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GPI-Anchored Aminopeptidase Is Involved in the Acrosome Reaction in Sperm of the Mussel *Mytilus edulis*

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ABSTRACT The sperm of the mussel *Mytilus* had hydrolytic activities against substrates for aminopeptidase. Acrosome reaction (AR) was suppressed in the presence of aminopeptidase substrate, Phe-4-methylcoumaryl-7-amide (MCA), and an aminopeptidase inhibitor, bestatin. Treatment of sperm with phosphatidylinositol-specific phospholipase C (PI-PLC) released aminopeptidase activity from sperm and suppressed AR. These results suggest that the enzyme is located on the sperm surface via glycosylphosphatidylinositol (GPI)-anchor and is involved in the AR. Immunoblot analysis showed that tyrosine residues of 40, 59, 68, and 72 kDa proteins were phosphorylated during induction of the AR. The 40 kDa protein was also recognized by anti-c-Src antibody by immunoblotting. The tyrosine phosphorylation of these proteins was inhibited when sperm were inseminated in the presence of Phe-MCA, and by PI-PLC treatment. Treatment of sperm with tyrosine kinase activator, 9,10-dimethyl-1,2-benzanthracene, induced AR, and its inhibitor, genistein, suppressed AR. These results suggest that tyrosine phosphorylation of 40, 59, 68, and 72 kDa proteins, induced by the interaction of GPI-anchored aminopeptidase with oocyte surface, triggers AR in *Mytilus* sperm. *Mol. Reprod. Dev. 67: 465–471, 2004.* © 2004 Wiley-Liss, Inc.

Key Words: fertilization; GPI-anchored protein; tyrosine kinase

INTRODUCTION

Glycosylphosphatidylinositol (GPI)-anchored proteins possess a covalently linked glycosylated phosphatidylinositol moiety which attaches the protein portion of the molecule to the cell surface lipid bilayer (Brown and London, 1998). Proteins linked to the cell surface via a GPI-anchor are involved in a wide variety of cellular functions, including T-cell activation, transduction of extracellular stimuli, hydrolysis of extracellular matrix proteins, cell–cell adhesion, and fertilization (Brown and London, 1998; Kasahara and Sanai, 2000; Simons and Toomre, 2000; Cherr et al., 2001). In mammalian sperm, GPI-anchored hyaluronidase (also known as PH-20) on the sperm surface appears to function as a receptor for hyaluronic acid (HA)-induced cell signaling, in addition to being a hyaluronidase itself (Cherr et al., 2001). It has also been demonstrated that the sea urchin sperm receptor for the egg ligand was found in detergent insoluble glycolipid fraction, which had at least four proteins involved in signal transduction, including a 63 kDa GPI-anchored protein (Ohta et al., 2000).

Acrosome reaction (AR) is essential for fertilization since it exposes proteins in the sperm plasma membrane that mediate the binding and fusion of this membrane with that of egg. In the marine bivalves, only the morphology of the AR has been studied (Longo, 1983), and the chemical nature of complementary recognition sites is poorly understood. In previous studies in the mussel *Mytilus edulis*, we have demonstrated that aminopeptidase-like protease released from oocytes at fertilization affects the oocyte surface and consequently suppresses AR of supernumerary sperm on the fertilized oocytes, resulting in establishment of polyspermy block (Togo et al., 1995; Togo and Morisawa, 1997). Our studies suggest that the molecules on the oocyte surface, which are recognized by aminopeptidase, are involved in induction of AR. We also have evidence from preliminary experiments that sperm also has aminopeptidase activity.

In the present study, we hypothesize that the interaction between aminopeptidase located on the sperm surface and the oocyte surface is involved in the induction of AR in *Mytilus*. We carried out experiments to determine whether the sperm aminopeptidase is involved in the induction of AR and found that GPI-anchored aminopeptidase on the sperm surface was required for the induction of AR.

Abbreviations: AMC, 7-amino-4-methylcoumarin; AR, acrosome reaction; FNSW, filtered natural seawater; HA, hyaluronic acid; GPI, glycosylphosphatidylinositol; MCA, 4-methylcoumaryl-7-amide; NSW, natural seawater; PI-PLC, phosphatidylinositol-specific phospholipase C; $R_{s/o}$, sperm–oocyte ratio.

