Identification of the sperm motility-initiating substance in the newt, *Cynops pyrrhogaster*, and its possible relationship with the acrosome reaction during internal fertilization

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ABSTRACT Motility initiation is a key event during internal fertilization of female-stored sperm, although the underlying mechanisms remain unclear. In internally fertilizing urodeles, quiescent sperm initiate motility on the surface of the egg-jelly, a thick extracellular matrix that accumulates around the egg in oviduct. By immunizing mice with egg-jelly extracts, we successfully generated an α34 monoclonal antibody (mAb) which neutralized sperm motility-initiating activity in the egg-jelly of the newt, *Cynops pyrrhogaster,* in a dose-dependent manner. The α34 mAb recognized an unglycosylated 34 kDa protein in the outermost of the six layers that comprise egg-jelly. Under nonreducing conditions, immunoblotting with α34 mAb produced many bands in addition to the 34 kDa protein, suggesting that the 34 kDa protein associates not only with the jelly matrix itself, but also with additional substances present in the matrix. Our current results are compatible with the supposed features of sperm motility-initiating substance (SMIS), indicating that the 34 kDa protein itself, or a complex consisting of the 34 kDa protein and some other molecules, is the SMIS in *C. pyrrhogaster*. Immunofluorescence staining further indicated that SMIS was distributed in a dot-like pattern in the outermost jelly layer and was fully covered with acrosome reaction-inducing substance (ARIS). Immunocytochemical and scanning electron microscopic examinations of the outermost jelly layer strongly suggests that the 34 kDa protein localized in granules (2 μm) and that ARIS was distributed covering the granules and in the sheet-like structure above the granules. These data suggest that the initiation of sperm motility is mediated by the acrosome reaction.

KEY WORDS: *sperm motility, acrosome reaction, internal fertilization, egg-jelly, newt*

Introduction

Sperm motility control is critical for successful fertilization in most animal species and occurs under species-specific fertilizing conditions in which various factors act as extracellular cues to induce motility initiation, activation, and chemotaxis (Ohtake, 1976; Morisawa and Suzuki, 1980; Suzuki *et al.*, 1981; Hansbrough and Garbers, 1981; Bradley and Garbers, 1983; Ward *et al.*, 1985; Yanagimachi *et al.*, 1992; Al-Anzi and Chandler, 1998). In externally fertilizing echinoderms and salmon fish, the initiation of sperm motility is mediated by several types of cations (Lee *et al.*, 1983; Morisawa and Suzuki, 1980), whereas it occurs via changes in

osmotic pressure in other species of seawater fish, freshwater fish, and anuran amphibians (Morisawa and Suzuki, 1980; Inoda and Morisawa, 1987; Takai and Morisawa, 1995). Egg-derived factors have also been shown to initiate sperm motility in the horseshoe crab and in herring (Clapper and Epel, 1985; Morisawa *et al.*, 1992; Yanagimachi *et al.*, 1992). All'of the factors described above specifically act in the spawning conditions and induce coordinated initiation of sperm motility.

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In internally fertilizing vertebrates, motility is initiated or activated in sperm stored in the female"genital tract (Suarez, 1987; Gist and Jones, 1989; Smith and Yanagimachi, 1991; Suarez *et al.*, 1991; Baskt, 1992; Birkhead *et al.*, 1993; Srinvas *et al.*, 1995). In mammals, the caudal isthmus of the oviduct is the sperm storage site (Smith and Yanagimachi, 1991) from which sperm are released into the lumen with hyperactivated motility (DeMott and Suarez, 1992). Progesterone is also a possible inducer of hyperactivation (Jaiswal *et al.*, 1999), whereas factors derived from the oviduct may contribute to hyperactivation through capacitation (King *et al.*, 1994; Anderson and Killian, 1994). In aves, sperm are stored in the mucosal crypts (Bakst, 1992), and the extracellular Ca^{2+} levels in the oviduct are thought to mediate temperature-dependent sperm motility in the oviduct (Ashizawa *et al.*, 2004). Our overall understanding of sperm motility control in the female genital tracts of vertebrates is still limited, however, because it is difficult to definitively evaluate any of the critical factors associated with the sperm during fertilization *in vivo*.

Amphibian egg-jelly is composed of a network of fibrils bound by diffusible globular materials (Bonnell *et al.*, 1996), both of which are secreted in the pars convoluta, the posterior region of the oviduct (Greven, 2003, in review). The pars convoluta is divided into several compartments, each of which secretes specific substances to form several morphologically and biochemically different layers in egg-jelly (Bonnell and Chandler, 1996; Okimura *et al.*, 2001). The egg-jelly plays a pivotal role in amphibian fertilization through a variety of active substances such as sperm chemoattractant in *Xenopus* (Al-Anzi and Chandler, 1998; Olson *et al.*, 2001), an inducer for capacitation-like change in *Bufo* sperm (Krapf *et al.*, 2007), a mediator for sperm binding to the vitelline envelope in *Cynops* (Hiyoshi *et al.*, 2007), a sperm acrosome-stabilizing factor in *Bufo* (Krapf *et al.*, 2006), and an acrosome reaction-inducing substance in *Cynops* (Sasaki *et al.*, 2002; Watanabe *et al.*, 2009).

