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Treatment of *Xenopus laevis* Coelomic Eggs With Trypsin Mimics Pars Recta Oviductal Transit by Selectively Hydrolyzing Envelope Glycoprotein gp43, Increasing Sperm Binding to the Envelope, and Rendering Eggs Fertilizable

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ABSTRACT *Xenopus laevis* coelomic (body cavity) eggs are not fertilizable until they pass through the pars recta oviduct. A secreted pars recta oviductal protease with trypsin-like activity, oviductin, selectively hydrolyzes egg envelope glycoprotein gp43 to gp41; this limited proteolysis is believed to render the egg fertilizable. The effects of trypsin as a substitute for oviductin in modifying envelope structure and function were examined. Trypsinolysis (5 mIU for 30 min at room temperature) selectively converted gp43 to gp41 without hydrolysis of other envelope glycoproteins, and rendered coelomic eggs fertilizable in the presence of a jelly water preparation. Chymotrypsin had no effect on the acquisition of fertilizability, indicating that the reaction was dependent on trypsin-like specificity. This was confirmed by the use of p-aminobenzamindine and leupeptin to inhibit the ability of trypsin preparations to induce fertilizability. A sperm binding assay revealed that trypsin treatment dramatically increased sperm binding to egg envelopes derived from both coelomic eggs and ovarian eggs. Jelly water was not required for sperm binding. Therefore, trypsin can mimic the biological action of oviductin, selectively cleaving egg envelope gp43 to generate or expose sperm binding sites, rendering the envelope penetrable by sperm and permitting fertilization. *J. Exp. Zool. 281:132–138, 1998.*

Eggs of anuran amphibians, obtained from the coelomic cavity (body cavity), cannot be fertilized due in part to the inability of sperm to penetrate the egg envelope (reviewed by Elinson, ’86; Katagiri, ’87; Hedrick and Nishihara, ’91). The envelope becomes penetrable as the egg passes through the first portion of the oviduct, the pars recta. Pars recta extracts and secretions have been used to render egg envelopes penetrable by sperm, and the process appears to involve a protease with trypsin-like substrate specificity (Miceli, ’86; Bakos et al., ’90a). This correlates with the observation that a specific egg envelope component is hydrolyzed during passage through the pars recta. The pars recta protease of *Xenopus laevis*, termed oviductin, has been purified and shown to cleave specifically egg envelope glycoprotein gp43 to gp41 (Hardy and Hedrick, ’92). This cleavage appears to be responsible for the overall envelope conformation change that alters the physical properties of the envelope, decreasing physical strength and making it more easily solubilized by heat or chemical agents (Nishihara et al., ’83; Bakos et al., ’90b). A question that remains is whether oviductin action by itself is sufficient to allow for sperm penetration of the egg envelope, or whether other pars recta oviductal factors are also required. For example, in *Bufo japonicus*, a pars recta factor trapped within the envelope structure has been indicated as inducing the sperm acrosome reaction (Katagiri et al., ’82; Takamune and Katagiri, ’87). Also, as detected by electron microscopy, a fibrous layer appears to be added to the inner aspect of the envelope during oviductal transit (Larabell and Chandler, ’89), although it is unknown whether this structure has a role in fertilization.

During our studies of envelope conversion by *Xenopus* oviductin, it was noted that selective processing of envelope gp43 could also be achieved

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using commercially available trypsin (Hardy and Hedrick, '92). This finding led us to ask the question of whether treatment of coelomic eggs with trypsin could induce fertilizability, thus testing the role of gp43 hydrolysis in envelope penetrability. A positive result would be very useful in a practical sense because it would provide a relatively quick and simple method for fertilizing eggs isolated from the coelomic cavity. Current experimental protocols usually include the use of (1) pars recta extracts to convert the envelopes; (2) removal of the envelopes with high concentrations of proteases or by manual dissection; or (3) the insertion of tagged, experimental eggs into the body cavity of a host female to allow for passage through the oviduct (Heasman et al., '91). Identification of a simple, nondamaging substitute for oviductal exposure would clearly be a valuable tool.