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MATERIALS AND METHODS

Preparation of Gametes

Specimens of the mature mussel *Mytilus edulis* were collected from November to April in the vicinity of Misaki Marine Biological Station (Kanagawa Prefecture), Otsuchi Marine Research Center, Ocean Research Institute, the University of Tokyo (Iwate Prefecture), Education and Research Center of Marine Bio-resources (Miyagi Prefecture), and Asamushi Marine Biological Station, Tohoku University (Aomori Prefecture). Specimens were kept in aquaria at 8°C. Spawning of oocyte and sperm was induced by transferring the mussels to warm natural seawater (NSW) at 25°C. When the mussels started spawning, they were returned to NSW at 8°C. Oocytes and sperm were washed with filtered NSW (FNSW) several times before use. Concentrations of oocyte were determined by counting the number of oocytes aspirated into 5-μl glass capillary tubes. Concentrations of sperm in the suspensions were determined using a hemocytometer by counting the number of sperm fixed with 1% formaldehyde. Sperm–oocyte ratio ($R_{s/o}$) in the medium at insemination was an absolute ratio. Artificial induction of AR was carried out by adding 10% (v/v) isotonic 0.33 M $CaCl_2$ to the sperm suspension (Tamaki and Osanai, 1985).

Fluorometric Measurement of Protease Activities

Sperm suspension at a concentration of 1×10^6/ml in FNSW and supernatant solution of sperm suspension were incubated with 1 μM peptidyl-4-methyl-coumaryl-7-amide (MCA) substrates (Peptide Institute, Osaka, Japan) for 1 hr at 25°C. The fluorescence due to liberated 7-amino-4-methylcoumarin (AMC) was measured using a Hitachi 650-10S fluorescence spectrophotometer with excitation and emission wavelength at 380 and 460 nm, respectively. The protease activity of the sperm was calculated as the amount of released AMC/hr $\times 10^6$ sperm.

PI-PLC Treatment

Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from Molecular Probes (Eugene, OR). This enzyme was supplied as a 100 U/ml solution in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.01% sodium azide, and 50% glycerol. A suspension of sperm (1×10^6/ml) was incubated either with 1.0 U/ml PI-PLC or vehicle alone (1/100 dilution) for 1 hr at 25°C. For protease activity assay, sperm suspension was centrifuged at 10,000*g* for 3 min to pellet sperm, and the supernatant solution was analyzed as described above. For the analysis of AR, sperm pellet was re-suspended in FNSW, and inseminated with unfertilized oocytes at $R_{s/o} = 3{,}000$.

Assay of AR

Sperm suspensions were inseminated with unfertilized oocytes at $R_{s/o} = 3{,}000$. They were fixed with 1% formaldehyde in FNSW 10 min after insemination. The head of acrosome-intact sperm pear-shaped, and the head becomes more round after AR (Togo et al., 1995). Furthermore, acrosome-intact sperm were easily removed from oocyte surface after fixation (Togo et al., 1995). An appropriate volume of the suspension was mounted on a glass slide, and the number of acrosome-reacted and -intact sperm in randomly selected fields (both bound and unbound on oocyte surface) was counted under a phase contrast microscope (OPTIPHOT, Nikon, Tokyo, Japan).

Assay of Sperm Motility

The sperm suspension (1×10^5/ml) was placed on a glass slide, and the percentage of motile sperm in randomly selected fields was evaluated under a phase contrast microscope (OPTIPHOT, Nikon).

Western Blotting

Oocytes were inseminated with sperm at $R_{s/o} = 1{,}000$ under the various conditions, and were removed by gentle centrifuge 3 min after insemination. Sperm suspension was then mixed with an equal volume of 2× SDS buffer (0.5 M Tris-HCl, pH 6.8, 2% glycerol, 2% β-mercaptoethanol, and 10% SDS) followed by vigorous vortexing. Membrane debris was pelleted by centrifuging at 15,000*g* for 15 min, and the supernatant containing solubilized sperm membrane proteins was collected. Solubilized sperm samples were mixed with 5× SDS–PAGE sample buffer and resolved on 7.5% polyacrylamide gel, then electroblotted to PVDF membrane (Millipore, Billerica, MA). The membrane was incubated for 1 hr with blocking solution (TBS, pH 7.2, 0.1% Tween-20, 1% BSA), and further incubated for 1 hr in blocking solution containing anti-phosphotyrosine antibody (Biomol Research Laboratory, Plymouth Meeting, PA). After washing with TBS (pH 7.2) containing 0.1% Tween-20, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 hr. Immunoreactive bands were detected by ECL Plus Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ). Blots were exposed immediately to film (X-OMAT AR; Kodak, Rochester, NY) and several exposures were obtained for each blot. The optical densities of the protein bands were quantified by NIH Image software.