During internal fertilization in urodeles, sperm motility is initiated on the egg-jelly surface (Ukita *et al.*, 1999; Watanabe *et al.*, 2003) on which the sperm are directly inseminated from the spermatheca, a sperm storage site at the dorsal wall of the female cloaca (Sever and Brizzi, 1998; Watanabe and Onitake, 2002). The outermost layer of the egg-jelly is crucial for internal fertilization in the Japanese newt, *Cynops pyrrhogaster* (Takahashi *et al.*, 2006) through both sperm motility-initiating (Ukita *et al.*, 1999; Watanabe and Onitake, 2003, Watanabe *et al.*, 2003) and acrosome reaction-inducing activities (Sasaki *et al.*, 2002; Watanabe *et al.*, 2009). The former of these activities is induced by the sperm motility-initiating substance (SMIS), a heat-stable proteinaceous factor (Mizuno *et al.*, 1999; Onitake *et al.*, 2000). The egg-jelly also contains an inactive SMIS with a larger molecular weight (Mizuno *et al.*, 1999), suggesting that a new mechanism for sperm motility initiation operates during internal fertilization in the newt. However, the nature of this mechanism has been poorly understood to date.

In our present study, we have investigated the mechanism underlying sperm motility initiation in the newt by generating a monoclonal antibody (mAb) α34 that neutralized the initiation of sperm motility in the egg-jelly extract in a dose-dependent manner. Immunoblotting analysis showed that this α34 antibody recognized a 34 kDa protein in the outermost layer of the egg-jelly. The 34 kDa protein in the newt appears to associate with other substances in the jelly matrix since it was detected under reducing conditions and, under nonreducing conditions, numerous bands

were detectable by immunoblotting with the α34 mAb. These finding is consistent with the results of previous studies (Ukita *et al.*, 1999; Mizuno *et al.*, 1999; Watanabe and Onitake, 2003; Watanabe *et al.*, 2003), showing that the 34 kDa protein itself, or a complex consisting of the 34 kDa protein and some other molecules is the SMIS in the *Cynops* egg-jelly. By immunostaining of the outermost layer of egg-jelly, SMIS was found to be distributed in the inner area as a dot-like pattern. Scanning electron microscopic observations further showed that the outermost layer was composed of many granules of about 2 μm in diameter that were fully covered with a sheet-like structure. The SMIS thus appeared to localize in the former structures, whereas the acrosome reaction-inducing substance (ARIS) showed expression in the latter. Our present results thus strongly suggest that sperm motility initiation is mediated by the acrosome reaction during the internal fertilization in the newt.

Results

Generation of a monoclonal antibody that neutralizes sperm motility initiation by egg-jelly in the newt

A monoclonal antibody, mAb α34 (IgG2a isotype), was obtained by screening of hybridomas for the neutralizing activity of their culture supernatants against sperm motility initiation by JE in the
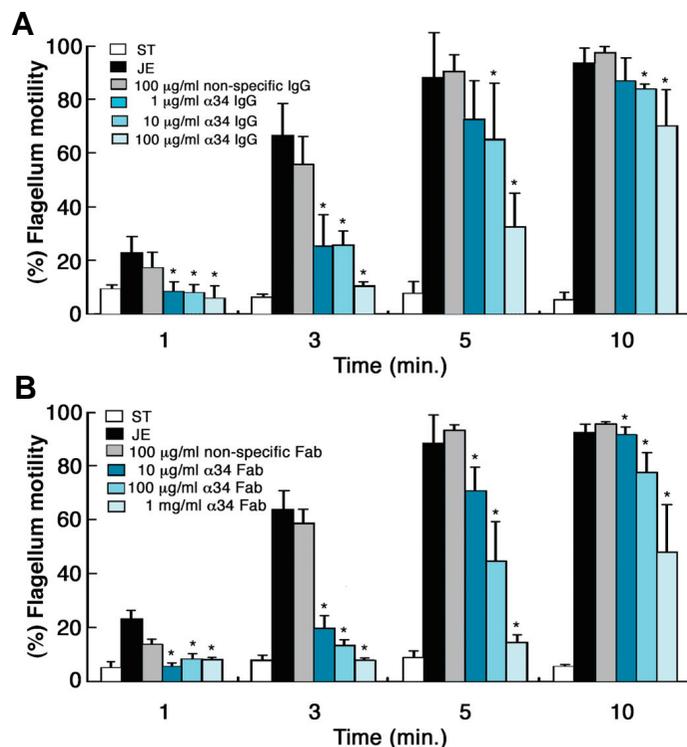

