the egg-raft-associated proteins

Rafts prepared as above (equivalent to 1 ml eggs) were concentrated and suspended with 400 μl of 50% Steinberg’s solution containing 29 mM NaCl, 0.33 mM KCl, 0.425 mM MgSO₄, 0.17 mM CaCl₂, 2.3 mM Tris–HCl, pH 7.4. The suspension was then mixed with 400 μl of 50% Steinberg’s solution supplemented with cathepsin B (0.2–10 U/ml) and incubated for the times indicated in the text at 21°C. After the treatments, the mixtures were diluted with H₂O and centrifuged at 300,000 x g for 10 min at 4°C. After centrifugation, the supernatants were collected as soluble fractions, and the pellets were collected as insoluble raft fractions. Proteolytic digestion of the raft-associated proteins was analyzed by silver stain and immunoblotting. In some experiments, protease treatments were done with Xenopus sperm extracts (see below) or heat-inactivated (95°C, 30 min) cathepsin B as a source of protease. Some protease experiments were performed in the presence of synthetic peptide or purified IgG.

Preparation of sperm extracts

Xenopus sperm in DeBoer’s solution were centrifuged at 1000 x g for 10 min at 4°C. The sediment sperm (2 x 10⁶) were suspended in 100 μl of 10% Steinberg’s solution and frozen once at –80°C. The thawed sample was centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was collected and centrifuged at 300,000 x g for 60 min at 4°C. The supernatant was collected, stored at –80°C, and used as the Xenopus sperm extracts.

Data processing and presentation

All results are representative of more than three independent experiments. Images were processed with the use of software Adobe Photoshop® and Adobe Illustrator® (Adobe Systems, WA, USA).

Results

Cathepsin B promotes tyrosine phosphorylation of xSrc, xPLCγ, and xUPIII in Xenopus egg rafts

It has been shown that the protease cathepsin B triggers a calcium transient that is similar to that induced by sperm (Mizote et al., 1999). The cathepsin-B-induced egg activation as well as normal fertilization can be blocked by synthetic cathepsin-B-inhibitor, indicating that sperm protease may be essential for signaling events of fertilization. Therefore, we wanted to examine whether cathepsin-B-induced egg activation accompanies tyrosine phosphorylation in the egg rafts. We performed immunoblotting with anti-phosphotyrosine antibody, PY99, of egg raft proteins that had been prepared from differently activated eggs. One tyrosine-phosphorylated protein at 30 kDa could be detected in fertilized egg rafts, while no band was visible in the rafts of unfertilized or A23187-activated egg samples, in which xSrc activation did not occur (Sato et al., 1999) (Fig. 1A, IB: pTyr). On the other hand, a strong signal for the 30-kDa protein was detected in egg samples activated by H₂O₂ that caused strong xSrc activation (Sato et al., 2002). Cathepsin B was also shown to promote tyrosine phosphorylation of the 30-kDa protein and some other proteins such as a 37-kDa protein and 55–60-kDa proteins in the egg rafts (Fig. 1A, IB: pTyr). Immunoprecipitation and immunoblotting confirmed that xUPIII, which migrated at 30 kDa on SDS-PAGE, was tyrosine-phosphorylated not only in fertilized eggs and H₂O₂-activated eggs, but also in cathepsin-B-treated eggs (Fig. 1A, IB: p-xUPIII). Tyrosine phosphorylation of xUPIII was always accompanied by tyrosine phosphorylation of xSrc Tyr₄¹⁸, an indicative of xSrc activation (Fig. 1A). Concomitantly, translocation of xPLCγ from non-rafts to the rafts and its tyrosine phosphorylation were also observed (Fig. 1A). Tyrosine phosphorylation of xUPIII in cathepsin-B-treated eggs occurred within 30 s of the treatment and declined after 5 min of activation (Fig. 1B). This was rapid as in H₂O₂-activated eggs (Fig. 1B). Its transient feature was similar to that seen in fertilized eggs (Fig. 1B). Thus, cathepsin B promotes phosphorylation of egg-raft-associated proteins including xUPIII as seen in fertilized eggs.