RESULTS

Aminopeptidase Activity of *Mytilus* Sperm

We first investigated protease activities of intact *Mytilus* sperm. Sperm suspensions were incubated with 1 μM peptidyl-MCA substrates, and the activities were measured by fluorometric method. Suspension of intact sperm mainly had hydrolytic activity against Phe-MCA, Leu-MCA, and Ala-MCA, substrates for aminopeptidase (Fig. 1A). The activities were 0.99 ± 0.06 ($n = 10$), 0.76 ± 0.18 ($n = 18$), and 0.21 ± 0.11 ($n = 3$) nmol/hr $\times 10^6$ sperm, respectively. Supernatant solution of sperm suspension also had hydrolytic activities against Phe-MCA (0.08 ± 0.02 nmol/hr $\times 10^6$ sperm,

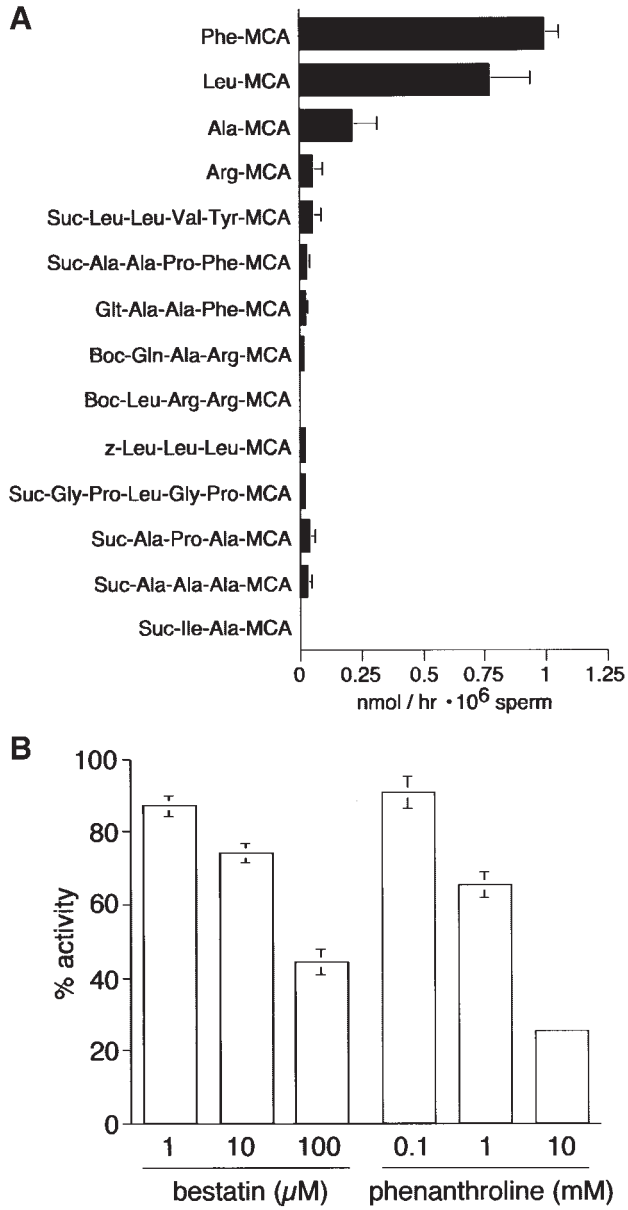

Fig. 1. A: Hydrolytic activities against peptidyl-4-methylcoumaryl-7-amide (MCA) substrates in the intact sperm of *Mytilus*. Sperm suspensions were incubated with 1 μM peptidyl-MCA substrates, and hydrolytic activities were measured as described in Materials and Methods. **B**: Effect of bestatin and *o*-phenanthroline on sperm aminopeptidase activity. Sperm suspensions were pre-incubated either with bestatin or *o*-phenanthroline for 15 min, and then 1 μM Phe-MCA was added. All values (mean ± SE) are represented as a percentage of the activity relative to that without inhibitors.

n = 31), but the activity was much lower than that of sperm suspension (Student's *t*-test, $P < 0.0001$). Sperm could also hydrolyze other protease substrates, but the activities were less than 0.05 nmol/hr × 10^6 sperm (Fig. 1A).

To investigate the effects of aminopeptidase inhibitors on sperm aminopeptidase activity, sperm suspensions were pre-incubated either with bestatin or *o*-phenanthroline for 15 min, and Phe-MCA (1 μM) was added. Bestatin inhibited the hydrolytic activity against Phe-MCA in a dose-dependent manner (Fig. 1B). The activity was decreased to 43.9 ± 3.4% (n = 11) at 100 μM bestatin. *o*-Phenanthroline also decreased the activity to 24.9 ± 1.3% (n = 3) at 10 mM. These results suggest that *Mytilus* sperm have an aminopeptidase activity.