Fig. 1. Inhibitory activity of α34 mAb against sperm motility initiation by jelly extract (JE). *JE was pretreated with whole IgG* **(A)** *or Fab fragment* **(B)** *of the α34 mAb at a concentration ranging from 1 μg/ml to 1 mg/ml for 30 min, and sperm were then suspended in the mixture. Sperm motility was observed by phase-contrast microscopy for up to 10 min. JE pretreated with non-specific whole IgG (A) or Fab fragment (B), intact JE and Steinberg's salt solution (ST) were used as controls. Values are indicated as means ± SD. Asterisks indicate statistical differences by Student t-test (P<0.01) against the percent of moving sperm in the JE pretreated with non-specific IgG (A) or Fab fragment (B).*

Fig. 2 (Left). Immunoblotting analysis of egg-jelly extracts with α34 mAb. *Jelly extract (JE, 100 µg) was treated with Laemmli's sample buffer with* **(A)** *or without* **(B)** *2-mercaptoethanol and electrophoresed using 10 % (A) or 2-15 % (B) polyacrylamide gels. This was followed by electrotransfer onto a PVDF membrane and incubation with α34 mAb. Under reducing conditions (A), the mAb produced a single band of 34 kDa (arrowhead). In addition to the 34 kDa protein (arrowhead), many larger bands were observed under nonreducing conditions (B). CBB, Coomassie brilliant blue staining; α34, immunostaining with α34 mAb; IgG, immunostaining with nonspecific mouse IgG.*
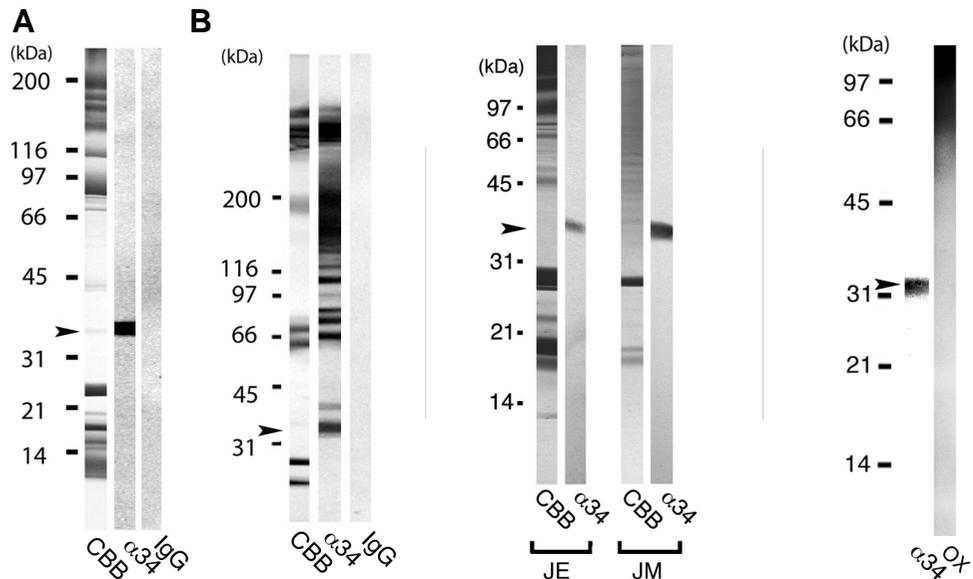

Fig. 3 (Middle). Immunoblotting of egg-jelly matrix with α34 mAb. *Jelly matrix solution (JM) was prepared by suspending the precipitated jelly matrices with the same volume of Steinberg's salt solution (ST) as that of obtained JE. The jelly extract (JE) and JM dissociated in the same volume as described in Materials and Methods were pretreated in reducing conditions, and 20 µl each was loaded on 15% acrylamide gels. After electrophoresis and immunoblotting with α34 mAb, both JE and JM produced a single band of 34 kDa. CBB, Coomassie brilliant blue staining; α34, immunostaining with α34 mAb.*

Fig. 4 (Right). Detection of carbohydrates in jelly extract (JE). *Electrophoresed JE proteins were electrotransferred onto a PVDF membrane, oxidized with periodate and then labeled with biotin hydrazide. The labeled substances were then treated with HRP-avidin and detected using ECL chemiluminescence. The 34 kDa protein (arrowhead) was not stained, suggesting that it is unglycosylated. α34, immunostaining with α34 mAb; OX, ECL detection of biotin-labeled hydrazide binding to JE substances oxidized with periodate.*

newt. To evaluate the neutralizing features of α34 mAb, JE was pretreated with α34 mAb and sperm were then suspended in this mixture. Sperm motility initiation was found to be inhibited in a dose-dependent manner at a concentration range of 1 to 100 µg/ml at 1, 3, and 5 min (Fig. 1A). At 10 min, the percentage of motile sperm increased in the JE that had been pretreated with 10 or 100 µg/ml α34 mAb (Fig. 1A). No inhibition was observed when 100 µg /ml non-specific IgG was used in a control experiment. A similar pattern of inhibition was observed when Fab fragments of α34 mAb were used at a dose range of 10 µg/ml to 1 mg/ml (Fig. 1B).