Cathepsin B promotes partial digestion of xUPIII that is sensitive to xUPIII-GRR peptide

Initial event of cathepsin-B-induced egg activation may be digestion of cell surface molecules. We, therefore, wanted to examine whether xUPIII could be a proteolytic target of cathepsin B. In Fig. 1, we did not see any proteolytic fragment of xUPIII in any egg activation condition. This could be due to that we performed immunoprecipitation of xUPIII, which would eliminate the recovery of the proteolyzed xUPIII fragments. By performing direct immunoblotting of raft fractions with anti-xUPIII antibody, we could conclude that this assumption was right. In vitro cathepsin B treatment of rafts resulted in digestion of some raft-associated proteins (Fig. 2A, silver stain). Among these proteins, xUPIII showed a partial digestion by cathepsin B (Fig. 2A, IB: xUPIII), while xSrc was not digested (Fig. 2A, IB: xSrc). It should be noted here that, in general, direct blot of xUPIII in the egg raft fractions shows multiple band appearance (Fig. 2A), while immunoprecipitation–immunoblot of xUPIII would result in an appearance of a discrete one (see Fig. 1A). We think this is because immunoprecipitation may enrich intact form of xUPIII; in fact, proteolyzed fragments of xUPIII were hardly detectable by immunoprecipitation (Fig. 1). Cathepsin B digested xUPIII in a dose-dependent manner, and heat-inactivated cathepsin B was without effect (Fig. 2B). Cathepsin B treatment produced anti-xUPIII-ED immunoreactive fragments at 21–27 kDa, which might reflect the extracellular portion of xUPIII. Consistently, an antibody against the carboxyl-terminus of xUPIII did not recognize these fragments (data not shown). Fragments of xUPIII were not detectable in the soluble/supernatant fractions (Fig. 2A, IB: xUPIII). This indicates that the extracellular fragments of xUPIII were associated with other raft molecules. Cathepsin-B-induced egg activation can be inhibited by synthetic substrates containing Arg–Arg sequence (Mizote et al., 1999), a preferential motif for proteolysis by cathepsin B. The extracellular domain of xUPIII contains this dibasic motif (Arg₁₈⁷–Arg₁₈₈) in a close proximity to the transmembrane domain. We found that a 15-amino-acid synthetic peptide that...
covers the Arg^{187}–Arg^{188} sequence, termed xUPIII-GRR peptide, completely inhibited the proteolysis of xUPIII (Fig. 2B) and other raft-associated proteins (data not shown) with cathepsin B.

**Inhibition of fertilization and cathepsin-B-induced egg activation by xUPIII-GRR peptide or anti-xUPIII ED antibody**

Results obtained in Figs. 2A and B prompted us to examine whether egg activation involves proteolysis of xUPIII and whether xUPIII-GRR peptide can affect egg activation. In this experiment, we employed immunoprecipitation–immunoblotting scheme to detect tyrosine phosphorylation of xUPIII and direct immunoblotting of xUPIII to detect its proteolysis. Tyrosine phosphorylation of xUPIII in fertilized eggs was inhibited by xUPIII-GRR peptide (Fig. 3A, IB: p-xUPIII). In fertilized eggs, however, proteolysis of xUPIII was not detected (Fig. 3A, IB: xUPIII). In H_{2}O_{2}-treated eggs, xUPIII-GRR peptide did not inhibit tyrosine phosphorylation of xUPIII, and no digestion of xUPIII was detected. On the other hand, cathepsin-B-induced egg activation involved tyrosine phosphorylation as well as partial proteolysis of xUPIII that could be inhibited by xUPIII-GRR peptide (Fig. 3A). Analysis of the 40-min activated samples demonstrated that xUPIII-GRR peptide inhibited egg activation of not only cathepsin-B-treated eggs but also fertilized eggs, as judged by the dephosphorylation of 42-kDa MAP kinase (Fig. 3A, IB: p-MAPK). Egg activation in the presence of xUPIII-GRR peptide was also analyzed by monitoring cortical contraction and first cell cleavage (Table 1). The inhibitory effect of xUPIII-GRR peptide on sperm- or cathepsin-B-induced egg activation was dose-dependent and specific because control peptide xUPIII-CT did not show such an effect (Table 1). H_{2}O_{2}-induced egg activation was again insensitive to xUPIII-GRR peptide (Fig. 3A and Table 1). Our previous study has demonstrated that the anti-xUPIII ED antibody inhibits fertilization, as judged by the occurrence of cortical contraction (Sakakibara et al., 2005). Consistently, the anti-xUPIII ED antibody inhibited sperm-induced tyrosine phosphorylation of xUPIII and dephosphorylation of MAP kinase (Fig. 3B, IB: p-xUPIII, p-MAPK). On the other hand, the anti-xUPIII ED antibody did not inhibit phosphorylation events by cathepsin B maybe because it could block proteolysis of xUPIII only partially (Fig. 3B).
Xenopus sperm possess protease activity that can digest xUPIII and is essential for fertilization