Effects of an Aminopeptidase Inhibitor and a Substrate on the AR

We next investigated the effect of an aminopeptidase inhibitor and a substrate on the AR (Fig. 2). When unfertilized oocytes were inseminated at $R_{s/o} = 3{,}000$, the rate of AR was 59.8 ± 4.2% (n = 5). When sperm suspensions were incubated with 100 μM bestatin for 15 min, and then unfertilized oocytes were added at $R_{s/o} = 3{,}000$, the rate decreased to 29.4 ± 3.9% (n = 8) (Student's *t*-test, $P = 0.0003$, compared with control). An aminopeptidase substrate also had a similar effect on the AR. When sperm suspension was incubated with 100 μM Phe-MCA for 15 min, and then unfertilized oocytes were added at $R_{s/o} = 3{,}000$, the rate was 15.3 ± 3.6% (n = 12) (Student's *t*-test, $P < 0.0001$, compared with control). When sperm suspension was incubated with mixture of phenylalanine and AMC (both 100 μM), liberated products of Phe-MCA, and inseminated as a control, the rate of AR was almost the same as normal insemination (59.7 ± 2.4%, n = 12). These results suggest that sperm aminopeptidase is required for induction of AR in *Mytilus*.

To determine if aminopeptidase inhibitor and substrate affected sperm motility, sperm suspensions were

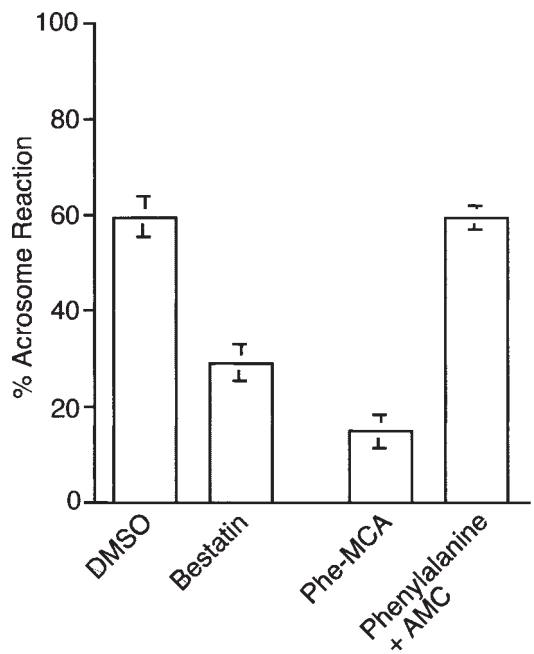

Fig. 2. Effect of an aminopeptidase inhibitor and a substrate on the acrosome reaction of *Mytilus* sperm. Sperm suspensions were incubated either with 100 μM bestatin, 100 μM Phe-MCA, or mixture of 100 μM phenylalanine and 100 μM AMC for 15 min, and then unfertilized oocytes were added to the sperm suspension. Values are mean ± SE of four experiments.

incubated either with 100 μM bestatin or 100 μM Phe-MCA for 15 min, and then the percentage of motile sperm was calculated. Bestatin and Phe-MCA did not affect sperm motility (data not shown). Since the aminopeptidase inhibitor and substrate had no deleterious effect on the sperm motility, inhibition of AR was not due to the inhibition of access to the oocyte.

AR of *Mytilus* sperm can be induced artificially by increasing external Ca^{2+} concentration (Tamaki and Osanai, 1985). The AR was almost completely induced when isotonic 0.33 M $CaCl_2$ was added (1/10 volume) in the presence of bestatin and Phe-MACA (data not shown). This indicates that AR can be still induced after the treatment with these reagents.

PI-PLC Treatment Releases Aminopeptidase Activity From Sperm and Suppresses AR

GPI-anchored proteins can be released from the cell surface by treatment with the specific enzyme PI-PLC (Low and Finean, 1978). To determine if the aminopeptidase was anchored to the sperm surface via GPI, sperm suspension was treated with 1.0 U/ml PI-PLC for 1 hr at 25°C, and the protease activity of the supernatant solution was analyzed (Table 1). When sperm suspension was treated with PI-PLC, an aminopeptidase activity was released to the supernatant solution. The activity was 0.31 ± 0.05 ($n = 7$) nmol/hr $\times 10^6$ sperm against Phe-MCA, but PI-PLC released only 31.3% of the total sperm aminopeptidase activity. As a control, sperm suspension was incubated with 1/100 dilution of vehicle for 1 hr at 25°C. The supernatant solution of the control experiment also had hydrolytic activity at 0.18 ± 0.04 ($n = 7$) nmol/hr $\times 10^6$ sperm, which is significantly lower than that of the supernatant solution of PI-PLC treated sperm (Student's *t*-test, $P = 0.0012$). Release of aminopeptidase activity into supernatant solution in control experiments could be due to release of a weakly associated aminopeptidase and/or a result of cell damage during long incubation and centrifugation. These results suggest that sperm aminopeptidase, at least in part, is anchored to the cell surface via GPI.