In newt sperm, the initiation of their motility is known to be spatiotemporally close to the acrosome reaction. Therefore, the α34 mAb was subjected to the investigation of its inhibitory activity against the acrosome reaction. The percentages of acrosome reacted sperm were 85 ± 6.3 in the JE pretreated 100 µg/ml α34 mAb, 81 ± 7.2 in JE, and 17 ± 2.5 in ST. In addition, the α34 mAb

did not show any binding to live sperm insofar as observed by indirect immunofluorescence microscopy (data not shown). These results indicate that the α34 mAb specifically neutralizes the activity of the SMIS in the JE through direct binding.

Characterization of antigen for the α34 monoclonal antibody

By immunoblotting, we found that the α34 mAb produced a single band of 34 kDa in the JE (Fig. 2A). This 34 kDa protein was more prominent in the precipitated jelly matrices compared with the soluble supernatant (Fig. 3). When JE was immunoblotted under nonreducing conditions with α34 mAb, numerous bands were observed in addition to the 34 kDa band (Fig. 2B). These results suggest that the 34 kDa protein exists in a soluble state in JE to some extent but dominantly associates with jelly components precipitated as the JM. Since 34 kDa protein was negative to periodate oxidation (Fig. 4), the protein itself was revealed to be

Fig. 5. Double staining of newt egg-jelly with α34 and anti-ARIS mAbs. *Frozen sections of egg-jelly were incubated with α34 mAb (IgG2a) and anti-ARIS mAb (IgM), followed by incubation with Alexafluor 488-conjugated anti-mouse IgG2a and Alexafluor 594-conjugated anti-mouse IgM as secondary antibodies.* **(A,B)** *Fluorescence views of egg-jelly stained with α34 mAb and anti-ARIS mAb, respectively. Both of these primary antibodies exclusively stained the outermost jelly layer.* **(C,D)** *Control reactions for (A,B), respec-*

tively. **(E,F)** *Higher magnification views of (A,B), respectively.* **(G)** *Merged image of (E,F), showing that the 34 kDa protein is distributed in a dot-like pattern and that ARIS resides on the outer surface of the egg-jelly. Bars, 200 and 75 µm in (A,E), respectively.*
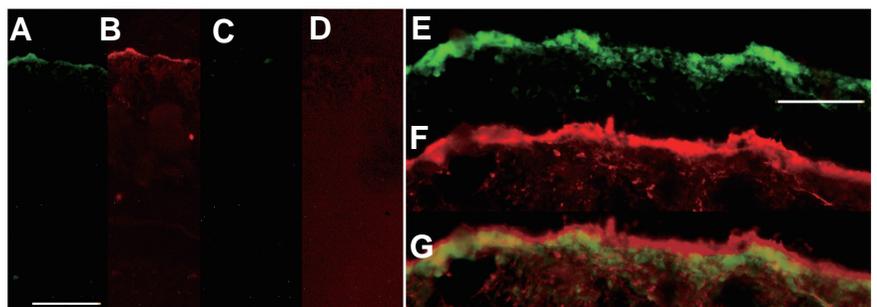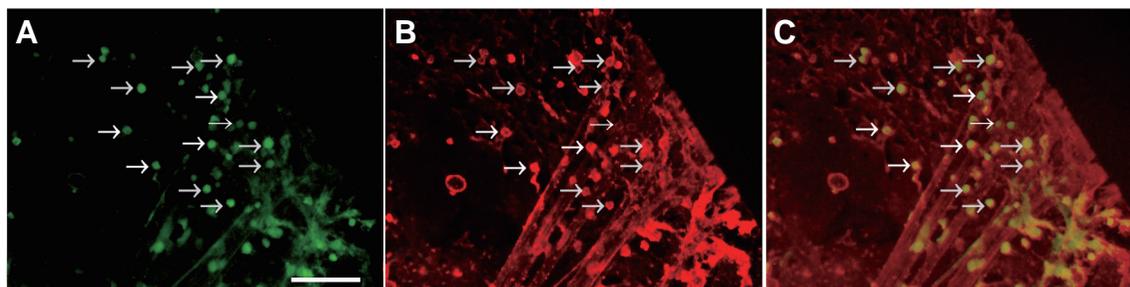
Fig. 6. Double staining of isolated outermost jelly layer with α34 and anti-ARIS mAbs. *The outermost layer of the egg-jelly was prepared on a glass slide, which was then incubated with α34 and anti-ARIS mAbs followed by Alexafluor 488-conjugated anti-mouse IgG2a and Alexafluor 594-conjugated anti-mouse IgM secondary antibodies. Immunostaining with α34 mAb* **(A)** *indicates that the 34 kDa protein is localized in the granules (arrows). The anti-ARIS mAb staining pattern* **(B)** *indicates that ARIS is distributed covering the granules (arrows) and in fibril structures beneath the granules.* **(C)** *A merged image of (A,B), showing a colocalization of the green and red fluorescent signals in all granules (arrows). Bar, 50 μm.*