In Figs. 3A and B, we failed to detect proteolysis of xUPIII in fertilized eggs. We assumed that this could be due to a localized nature of the xUPIII digestion in fertilized eggs, while cathepsin B could promote it on the entire egg surface. To evaluate this assumption, we performed in vitro proteolysis assay with use of crude extracts of *Xenopus* sperm. We employed the sperm extract instead of intact sperm in this experiment because we thought that sperm-derived protease, if present, would be efficiently analyzed after extraction in a concentrated manner. As shown in Fig. 4A, the sperm extracts promoted proteolysis of xUPIII as cathepsin B did; fragments obtained were 21, 23.5, and 27 kDa, which were similar to those obtained with cathepsin B. In general, proteolysis of xUPIII by the sperm extract was not effective as that by cathepsin B. In fact, proteolysis of whole egg raft proteins by the sperm extract, evaluated by silver stain as in Fig. 2A, was hardly detectable (data not shown). As in the case of cathepsin B, however, sperm-extract-mediated digestion of xUPIII was completely inhibited by xUPIII-GRR peptide (Fig. 4A), while control peptide (xUPIII CT peptide) did not show such an effect. Anti-xUPIII ED antibody, which could partially block cathepsin-B-mediated proteolysis of xUPIII, but not anti-xUPIII CT antibody, was also shown to block proteolysis of xUPIII by the sperm extract (Fig. 4A). These results strongly argue that the sperm extracts contain a protease activity that can digest xUPIII in a similar manner to cathepsin B.

We next performed insemination of eggs with sperm pretreated with one of several different protease inhibitors that had been reported to be inhibitory or non-inhibitory toward normal fertilization in *Xenopus* (Mizote et al., 1999). We evaluated the effect of these protease inhibitors on tyrosine phosphorylation of xUPIII and dephosphorylation of MAPK, which should occur early (within minutes of insemination, see Fig. 1B) and late (within 40 min of insemination, see Fig. 3) upon successful fertilization, respectively. As shown in Fig. 4B, both of the sperm-induced egg activation events were inhibited by Boc-Gly-Arg-Arg-MCA, a competitive inhibitor/substrate of cathepsin B, and the serine protease inhibitors aprotinin, APMSF, and leupeptin, and an aspartic protease inhibitor pepstatin. On the other hand, chymostatin, another serine protease inhibitor, which had been shown to be inert in fertilization (Mizote et al., 1999), did not show such an inhibitory effect (Fig. 4B). These results demonstrate that sperm-derived protease activity essential for normal fertili-
tion is required for xUPIII phosphorylation and MAPK dephosphorylation.

xUPIb, a tetraspanin molecule, is in association with xUPIII in Xenopus egg rafts

In mammals, UPIII is present as a heterodimer with uroplakin Ib (UPIb), a tetraspanin member of UPs (Liang et al., 2001; Tu et al., 2002). To test whether Xenopus UPIb (xUPIb) is expressed in egg rafts, we performed immunoblotting of raft and non-raft fractions of unfertilized eggs. xUPIb was expressed as a 20-kDa protein, and a large part of it co-localized with xUPIII in the raft fractions (Fig. 5A). Immunoprecipitation study demonstrated that xUPIb was in association with xUPIII (Fig. 5B). This interaction is specific and not due to the co-precipitation of insoluble raft molecules because prohibitin, a 28-kDa protein enriched in the egg rafts (data not shown), was not detected in the same anti-xUPIII immunoprecipitates (Fig. 5B).