We present here the results of our studies on the effects of trypsin on fertilizability of coelomic eggs, on envelope glycoprotein structure, and on sperm binding to the egg envelope.

MATERIALS AND METHODS

All enzymes and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

Enzyme assays

Proteases tested were crude trypsin from porcine pancreas (Type II), and sequencing grade trypsin and α-chymotrypsin from bovine pancreas. Protease stock solutions were prepared as 1 mg/ml in reaction buffer (200 mM NaCl, 1 mM $CaCl_2$, 25 mM Tris HCl pH 8.0). Trypsin activity was measured spectrofluorometrically using the substrate N-tert-butoxycarbonyl-Phe-Ser-Arg-7-amido-4-methylcoumarin (Boc-Phe-Ser-Arg-MCA). For chymotrypsin activity measurements, the substrate used was N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (Suc-Ala-Ala-Pro-Phe-MCA). The assay mixture consisted of 300 μl reaction buffer, 2 μl sample (usually a 1:100 dilution of the protease stock solution), and 1 μl substrate stock solution (5 mM in dimethyl sulfoxide). Fluorescence was measured at 380 nm excitation and 460 nm emission. A standard curve was constructed using known concentrations of 7-amino-4-methylcoumarin. Alternatively, a spectrophotometric assay was used for measurement of trypsin activity, using the substrate Nα-benzoyl-L-arginine-ethyl ester (BAEE), with an assay mixture of 965 μl reaction buffer, 25 μl sample, and 10 μl substrate stock solution (100 mM in water); the absorbence was measured at 253 nm. Enzyme units were calculated using a molar absorptivity value of 1150 M^{-1} cm^{-1} (Sarath et al., '89).

Egg materials

Oviposited eggs were obtained by inducing female frogs to shed eggs by an injection of 35 IU of pregnant mare serum gonadotropin (PMSG) followed 4 days later by injection of 1000 IU of human chorionic gonadotropin (hCG, from Scripps Laboratories, San Diego, CA) (Hedrick and Hardy, '91). Eight to 12 h after the final injection, eggs were stripped from females. These eggs were dejellied using mercaptoethanol as described previously (Wolf and Hedrick, '71), and rinsed in DeBoers solution (DB: 110 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, to pH 7.2 with $NaHCO_3$). Coelomic eggs were obtained by ligating the animal's oviducts prior to hormonal stimulation, to prevent ovulated eggs from entering the oviducts (Hedrick and Hardy, '91). Eggs were then obtained from the body cavity surgically, 10–12 h post-hCG, and rinsed in DB. Ovarian eggs were obtained from PMSG-stimulated animals four days after injection, by removing the ovary and releasing eggs from the tissue using a meat grinder (Hedrick and Hardy, '91).

Egg envelopes from oviposited eggs (vitelline envelopes), coelomic eggs (coelomic envelopes), and ovarian eggs (ovarian envelopes) were isolated as described by Hedrick and Hardy ('91). This process consisted of passing eggs through an 18-gauge syringe needle, and sieving the egg lysate over a 102-μm nylon mesh to collect the envelopes. Envelopes were washed on the screen with water, pelleted by centrifugation, and stored in high-salt solution at 4 C to solubilize contaminating yolk, as described previously (Lindsay and Hedrick, '89). Sodium azide (0.02%) was added to the high-salt solution if envelopes were stored longer than overnight. Before use the envelopes were washed 3 times by repeated pelleting and resuspension in water.