To investigate the function of GPI-anchored protein in AR, sperm were treated with 1.0 U/ml PI-PLC for 1 hr at 25°C and then inseminated. PI-PLC treatment significantly suppressed AR (Student's *t*-test, $P < 0.0001$, compared with control) (Table 1). The percentage of AR was $26.2 \pm 3.1\%$ ($n = 11$), whereas the rate was $50.6 \pm 3.5\%$ ($n = 11$) in the control. Long incubation of sperm at 25°C slightly affected sperm motility; 20% of sperm on average became immotile during incubation (data not shown). AR was almost completely induced by adding isotonic 0.33 M $CaCl_2$ (1/10 volume) in PI-PLC treated sperm. These results suggest that GPI-anchored proteins, including aminopeptidase, are required for AR in *Mytilus* sperm.

TABLE 1. Effects of PI-PLC on Sperm Aminopeptidase Activity and AR

Concentration (U/ml)	Activity (nmol/hr $\times 10^6$ sperm)	AR (%)
0	0.18 ± 0.04 ($n = 7$)	50.6 ± 3.5 ($n = 11$)
1.0	0.31 ± 0.05 ($n = 7$)	26.2 ± 3.1 ($n = 11$)

PI-PLC, phosphatidylinositol-specific phospholipase C; AR, acrosome reaction; FNSW, filtered natural seawater; $R_{s/o}$, sperm–oocyte ratio.
Sperm suspension (1×10^6/ml) was incubated with or without 1.0 U/ml PI-PLC for 1 hr at 25°C. For protease activity assay, sperm suspension was centrifuged at 10,000*g* for 3 min to pellet sperm, and the supernatant solution was analyzed as described in Materials and Methods. For the analysis of AR, sperm pellet was re-suspended in FNSW, and inseminated with unfertilized oocytes at $R_{s/o} = 3{,}000$. Acrosome-reacted sperm were counted 10 min after insemination.

Tyrosine Phosphorylation in Sperm During Induction of AR

It has been demonstrated that GPI-anchored proteins can mediate cell signaling through tyrosine phosphorylation (Harder and Simons, 1999). Tyrosine phosphorylation of sperm was analyzed by immunoblotting of SDS-solubilized sperm extracts with an anti-phosphotyrosine antibody, and a difference in banding patterns and optical densities was measured between the treatments (Figs. 3 and 4). Insemination significantly increases tyrosine phosphorylation of 40, 59, 68, and 72 kDa proteins in sperm (Fig. 3A, black and white arrowheads, compare lanes 1 and 2; Fig. 4). The other bands had no significant differences between noninseminated and inseminated sperm (data not shown). PI-PLC-treatment inhibited tyrosine phosphorylation of these proteins (Fig. 3A, lane 3; Fig. 4). When sperm were inseminated in the presence of aminopeptidase substrate Phe-MCA, tyrosine phosphorylations of the proteins was also inhibited (Fig. 3A, lane 4; Fig. 4). Finally, a tyrosine kinase inhibitor, genistein, inhibited the increase in tyrosine phosphorylation during induction of the AR (Fig. 3A, lane 5; Fig. 4). The specificity of anti-phosphotyrosine immunoblotting was verified by the fact that the most of bands could be eliminated in the presence of L-phosphotyrosine (Fig. 3B). It appears that prominent bands between 75 and 105 kDa were not due to tyrosine phosphorylation. These results suggest that GPI-anchored aminopeptidase on the sperm surface is required for tyrosine phosphorylation of 40, 59, 68, and 72 kDa proteins during induction of AR. The 40 kDa band in Figure 3A (white arrowhead) might be related to the phosphorylation of Src-family tyrosine kinase, since anti-c-Src antibody recognized the same band (Fig. 3C, white arrowhead).