unglycosylated. This finding is supported by our additional finding that the mobility of the 34 kDa protein by electrophoresis did not alter before and after periodate oxidation of JE (data not shown).

Localization of 34 kDa protein in egg-jelly

The sperm motility-initiating activity in *Cynops* localizes in the outermost layer of the egg-jelly, which consists of six distinct layers in this species (Watanabe *et al.*, 2003). Indirect immunofluorescence observations of frozen sections of egg-jelly in our present analyses revealed a unique pattern of distribution for the 34 kDa protein and ARIS. The 34 kDa protein showed a dot-like expression pattern and was found to localize in the outermost layer (Fig. 5 A,C,E). ARIS was observed to cover the surface of outermost layer with overlapping the dot-like pattern of the 34 kDa protein (Fig. 5 B,F,G). These distributions were further analyzed using outermost jelly layer preparations isolated on glass. The 34 kDa protein was found to be distributed in granules of less than 5 μm in diameter in unfixed specimens (Fig. 6A), whereas ARIS was present in the jelly matrices beneath the granules as well as the granules themselves (Fig. 6B). It should be emphasized that all of the granules containing 34 kDa protein were also covered with ARIS (Fig. 6C).

Scanning electron microscopy

Egg-jelly is a thick extracellular matrix that surrounds the egg, and comprises high molecular weight glycoconjugates that form a stable network with globular proteins (Bonnell *et al.*, 1994; Bonnell *et al.*, 1996). To morphologically confirm the presence of the

granules in the outermost jelly layer, scanning electron microscopic examinations were carried out. As shown in Fig. 7, the J1, J3, and J5 layers were found to be uniformly constructed, whereas the J2 and J4 layers were formed with accumulated laminae. Each layer was fundamentally composed of large fibrils. In contrast to these morphologies, specific structures were observed in the outermost J6 layer. The outer surface was covered with a sheet-like structure, under which many granules were present and which was sustained by a network of thick and flattened structures. The approximate diameter of the granules was 2 μm in methanol-fixed specimens. The localization patterns of these granules and sheet-like structure are consistent with the results obtained by indirect immunofluorescence analyses with α34 and anti-ARIS mAbs (Figs. 5, 6), although the approximate diameter of the granules measured by scanning electron microscopy was smaller than that measured by immunostaining of unfixed samples. This indicates that the 34 kDa protein localizes in the granules, whereas the ARIS is localized in the outer surface of the granules and the sheet-like structure. In inseminated egg-jelly, the granules are sometimes exposed to the outside by the disruption of the sheet-like structure (Fig. 7C).

Discussion

Motility initiation is induced in the newt egg-jelly for the sperm quiescently stored in the female spermatheca (Watanabe and Onitake, 2002; 2003). It is caused by a proteinaceous factor, SMIS,
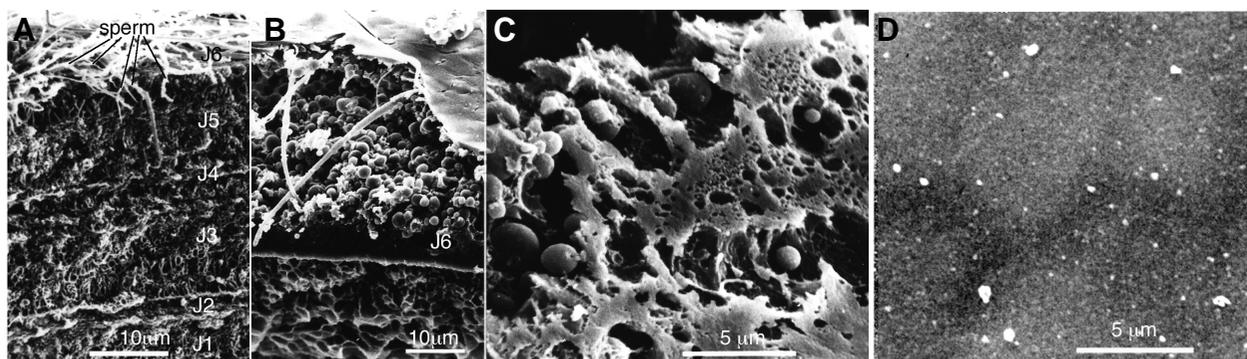