Egg and envelope treatments

For protease treatment and fertilization of coelomic eggs, rinsed eggs were placed into 1-cm wells of a glass spot plate. The DB was replaced with a given enzyme solution and allowed to incubate at room temperature for 30 min, after which the eggs were rinsed with four changes of 1/3 DB and fertilized. A sperm suspension was prepared in jelly water as described by Heasman et al. ('91) and used immediately to fertilize eggs. After about 30 min, the eggs were rinsed with 1/3

DB to remove excess sperm and testicular material. Fertilization was determined by development to the 8 to 16 cell stage.

For protease treatment of isolated envelopes, 20–50 μl of washed, pelleted envelopes were resuspended in 1 ml of trypsin solution and incubated at room temperature for 30 min. Envelopes were then washed 3 times with water and either used in sperm binding assays or analyzed by SDS-PAGE under reducing conditions as described previously (Hardy and Hedrick, '92).

Sperm binding assays

Sperm were collected as described above, in either jelly water or 1/3 DB, and then mixed with an equal volume of isolated egg envelopes suspended in 1/3 DB (usually 200 μl each). After 5–10 min, the unbound sperm were separated from the envelopes by passage over a 102-μm nylon mesh screen followed by gentle rinsing with 1/3 DB. Care was taken to keep the envelopes suspended in buffer during the washing process. The envelopes were then pipetted from the screen and added to an equal volume of fixative (2.5% glutaraldehyde in 1/3 DB). This mixture was transferred to a microscope slide and a coverslip was applied. In an alternate procedure to remove envelopes at specific time points after sperm addition, an isolated envelope with attached sperm was picked from the incubation mixture using fine-tipped forceps, and unbound sperm were washed from the envelope by swirling the tip of the forceps holding the envelope in a dish containing 1/3 DB. The envelope was then released into a drop of fixative on a microscope slide and a coverslip was applied.

RESULTS

Effects of trypsin treatment on fertilizability of eggs

Hardy and Hedrick ('92) reported that treatment of isolated coelomic envelopes with 2.5 mIU/ml oviductin or trypsin (Boc-Phe-Ser-Arg-MCA as the substrate) for 30 min resulted in the complete and selective hydrolysis of envelope gp43 to gp41. Therefore, we used the same conditions for testing the ability of trypsin to render coelomic eggs fertilizable. Table 1 shows that this treatment resulted in about 40% fertilization, which was essentially identical to the level of fertilization observed for dejellied, oviposited eggs, which were used as a control. When the concentration of trypsin was reduced, the percentage of fertilized eggs decreased dramatically, and fertilization was never observed when trypsin was eliminated altogether. Using 5.0 mIU/ml of trypsin (2×) gave a substantial rise in the number of fertilized eggs, to around 70%. This level of trypsin (5.0 mIU/ml) did not appear to damage the envelopes, since treated, isolated envelopes appeared normal when viewed using phase-contrast microscopy, and an SDS-PAGE gel of the envelopes showed that only gp43 was hydrolyzed to gp41 while all other components remained intact (Fig. 1). In fact, envelopes treated with 125 mIU/ml trypsin (50×) gave identical results (data not shown), indicating that the envelope structure is highly resistant to cleavage by trypsin, with the only sensitive bond being within gp43 to yield gp41.

The experiments described above utilized a crude trypsin preparation. Since the preparation also contained up to 1% chymotrypsin, inhibitors of trypsin were tested for the ability to prevent the acquisition of fertilizability of coelomic eggs treated with the crude trypsin preparation. As shown in Table 1, fertilizability was reduced to zero by the trypsin inhibitors leupeptin and p-aminobenzamidine, indicating that trypsin was the active agent within the crude preparation. This conclusion was supported further by the ability of homogeneous preparations of trypsin (sequencing grade) to induce fertilizability, and the