Effects of Tyrosine Kinase Activator and Inhibitor on AR

It has been demonstrated that 9,10-dimethyl-1,2-benzanthracene (DMBA) activates tyrosine kinases in T-cells (Archuleta et al., 1993). To confirm the involvement of tyrosine kinase on the AR of *Mytilus* sperm, sperm were treated with DMBA for 10 min, and acrosome-reacted sperm were counted after the fixation with 1% formaldehyde in seawater. As shown in Figure 5A, treatment with DMBA induced AR in a dose-dependent manner. DMBA (20 μM) induced AR of

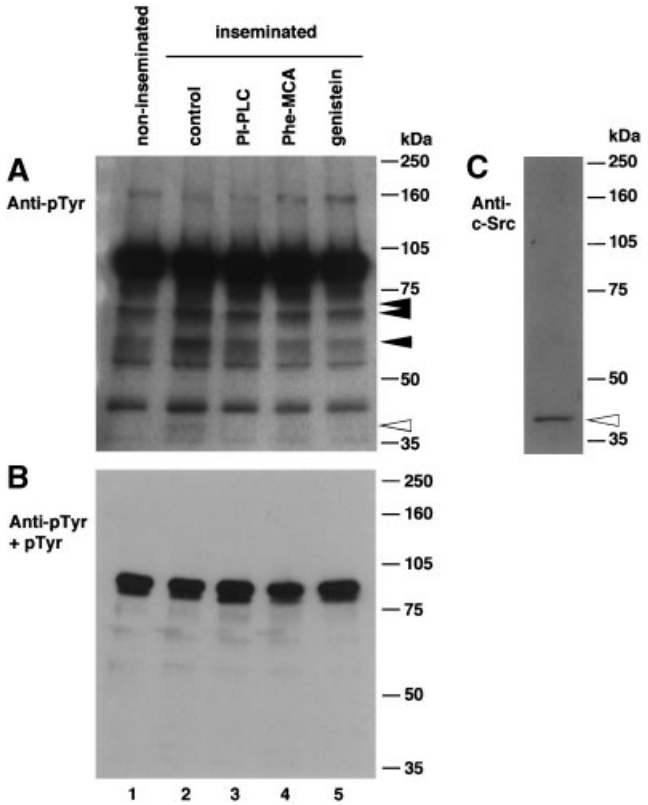

Fig. 3. Tyrosine phosphorylation of *Mytilus* sperm in response to insemination. SDS-solubilized extract was prepared from *Mytilus* sperm that had been either inseminated in the absence or presence of Phe-MCA or treated with phosphatidylinositol-specific phospholipase C (PI-PLC) or genistein. Sperm proteins were separated by 7.5% SDS–PAGE, and analyzed by immunoblotting either with anti-phosphotyrosine antibody (**A**), anti-phosphotyrosine antibody plus 10 mM L-phosphotyrosine (**B**), or anti-c-Src antibody (**C**). Samples in (A) and (B) are: noninseminated sperm (**lane 1**), inseminated sperm (**lane 2**), sperm treated with PI-PLC and inseminated (**lane 3**), inseminated sperm in the presence of Phe-MCA (**lane 4**), and genistein treated and inseminated sperm (**lane 5**). Black and white arrowheads indicate the positions of tyrosine phosphorylated proteins during induction of AR. The protein indicated by white arrowhead in (A) is also recognized by anti-c-Src antibody as indicated in (C).