Discussion

Multiple processes of fertilization may involve sperm or egg protease activity (for review, see Iwao, 2000): 1) acrosome reaction in the sperm (Infante et al., 2001; Ueda et al., 2002); 2) sperm binding to and/or passage through the egg jelly and/or the vitelline coat (Lindsay and Hedrick, 2004; Lindsay et al., 1999; Tian et al., 1997); 3) egg–sperm membrane contact and/or fusion (Mizote et al., 1999; Shilling et al., 1997); and 4) post-egg activation events (Haley and Wessel, 2004; Lindsay and Hedrick, 1989). Of particular interest is the report by Mizote et al. (1999) who showed that cathepsin B and sperm protease(s) were capable of activating Xenopus eggs. In that report, however, molecular details of the protease action remained unclear. In the present study, we show that cathepsin B promotes rapid and transient tyrosine phosphorylation of

Table 1

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<tr>
<th>Peptides</th>
<th>% egg activation (activated eggs/eggs examined)</th>
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<tr>
<td></td>
<td>10⁶ sperm/ml</td>
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<tr>
<td>xUPIII-GRR</td>
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<tr>
<td>0.1 mM</td>
<td>83 (25/30)</td>
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<tr>
<td>1.0 mM</td>
<td>27 (8/30)</td>
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<tr>
<td>xUPIII-CT</td>
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<tr>
<td>0.1 mM</td>
<td>90 (27/30)</td>
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<tr>
<td>1.0 mM</td>
<td>93 (28/30)</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>97 (29/30)</td>
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* X. laevis unfertilized eggs were inseminated and parthenogenetically activated in the absence (PBS alone) or the presence of synthetic peptide dissolved in PBS. Successful egg activation was scored by the occurrence of cortical contraction, a cytological marker that could be observed under dissecting microscope.

b Five unit/ml of cathepsin B was used.

c Ten micromolars of H₂O₂ was used.

d N.D., not determined.
xUPIII concomitantly with phosphorylation of xSrc and xPLCγ in egg rafts, as sperm do in normal fertilization. Mizote et al. (1999) have also demonstrated that sperm protease essential for fertilization is not cathepsin B because of their different sensitivity to inhibitors. Under this background, we employed further cathepsin B as a model system to learn what kind of molecular events is operating in protease-induced egg activation in *Xenopus*, with a special focus on xUPIII. Results obtained revealed that xUPIII could be proteolyzed by both cathepsin B and sperm-derived protease(s) and that both proteolytic events could be blocked by a synthetic xUPIII-GRR peptide. Importantly, the xUPIII-GRR peptide could inhibit normal egg fertilization as well as cathepsin-B-induced egg activation. Several protease inhibitors, which had been shown to be inhibitory toward normal fertilization, were shown to block tyrosine phosphorylation of xUPIII in response to insemination as well as egg activation as judged by dephosphorylation of MAPK. Interestingly, as inferred before by Mizote et al. (1999), not only cathepsin-B-like enzyme but also several other kinds of protease seem to be important for sperm-induced tyrosine phosphorylation of xUPIII and dephosphorylation of MAPK. Protease activity of sperm essential for fertilization may include serine, aspartic, and/or maybe cysteine protease, and their activity is essentially trypsin-based. Taken together, our results suggest that *Xenopus* fertilization involves an action of sperm protease(s), one of the crucial targets of which can be xUPIII (Fig. 6). We do not have experimental data as to where sperm protease is actively present for egg activation. It is likely that the protease is present in the acrosomal contents and/or on the post-acrosomal membrane because prior to contact with egg plasma membrane sperm must undergo acrosome reaction (Ueda et al., 2002).