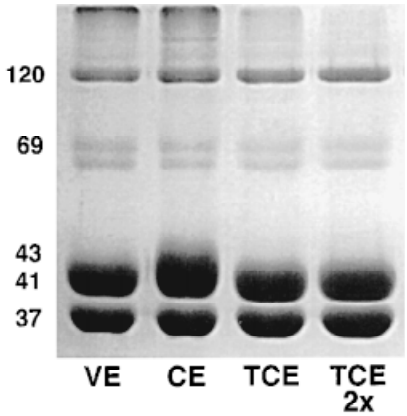

Fig. 1. SDS-PAGE (8.75% gel) of isolated egg envelopes, showing selective hydrolysis of gp43 by trypsin. The hydrolysis of gp43 to gp41 is observed as a sharpening and increase in band mobility, most evident on this particular gel at the top of the band. VE = vitelline envelopes isolated from dejellied, oviposited eggs as a control demonstrating in vivo gp43 hydrolysis; CE = envelopes from coelomic eggs; TCE = envelopes from coelomic eggs treated with 2.5 mIU/ml trypsin, or 5 mIU/ml (TCE 2×). Apparent molecular masses (in kDa) of envelope glycoproteins are given to the left of the gel. Twenty micrograms of protein were loaded into each lane.

TABLE 1. Effects of proteases on fertilizability of coelomic eggs

Treatment[1]	% fertilization[2]	n[3]	Total no. of eggs examined
Controls			
Buffer only	0 ± 0	9	197
Oviposited eggs[4]	42 ± 33	5	107
Coelomic eggs + crude trypsin			
5.0 mIU/ml	69 ± 21	3	106
2.5 mIU/ml	41 ± 22	9	323
1.3 mIU/ml	11 ± 3	3	47
0.25 mIU/ml	0 ± 0	3	53
+ PABA[5]	0 ± 0	6	103
+ leupeptin[5]	0 ± 0	3	73
Coelomic eggs + purified trypsin			
5.0 mIU/ml	70 ± 11	2	91
2.5 mIU/ml	29 ± 13	7	160
Coelomic eggs + purified chymotrypsin[6]			
5.0 mIU/ml	0 ± 0	2	44

[1]Coelomic eggs were treated for 30 min at room temperature with the designated amount of protease preparation, with trypsin enzyme units determined using Boc-Phe-Ser-Arg-MCA as the substrate.
[2]Mean ±S.D.
[3]Number of females used as sources of eggs.
[4]As a positive control, oviposited eggs were dejellied and then fertilized as described for the coelomic eggs. In two of the experiments, both coelomic and oviposited eggs were obtained from the same female by ligating only one of the oviducts.
[5]Inhibitors were used with 2.5 mIU/ml crude trypsin preparations.
[6]Enzyme activity was determined using Succ-Ala-Ala-Pro-Phe-MCA as the substrate.

inability of purified chymotrypsin to render eggs fertilizable.

Effects on sperm binding

A sperm binding assay was developed to study the mechanism of increased fertilizability of coelomic eggs upon treatment with trypsin, using isolated envelopes to avoid the inconvenience associated with using dejellied eggs. A time course of sperm-envelope binding showed that the number of bound sperm reached a maximum at around five minutes, and stayed at this level for at least 45 min (Fig. 2). Thus sperm did not appear to detach or fall off the envelope in this time period. Also, the time course and level of sperm binding was essentially identical in the presence or absence of jelly water (Fig. 2). From these data, we chose to use routine experimental conditions of a 5–10 min incubation period, in the absence of jelly water.

As shown in Figure 3, coelomic envelopes bound very few sperm, while vitelline envelopes exhibited a high level of sperm binding, as expected. Once coelomic envelopes were treated with the same amount of trypsin that gave high levels of

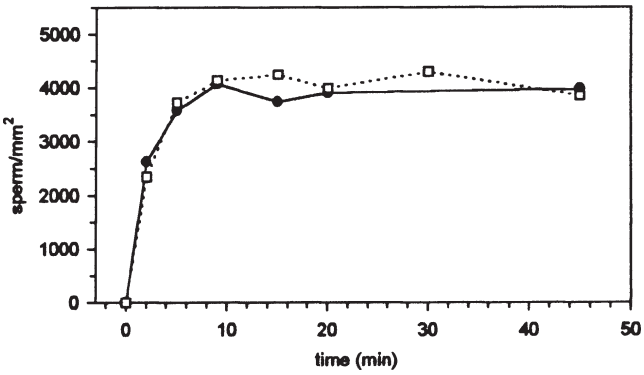

Fig. 2. Time course of sperm binding to isolated vitelline envelopes, in the presence of jelly water (filled circle), or in buffer only (open square). The data are from one representative experiment.