Fig. 7. Scanning electron microscopic images of egg-jelly. (A) *Whole image of the cut face of egg-jelly. J1 to J6 indicate each layer of the egg-jelly from inner to outer.* **(B)** *High-magnification view of the J6 layer. A sheet-like structure covers the outer surface, and many granular structures are present beneath it.* **(C)** *Outer surface of inseminated egg-jelly. The sheet-like structure is partly disrupted, and granular structures are exposed to the exterior.* **(D)** *Outer surface of the sheet-like structure in uninseminated egg-jelly, as a control of (C).*
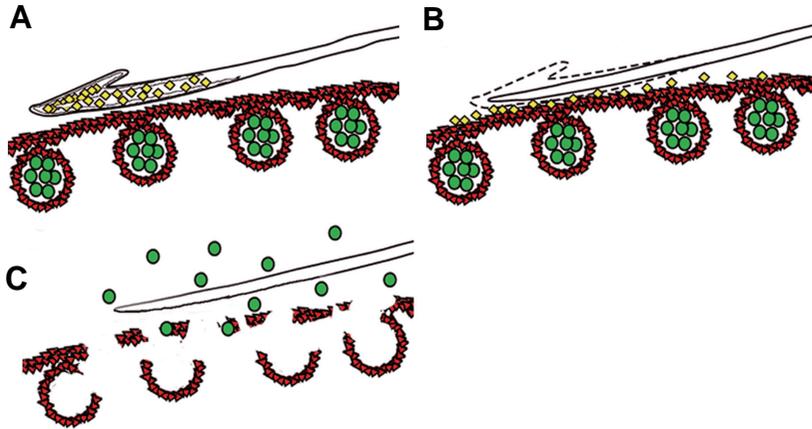
A

B

C

Fig. 8. A working model for sperm motility initiation mediated by the acrosome reaction on the egg-jelly surface in newts. (A) *The sperm are inseminated on to the surface of the egg-jelly directly from the spermatheca.* **(B)** *The acrosome reaction is induced by the ARIS (red triangles) which is present as a sheet-like structure on the surface, and the acrosomal enzymes (yellow squares) are released onto this structure.* **(C)** *The acrosomal enzymes digest the sheet-like structure and the SMIS (green circles) is thereby released from the granules to the sperm where it initiates motility.*

which is less than 50-kDa and more than 500-kDa in its active and inactive forms, respectively (Ukita *et al.*, 1999; Mizuno *et al.*, 1999). Because of this unique feature of sperm activity control, we speculated that a novel mechanism of motility initiation might function during internal fertilization in urodeles. In our present study, we generated a mAb that neutralized the sperm motility-initiating activity of newt egg-jelly and found the SMIS in this species as a 34 kDa unglycosylated protein (Figs 1, 2 and 4). A portion of the expressed 34 kDa protein is present in an unbound form in JE whereas the most of them are associated with the jelly matrices (Figs. 2 and 3), both of which are distributed exclusively in the outermost jelly layer (Fig. 5). Our present findings are fully consistent with the results of previous studies (Mizuno *et al.*, 1999; Watanabe *et al.*, 2003), thus indicating that the 34 kDa protein itself, or a complex consisting of the 34 kDa protein and some other molecules is the SMIS in *C. pyrrhogaster*. This is the first identification of an egg-derived factor that promotes sperm motility initiation in amphibians.

Sperm-activating peptides (SAPs) with a species-specific amino acid sequence of 10 to 15 residues are present in sea urchin egg-jelly (Suzuki, 1995). At spawning, these peptides diffuse into the seawater and can activate motility in swimming sperm (Suzuki *et al.*, 1981; Hansbrough and Garbers, 1981) and also act as a chemoattractant (Ward *et al.*, 1985). In *Xenopus*, allurin, an unglycosylated, heat-stable 21-kDa protein, localizes in the outer layers of the egg-jelly as a sperm chemoattractant (Olson *et al.*, 2001; Xiang *et al.*, 2004). Although the molecular features and the localization of allurin in *Xenopus* egg-jelly seem to be similar to those of the newt SMIS (Mizuno *et al.*, 1999; Figs. 4-6), allurin is highly diffusible and does not activate sperm motility (Olson *et al.*, 2001; Xiang *et al.*, 2005). We show from our current data that the SMIS in newts, unlike allurin and SAPs, associates with other jelly substances and does not easily diffuse into the medium, though it may be diffusible in itself (Figs. 2, 3). The association of newt SMIS with jelly substances should contribute to its rigid localization in the granular structures (Figs. 5-7). Furthermore, it may also contribute to the inactivation of SMIS (Mizuno *et al.*, 1999). In the present