The fact that xUPIII-GRR peptide, but not a control peptide (xUPIII-CT peptide), inhibits proteolysis of xUPIII suggests that the proteolytic event occurs in the amino acid sequence containing the Gly186–Arg187–Arg188 motif, which locates just near the extracellular face of the transmembrane domain of xUPIII. This motif resides in the ‘conserved domain’ of UPIII (Deng et al., 2002), which is highly conserved among UPIIs identified in different species (human, bovine, mouse, rat, and

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**Fig. 4.** *Xenopus* sperm possess protease activity toward xUPIII that is essential for tyrosine phosphorylation of xUPIII and egg activation. (A) In vitro proteolysis of the raft-associated xUPIII by cathepsin B or *Xenopus* sperm extracts. In the left panel, unfertilized egg rafts (2 μg/assay) were subjected to in vitro cathepsin B treatment (5 U/ml, 30 min) in the absence or the presence of one of the indicated synthetic peptides (each at 1 mM) or antibodies (each at 400 μg/ml). After the treatments, the raft fractions were collected and analyzed by immunoblotting with anti-xUPIII ED antibody. In the right panel, a similar experiment as in left panel was done with use of sperm extracts (1 mg/ml, 12 h). (B) Inhibition of sperm-induced egg activation events by a synthetic cathepsin B substrate and protease inhibitors. Fertilization was performed in the absence or the presence of several protease inhibitors: aprotinin (10 μM), APMSF (5 mM), chymostatin (1 mM), leupeptin (2 mM), or pepstatin (1 mM). In the upper panel, tyrosine phosphorylation of xUPIII was analyzed as in Fig. 1A using the raft fractions (10 μg/lane) prepared from 5-min inseminated eggs. In the lower panel, tyrosine phosphorylation of p42 MAPK was analyzed as in Fig. 3 by using non-raft samples (50 μg/lane) prepared from 40-min inseminated eggs.

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**Fig. 5.** Co-localization of xUPIII and xUPIb in the rafts of *Xenopus* eggs. (A) Unfertilized *Xenopus* eggs were subjected to raft preparation, and fractions obtained (no. 1 – 12, 20 μl/lane that is equivalent to about 5 eggs/lane) were analyzed by immunoblotting with antibodies against xUPIb or xUPIII. Arrowheads indicate the positions of each protein band. (B) Raft fractions (10 μg/lane) were solubilized by 0.1% SDS and immunoprecipitated with either preimmune serum (Preimm) or anti-xUPIII antibody. The immunoprecipitates were analyzed by immunoblotting with the antibodies indicated. Input, raft fractions (1 μg/lane). NRS, normal rabbit serum.
Until now, however, no direct evidence has been available as to its functional significance (Wu and Sun, 1993). Thus, our present study has suggested for the first time functional role of the Gly186–Arg187–Arg188 motif as a proteolytic segment in xUPIII molecule. It should be noted, however, that proteolysis of xUPIII by cathepsin B or sperm extract produced multiple fragments of 21–27 kDa, indicating that proteolysis occurred in multiple regions. In the extracellular domain of xUPIII, 13 potential tryptic cleavage sites (arginine or lysine residue) are present other than the Gly186–Arg187–Arg188 segment; one of which is another dibasic segment Ala74–Lys75–Lys76 (Sakakibara et al., 2005). Moreover, in the light of the fact that the cathepsin B substrates Boc-Gly-Arg-Arg-MCA and xUPIII-GRR peptide are inhibitory toward cleavage as well as tyrosine phosphorylation of xUPIII, it should also be noted that another kind of the serine protease, such as plasminogen activator, can be involved in the proteolysis (Andreasen et al., 1997). Further study should clarify molecular identity of sperm protease and which segment(s) is digested on xUPIII molecule.

Tyrosine phosphorylation but not proteolysis of xUPIII was detectable in fertilized eggs. Therefore, we suggest that proteolysis of xUPIII, if any, is relatively minor event limited at the egg–sperm contact site and that tyrosine phosphorylation of intact xUPIII is a downstream propagative event of proteolysis-mediated xSrc activation (Fig. 6). Protease activity used for egg activation in normal fertilization seems to be much lower than that used in cathepsin-B-induced egg activation (5 U/ml). Under this condition, however, the extent of tyrosine phosphorylation of xUPIII in cathepsin B-treated eggs was comparable to that detected in fertilized eggs, where no xUPIII proteolysis was detectable. Thus, protease action cannot be solely responsible for triggering tyrosine phosphorylation of xUPIII. We suggest that sperm may employ a mechanism(s) to effect its protease action as it has been reported that sperm–egg interaction at the plasma membranes may involve disintegrin–integrin molecular interaction (Iwao and Fujimura, 1996; Sato et al., 1999; Shilling et al., 1997, 1998). Thus, it is attractive to suggest that sperm protease action is reinforced by a rigid molecular interaction between sperm and egg plasma membranes, which enables the protease activity from a single spermatozoon to be sufficient for initiation of egg activation.