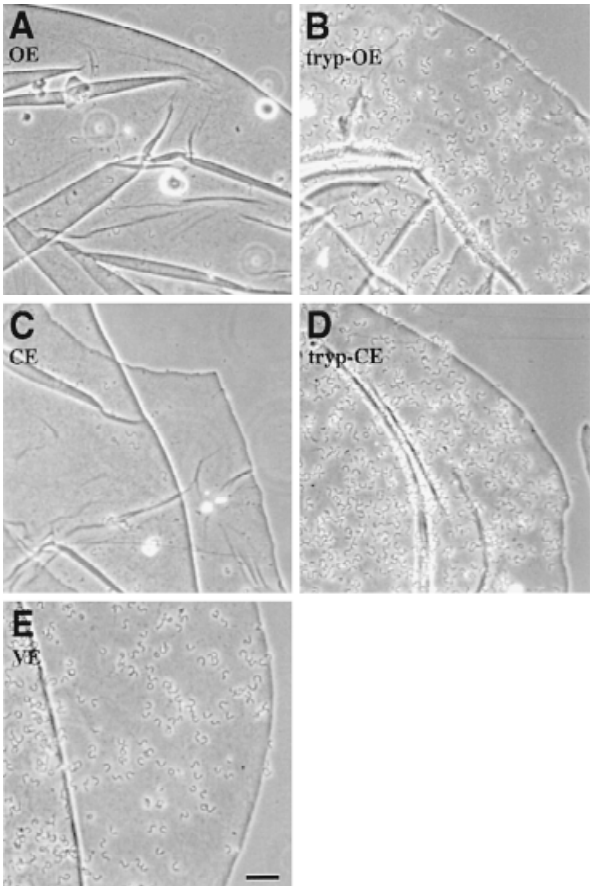

Fig. 3. Sperm binding to isolated envelopes. (**A**) An untreated ovarian envelope; (**B**) an ovarian envelope treated with trypsin (5 mIU/ml) as described under Materials and Methods; (**C**) an untreated coelomic envelope; (**D**) a trypsin treated coelomic envelope (5 mIU/ml); and (**E**) an untreated vitelline envelope. Bar = 25 μm.

fertilization of coelomic eggs (5 mIU/ml), sperm binding increased dramatically. Quantification of binding (Table 2) showed that the trypsin treatment actually resulted in levels of sperm binding exceeding that observed to vitelline envelopes, which correlated with the higher levels of fertilization observed with 5 mIU/ml trypsin treatment (about double in both cases). Envelopes from ovarian eggs were also examined, to test whether oocyte maturation has any effect on the ability of trypsin to increase sperm binding. We found that ovarian envelopes responded essentially as well to trypsin treatment as coelomic envelopes (Fig. 3, Table 2).

DISCUSSION

The results presented here show that trypsin can mimic the reactions associated with pars recta oviductal transit in *Xenopus* by selectively cleaving egg envelope gp43 to gp41 and rendering coelomic eggs fertilizable. Trypsin treatment also dramatically increased sperm binding to egg envelopes, which has also been observed using purified oviductin protease (Lindsay and Hedrick, in press). This suggests a limited proteolytic mechanism by which fertilizability of eggs is achieved through an increase in sperm binding. Therefore, we propose that the limited proteolysis of gp43 causes conformational changes that produce a rearrangement of envelope glycoproteins, thus exposing sperm binding sites. Our laboratory has previously documented such envelope conformational changes for *Xenopus* (Nishihara et al., '83; Bakos et al., '90b). In addition to revealing sperm binding sites as shown here, it is known that these conformational changes alter the physical properties of the envelope, perhaps inferring a greater sensitivity of the envelopes to sperm lysin, a protease involved in envelope penetration, as has been shown for *Bufo japonicus* (Takamune et al., '86) and *B. arenarum* (Miceli, '86).