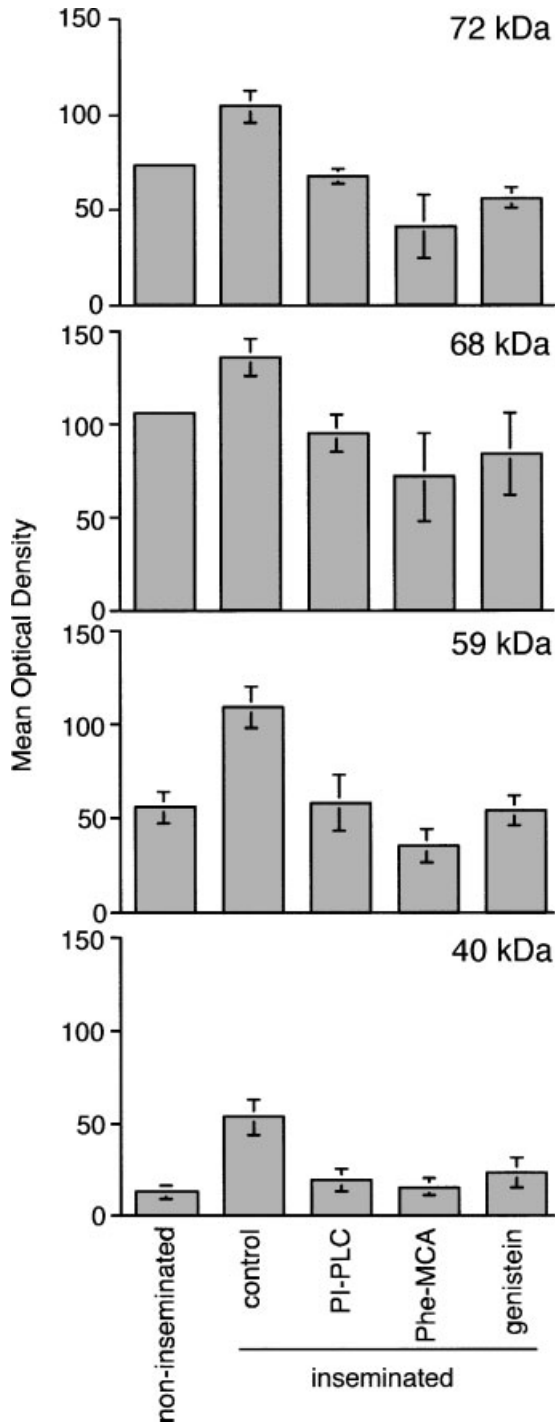

Fig. 4. Mean optical densities of tyrosine phosphorylated protein bands. Values are given as mean ± SE (n = 4).

52.3 ± 3.1% (n = 3) of total sperm. On the other hand, AR was inhibited when sperm were treated with tyrosine kinase inhibitor, genistein before insemination (Fig. 5B). The rates of AR were suppressed to 9.6 ± 2.5 (n = 3) and 1.2 ± 0.5% (n = 3) when sperm were treated with 50 and 100 μM genistein, respectively. The treatment with genistein did not affect sperm motility (data not shown). We also could induce AR by adding isotonic 0.33 M $CaCl_2$ (1/10 volume) in the presence of this reagent. These results suggest that tyrosine phosphorylation is required for AR in *Mytilus* sperm.

DISCUSSION

Aminopeptidases belong to a group of exopeptidases that catalyze the cleavage of an amino terminal residue of proteins and are known to be a metalloenzyme containing Zn^{2+} as essential metal ions. They are widely found throughout the animal and plant kingdoms, and are localized in many subcellular organelles, in cytoplasm and as membrane components. The existence of aminopeptidase in sperm has been described in mammals and sea urchins (Yasuhara et al., 1983, 1990, 1991; Arienti et al., 1997; Schaller and Glander, 2000), and it has been suggested that it contributes to sperm respiration in the sea urchin.

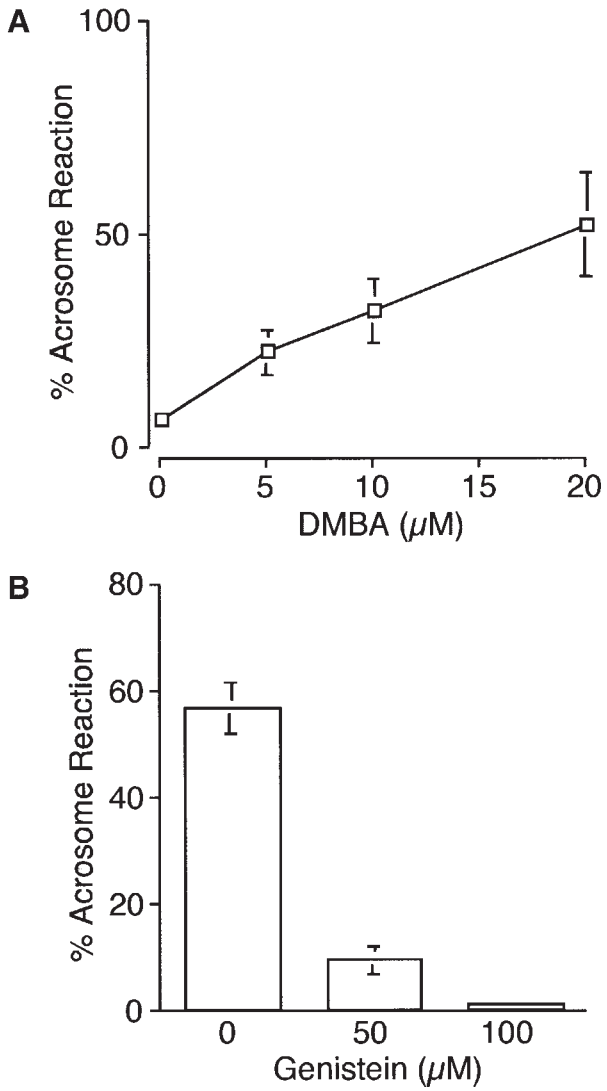

Fig. 5. Effects of tyrosine kinase activator 9,10-dimethyl-1,2-benzanthracene (DMBA) and inhibitor genistein on the acrosome reaction of *Mytilus* sperm. **A**: Sperm were treated with various concentrations of DMBA for 10 min and acrosome-reacted sperm were counted under the microscope. **B**: Sperm suspension was incubated with genistein for 10 min, and unfertilized oocytes were added to the suspension. The rate of acrosome reaction (AR) was assayed 10 min after the insemination.