study, the inactivated SMIS is suggested to be activated since most sperm suspended in the JE finally showed motility regardless of the presence of the antibody (Fig. 1). The JE contains ARIS (Watanabe *et al.*, 2009) whose activity is not inhibited by the α34 mAb and the ARIS colocalizes with SMIS in the outermost layer of egg-jelly (Figs. 5, 6). Probably, ARIS involves in an activation process for the inactive SMIS. Both the localization and inactivation of the SMIS in the egg-jelly are specific to the mechanisms underlying sperm motility initiation in the newt. Further studies concerning the molecular structure of SMIS will be needed using recombinant proteins to more fully elucidate the unique features of this novel inducer for sperm motility initiation.

The outermost layer of the egg-jelly is the site for sperm motility initiation at the beginning of internal fertilization in *C. pyrrhogaster* (Watanabe *et al.*, 2003). This layer harbors the acrosome reaction-inducing activity (Sasaki *et al.*, 2002), and is crucial for the success of internal fertilization (Takahashi *et al.*, 2006). Based upon our present data, the SMIS-localizing granular structures are covered with a sheet-like structure in which ARIS localizes (Figs. 5-7). The localization patterns of SMIS and ARIS suggest that, during internal fertilization in *C. pyrrhogaster*, the acrosome reaction is first induced in the inseminated sperm on the surface of the outermost layer of the egg-jelly, whereas motility initiation cannot be induced unless the sheet-like structure is disrupted as shown in Fig. 7C. The acrosomal enzymes may directly disrupt the sheet-like structure, an idea which is compatible with the fact that in *C. pyrrhogaster*, the acrosome reaction must occur on the outermost layer to obtain a high fertilization rate (Takahashi *et al.*, 2006). Based on the possible involvement of the acrosome reaction in sperm motility initiation in the newt as described above, a working model of these processes is presented in Fig. 8.

A remarkable feature of this mechanism in *C. pyrrhogaster* is that the acrosome reaction is first induced in quiescent sperm, in contrast to the generally accepted hypothesis that motile sperm undergo the acrosome reaction in all the animal species. This mechanism of sperm motility initiation mediated by the acrosome reaction may thus be exclusive to internal fertilization in urodeles, in which sperm do not need to access ovulated eggs. Our recent data suggest that molecules homologous to SMIS in egg-jelly may initiate or activate sperm motility in the external fertilization of a primitive species of anuran amphibian, *Discoglossus pictus* (Watanabe *et al.*, unpublished data) and of an urodele amphibian, *Hynobius lichenatus* (Ohta and Watanabe, unpublished data). Furthermore, acrosome reaction-inducing activity is localized in the outermost layer of *Discoglossus* egg-jelly (Campanella *et al.*, 1997), suggesting that a similar mechanism of sperm motility initiation mediated by the acrosome reaction may exist in other amphibian species regardless of their modes of fertilization. On the other hand, this mechanism does not operate in fertilization in *X. laevis* or *B. japonicus*, in which the acrosome reaction is induced on the vitelline envelope after their sperm pass through the jelly layer (Yoshizaki and Katagiri, 1982; Ueda *et al.*, 2002). We thus conclude that different mechanisms of sperm motility initiation are required for the success of various modes of fertilization in

amphibians.

Materials and Methods

Gametes

Ovulation was induced in females of sexually mature newts, *Cynops pyrrhogaster*, by two injections with gonadotropin (Teikoku Zoki) at a dose of 50 IU at a 24 h interval. Eggs were obtained from the posterior most portion of the oviduct, the uterus at 2-3 days after the last injection and stored in a moist chamber. Sperm were obtained from the vasa deferentia of male newts and stored in a moist chamber at room temperature until use.

Jelly extracts

Mature newt eggs were immersed in Steinberg's salt solution (ST) (58.2 mM NaCl, 0.67 mM KCl, 0.34 mM CaCl$_2$, 0.83 mM MgCl$_2$, 10 mM Tris-HCl; pH 8.5) at a volume of 20 μl per egg and shaken at 4°C for 1 h. The solution was then centrifuged at 16000 x g at 4°C for 30 min and the supernatant was collected as jelly extract (JE). The pellet was washed twice and a volume of ST equal to that of the JE was added as jelly matrix solution (JM). These JE and JM preparations were stored at -30°C.

Induction of sperm motility

Sperm motility experiments were performed according to the method described previously (Ukita *et al.*, 1999). Briefly, dry sperm (1 μl) were suspended in JE for up to 10 min and sperm motility was calculated at 1, 3, 5, and 10 min as the percentage of sperm moving their undulating membrane. As a control, ST was substituted for JE.