We found that a trypsin concentration of 2.5 mIU/ml (30 min incubation), which was the amount of pars recta oviductin activity required to completely process gp43 (Hardy and Hedrick, '92), rendered coelomic eggs fertilizable. Doubling the trypsin concentration to 5 mIU/ml almost doubled the fertilization rate, which is puzzling considering that 2.5 mIU/ml was sufficient to completely process gp43 and to give a level of fertilization comparable to the control eggs that represented the in vivo processing. It is unlikely that the higher trypsin concentration compromised the integrity of the envelope thus allowing sperm direct access to the egg surface because the envelopes appeared identical to untreated envelopes when viewed microscopically and by SDS-PAGE. It is possible that the use of marginal amounts of protease (2.5 mIU/ml) to hydrolyze gp43 is not sufficient to allow for the complete reorganization of the envelope structure to expose a maximal number of sperm binding sites. If this is the case, then the processing that occurs in vivo must not be complete, but certainly adequate to enhance sperm binding enough to allow for fertilization. In fact, limiting the amount of sperm binding may be important toward preventing polyspermy. This interpretation of our results might also explain results obtained in studies of *B. japonicus*, where it was shown that limited hydrolysis of envelope glycoproteins by pars recta extracts was required but not sufficient for fertilization (Takamune and Katagiri, '87; Takamune et al., '87). If their pars recta preparations contained minimal amounts of proteolytic activity to achieve hydrolysis of the envelope, then it is possible that the envelope had not undergone sufficient conformational change to permit fertilization. It will be interesting to determine whether there is a correlation between increasing protease concentration, gp43 processing, the degree of envelope conformational change, and the amount of sperm binding.

A trypsin-induced increase in sperm binding was also described for *B. japonicus* in a recent study by Omata and Katagiri ('96). They noted that a short treatment of coelomic envelopes with 0.001% trypsin increased sperm binding and selectively cleaved envelope component gp40-52 to gp36-39 (analogous to *Xenopus* gp43 to gp41). This trypsin-induced change was identical to the envelope processing that occurs in vivo during pars recta oviductal transit (Takamune et al., '86). Based on our results with *Xenopus*, we predict that trypsin treatment of *Bufo* coelomic eggs will also render eggs fertilizable. Consistent with this prediction is an earlier observation made by Elinson ('73)

TABLE 2. Effects of trypsin on sperm binding to isolated egg envelopes

Envelope	Trypsin treatment*	Sperm/mm^2	% binding
Vitelline	–	2280 ± 880	100
Ovarian	–	140 ± 50	6
Ovarian	+	4340 ± 660	190
Coelomic	–	98 ± 42	4
Coelomic	+	5470 ± 1290	240

*5 mIU/ml, 30 min.

using *Rana pipiens* eggs. Brief treatment (60 sec) of coelomic eggs with 0.001% trypsin enhanced their fertilizability. It will be interesting to determine whether other species also exhibit similar egg envelope structural and functional changes in response to trypsin.