As discussed above, proteolysis of xUPIII generated multiple fragments derived from the extracellular domain. At the same time, it should also generate fragments that involve the transmembrane as well as cytoplasmic domain. However, such fragments were hardly detectable in any conditions that involved digestion of xUPIII (data not shown). Thus, we suggest that the carboxyl-terminal fragment(s) of xUPIII is unstable and/or translocating to non-raft areas. We now prefer the former possibility because we were unable to detect smaller carboxyl-terminal fragment(s) of xUPIII in the non-raft fractions (data not shown). As opposed to the cases in fertilized or protease-activated eggs, tyrosine phosphorylation of xUPIII in H2O2-activated eggs was not inhibited by xUPIII-GRR peptide. We suggest that this is because xSrc is directly activated by H2O2 without participation of membrane-bound receptor machinery and more specifically xUPIII. In fact, proteolysis of xUPIII was not detectable in H2O2-activated eggs. More importantly, the results with H2O2-activated eggs indicate that tyrosine phosphorylation of xUPIII can occur independently of proteolysis of xUPIII.

A potentially similar mechanism of signal transduction involving proteolysis of cell surface molecule and subsequent intracellular protein phosphorylation has been found for the

*Xenopus*: see Sakakibara et al., 2005).
function of CD44, a transmembrane protein. CD44 is a proteolytic target of multiple proteases that produce an extracellular fragment as well as a cytoplasmic fragment containing phosphorylated serine residues (Nagano and Saya, 2004). Other examples include thrombin receptor (Vu et al., 1991) and protease-activated receptor systems (Sabri et al., 2003), both of which seem to involve Src-dependent tyrosine phosphorylation of cytoplasmic molecules to exert their cellular functions. Molecular events learned from these different systems will be certainly of interest to examine in fertilization system of Xenopus (see also below).

Our previous study has demonstrated that an antibody against the extracellular domain of xUPIII (anti-xUPIII ED antibody) inhibited normal fertilization (Sakakibara et al., 2005). In the present study, we showed that proteolytic action of cathepsin B and sperm-induced tyrosine phosphorylation of xUPIII could be blocked by the same antibody, but not by anti-xUPIII-CT antibody. Together, it is suggested that inhibition of fertilization by the antibody is due to the failure of the sperm protease to digest xUPIII. While the anti-xUPIII ED antibody could block efficiently sperm-induced tyrosine phosphorylation of xUPIII as well as egg activation, the effect of the same antibody on cathepsin-B-induced egg activation events was limited. This may be because that cathepsin B used in our experiments was in excess to overcome the presence of the antibody. In fact, the inhibitory effect of the same antibody on normal fertilization and egg activation was also dependent on sperm concentration (Sakakibara et al., 2005).

A question arises as to how proteolysis of xUPIII contributes to egg activation. In general, protease-dependent cell surface events involve: 1) release of soluble ligands (e.g. ADAM12, thrombin receptor: Kang et al., 2000; Sabri et al., 2003; Vu et al., 1991); 2) ligand-like actions of the protease itself (e.g. disintegrins: Iwao and Fujimura, 1996; Shilling et al., 1998); and 3) modulation of the molecular interactions (e.g. urokinase/co-receptors: Andreasen et al., 1997; Guerrero et al., 2004). We expect that the extracellular fragment of xUPIII may act as a soluble ligand to interact with another egg-raft-associated molecule(s) and that proteolysis contributes to the modulation of the molecular interactions involving xUPIII and its binding partner(s) (e.g. xUPIb, see below). Currently, however, we cannot exclude the possibility that egg surface proteins other than xUPIII could also be important targets of protease(s). Further study should clarify this issue.

We observed that xUPIb was in association with xUPIII in egg rafts. In mammalian cells, association of UPIII and UPIb is a prerequisite for UPIII to localize to the plasma membranes (Tu et al., 2002). Further study will evaluate the role of xUPIII/xUPIb complex in activation of the raft-associated xSrc upon fertilization.

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