Recent studies have suggested that aminopeptidase functions as a receptor protein in addition to a protease itself. For instance, it has been shown that coronaviruses and a Herpes virus use aminopeptidase as a receptor in their target tissue (Delmas et al., 1992; Yeager et al., 1992; Soderberg et al., 1993; Kolb et al., 1998). GPI-anchored aminopeptidase has also been recognized as a receptor or specific binding protein for an insecticidal toxin of *Bacillus thuringiensis* (Knight et al., 1995; Hua et al., 1998; Yaoi et al., 1999).

The process of fertilization requires interaction between complementary molecules on the sperm and the extracellular coat of oocyte. Interaction between an enzyme and its substrate has been implicated as a possible mechanism for sperm–oocyte recognition in ascidians and mammals. In ascidians, fucosidase and *N*-acetylglucosaminidase located on sperm surface function as a recognition protein for the sperm receptor on the vitelline coat, which is considered to be a substrate of these enzymes (Hoshi, 1984, 1986; Godknecht and Honegger, 1991, 1995; Matsumoto et al., 2002). In mice, sperm–oocyte recognition and induction of the AR are mediated by ZP3, one of three glycoproteins in the zona pellucida of the oocyte, and enzymes and proteins located on the sperm surface have been proposed as candidates for a ZP3 recognition site (for review: Wassarman et al., 2001).

In this study, we have obtained several lines of evidence suggesting that the interaction between sperm aminopeptidase and molecules on the oocyte surface may play a role in the induction of AR in mussel sperm; (1) sperm had hydrolytic activities against aminopeptidase substrates, and activity of the enzyme was inhibited by aminopeptidase inhibitors, bestatin and *o*-phenanthroline (Fig. 1), (2) bestatin and an aminopeptidase substrate, Phe-MCA, inhibited AR without a deleterious effect on the sperm (Fig. 2). These results suggest that the recognition site for oocyte surface may lie at or near the active site of aminopeptidase molecules on sperm.

We further found that treatment of sperm with PI-PLC released aminopeptidase activity and suppressed AR (Table 1), suggesting that GPI-anchored aminopeptidase is involved in induction of the AR. The clustering or ligation of GPI-anchored proteins to specific membrane regions can trigger transmembrane signal transduction (Brown and London, 1998; Simons and Toomre, 2000). So far the GPI-anchored proteins involved in signaling are associated with signaling molecules that are typically bound to the cytoplasmic leaflet of the plasma membrane. These proteins include the Src-family of tyrosine kinases. It has been demonstrated that the patches that form around the aggregated GPI-anchored proteins are enriched in tyrosine phosphorylated proteins as well as tyrosine kinases in BHK cells and Jurket T-lymphoma cells (Harder et al., 1998). In macaque sperm, GPI-anchored hyaluronidase, PH-20, on the sperm surface appears to be a receptor for HA-induced cell signaling (Cherr et al., 2001). It has been shown that treatment of sperm with HA or anti-PH-20 antibody induces tyrosine phosphorylation of the 92-kDa protein (Cherr et al., 2001). It has also been demonstrated that tyrosine phosphorylation of proteins plays an important role in AR of mammalian sperm (for review: Baldi et al., 2002; Breitbart, 2002; Urner and Sakkas, 2003).

In the present study, immunoblot analysis showed that tyrosine residues of 40, 59, 68, and 72 kDa proteins of *Mytilus* sperm were phosphorylated during induction of the AR, and that the phosphorylations were inhibited when sperm were inseminated in the presence of Phe-MCA, and by PI-PLC treatment (Figs. 3 and 4). We also found that the 40 kDa protein may be related to Src-family kinase since anti-c-Src antibody recognized the

same 40 kDa band in Western blot analysis (Fig. 3). Furthermore, activation of tyrosine kinases induced the AR, and inhibition of the kinases suppressed the AR (Fig. 5). Therefore, we speculate that tyrosine phosphorylation of 40, 59, 68, and 72 kDa proteins, which is induced by the interaction of GPI-anchored aminopeptidase with oocyte surface, triggers the AR in *Mytilus*. Some molecules, for example, aminopeptidase substrate, on the oocyte surface that are recognized by aminopeptidase may function as AR-inducing substance in *Mytilus*, although the nature of this substance is still unknown.

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