The sperm-envelope binding assay used by Omata and Katagiri ('96) for *B. japonicus* was very similar to that used in our study of *Xenopus* sperm binding, and the levels of binding were comparable, about 1350 sperm/mm^2 for *Bufo* vitelline envelopes, and 2300 sperm/mm^2 for *Xenopus*. Our larger value possibly reflects the fact that *Xenopus* sperm may have bound to both sides of the envelope. It was difficult to distinguish one envelope side from the other because of the thinness of the envelopes (1 μm) compared to the size of the sperm heads (10 μm). This is in contrast to the much thicker *Bufo* envelope, which allows for focusing of the microscope on one side of the envelope or the other for sperm counting. Recently, a study of sperm-egg envelope binding in *Xenopus* was reported by Tian et al. ('97a), in which intact (dejellied) eggs were used as opposed to isolated envelopes. They observed that sperm binding appeared to reach a maximum of about 1,500 sperm per egg, which converts to about 300 sperm/mm^2, roughly one-fifth to one-tenth the level of binding that we observed in our study. Other than the use of dejellied eggs versus isolated envelopes, the only significant difference in assay conditions was the presence of jelly water in the samples of Tian et al., whereas our experiments omitted jelly water. But, as both groups observed, jelly water had no effect on the level of sperm binding. This finding is interesting because jelly water is required for fertilization, but apparently for a step downstream from sperm binding. The discrepancy in sperm binding values may be due to differences in the states of the envelopes (in situ vs. isolated) or in the washing conditions. Or perhaps our numbers reflect the presence of a larger number of sperm binding sites on the inner surface of the envelope. This possibility might be addressed using scanning electron microscopy to distinguish one envelope surface from another.

Our hypothesis that gp43 hydrolysis triggers conformational changes in envelope glycoproteins which expose sperm binding sites is supported by recent results of Tian et al. ('97a,b). Using competitive sperm binding assays and isolated envelope glycoproteins or antibodies, they determined that gp69 was the egg envelope ligand for sperm binding and, as determined by antibody binding experiments, gp69 was inaccessible in the CE but was accessible in the VE. The change in accessibility of gp69 to antibodies and sperm was proposed to be due to the processing of gp43 to gp41 and accompanying conformational changes, and now our results confirm this view. As mentioned above, an oviduct proteolytic processing event similar to that in *Xenopus* has been observed in *B. japonicus* egg envelopes (gp40-52 to gp36-39), which correlates with increased sperm binding. However, in *B. japonicus*, the envelope ligand for sperm binding was identified as gp36-39 itself (Omata and Katagiri, '96), in contrast to the results of Tian et al. with *Xenopus* where a separate envelope component, gp69, was the ligand for sperm. The *B. japonicus* results are intriguing from an evolutionary perspective, since gp40-52 is thought to be the structural homologue of *X. laevis* gp43 (Takamune et al., '87), and from sequence identity, gp43 is homologous to the egg envelope (zona pellucida) ligand for sperm in mammals, ZPC or ZP3 (Kubo et al., '97; Yang and Hedrick, '97). Thus, there may be a phylogenetic conservation of structure and binding function of this envelope glycoprotein. The proposal of Tian et al. ('97a) that gp69 is the ligand for sperm binding in *Xenopus* presents an apparent conflict with this evolutionary perspective. However, phylogenetic comparisons of structure-function relations is speculative at this point because *Xenopus* gp69 and *B. japonicus* gp40-52 have not been characterized with respect to cDNA/amino acid sequence nor in terms of oligosaccharide structures. Additional structure-function information is clearly required to resolve this apparent conflict.

The ability of trypsin to render *Xenopus* coelomic eggs fertilizable should be useful to investigators interested in fertilizing experimental oocytes that have been matured in vitro. Trypsin appears to be an ideal choice for mimicking the effects of oviductin because it has only a limited ability to digest egg envelope glycoproteins. This allows for processing of gp43 without compromising envelope integrity, in contrast to the almost complete digestion of the envelope as occurs with the commonly used protease pepsin (Heasman et al., '91). Our results showing that ovarian egg envelopes treated with trypsin bind sperm as well as treated coelomic envelopes suggest that trypsin treatment may allow for fertilization of in vitro matured oocytes.

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