Preparation of monoclonal antibodies

Mice were immunized with JE as described previously (Watanabe *et al.*, 2009). The lymph node cells from the immunized mice were fused with PAI myeloma cells as described previously (Katagiri *et al.*, 1999). The first selection of hybridomas was performed by assaying the neutralizing activity of the culture supernatants against the sperm motility-initiating activity in JE as follows. JE was mixed with a one-third volume of culture supernatant and allowed to sit for 30 min at room temperature. Sperm motility-initiating activity was estimated as described above. The culture supernatants were also selected by western blotting of the JE. Briefly, JE in Laemmli's sample buffer containing 2-mercaptoethanol was electrophoresed using a 10% polyacrylamide gel, followed by electrotransfer onto a polyvinylidene difluoride (PVDF) membrane (Millipore) according to the method of Towbin *et al.* (1979). The membrane was blocked with 5% skim milk in PBS and incubated with the culture supernatants for 1 h and then with a peroxidase-conjugated anti-mouse Ig (GE Healthcare) at a 1:1000 dilution in 1% skim milk in PBS. The reactions were developed using chemiluminescence. The isotype of the produced mAbs was determined using a mouse monoclonal antibody isotyping kit (GE Healthcare).

The hybridoma clone (2x10^7 cells per mouse) that generated the α34 mAb was implanted into the body cavities of pristane (2,6,10,14-tetramethylpentadecane)-primed nude mice, and the ascites fluid was obtained at 14 days after that implantation. The antibody in the ascites fluid was purified with an Affi-Gel Protein A MAPS II Kit (BioRad) according to the manufacturer's instructions. Fab fragments were prepared using an ImmunoPure Fab Preparation Kit (Pierce Chemical).

Induction of acrosome reaction

Acrosome reaction was induced according to the method described previously (Sasaki *et al.*, 2002; Watanabe *et al.*, 2009). JE was pretreated with 100 μg/ml α34 mAb for 30 min. Dry sperm (1μl) were suspended in the JE and allowed to sit for 5 min. They were fixed in 2.5% glutaraldehyde in PBS. Acrosome reaction was evaluated in the sperm by microscopic observation. JE without treating with the mAb or ST was substituted to the mAb-pretreated JE as controls.

Detection of carbohydrates

Carbohydrate moieties of glycoprotein in JE were labeled with biotin hydrazide using an ECL glycoprotein detection system (GE Healthcare) according to manufacturer's instructions. Substances in the JE that had been electrophoresed using a 2-15% polyacrylamide gel and electrotransferred onto a PVDF membrane were oxidized for 20 min with 10 mM sodium metaperiodate in 100 mM acetate buffer (pH 5.5) in the dark. After brief rinsing with PBS, the aldehyde group formed was biotinylated with 5 mM biotin hydrazide. The membrane washed was incubated with peroxidase-conjugated streptoavidin, and the bands were visualized by ECL detection.

Immunostaining

Mature eggs were embedded in OCT compound, and frozen 20 μm sections were prepared. The outermost layer of newt egg-jelly was obtained from mature eggs following adherence to glass slides. The slides were then washed with PBS, blocked with 5% skim milk in PBS, and treated at room temperature for 1 h with the α34 mAb (IgG2a) diluted to a 1 μg/ml concentration using the culture supernatant of the hybridoma clone that produced the anti-ARIS mAb (IgM) (Watanabe *et al.*, 2009). Mouse monoclonal IgG2a and IgM (Sigma) diluted at 1 μg/ml each in fresh HAT medium were used as the control. After washing with PBS and blocking with 5% skim milk in PBS, the sections were treated with Alexafluor 488-conjugated anti-mouse IgG2a (Molecular Probes) and Alexafluor 594-conjugated anti-mouse IgM (Molecular Probes) at room temperature for 1 h to enable indirect immunofluorescence microscopic examination (BH2-frk; Olympus).

For immunostaining of sperm, sperm were suspended in PBS followed by brief centrifugation at 1500 x g. This washing was repeated several times. The washed sperm blocked with 5% skim milk in PBS were subjected to immunoreaction. The sperm were treated at room temperature for 1 h with the α34 mAb (IgG2a) diluted to a 1 μg/ml in PBS. After washing and blocking, they were treated with Alexafluor 488-conjugated anti-mouse IgG2a.

Scanning electron microscopy

Egg-jelly was fixed in chilled methanol to prevent hydration-induced swelling of the jelly matrix or disruption of the structure at the surface layer. This was followed by freeze-drying and dissection with a fine blade. On the cut face of the egg-jelly, the morphological features of each layer were well preserved. The specimens were next coated with platinum and observed using a scanning electron microscope (JSM-5400; JEOL).

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