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Vitellogenin C-terminal fragments participate in fertilization as egg-coat binding partners of sperm trypsin-like proteases in the ascidian *Halocynthia roretzi*

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ABSTRACT

Sperm trypsin-like proteases are known to play important roles in fertilization, but their detailed functions are still unknown. We previously explored the binding partners of sperm trypsin-like proteases, HrProacrosin and HrSpermosin, in the ascidian *Halocynthia roretzi*, and we isolated several candidate proteins on the vitelline coat. We found that some of these proteins are identical to the C-terminal coding region (CT) and von Willebrand factor type D (vWF-D) domain of vitellogenin. We also found that CT on the vitelline coat disappears after fertilization. Vitellogenin is a large lipid transfer protein that is enzymatically processed during vitellogenesis. Although the processed domains including phosvitin and lipovitellin are known to function as yolk nutrient proteins, the roles of the CT and vWF-D domain remain elusive. Our results showed that the CT and vWF-D domain of vitellogenin are processed and attached to the vitelline coat, which in turn participate in fertilization as the binding partners of sperm proteases.

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Introduction

Fertilization is a key event in sexual reproduction, which is essential for living organisms to achieve genetic diversity in the next generation. For successful fertilization, sperm must bind to and penetrate through the extracellular glycoprotein matrix surrounding the egg, which is called the zona pellucida (ZP) in mammals and the vitelline coat (VC) in marine invertebrates [1,2]. In mammals, an acrosomal trypsin-like protease, acrosin [EC 3.4.21.10], had long been believed to be a lytic agent, lysin, which makes a small hole for sperm penetration through the ZP of the ovum [3–5]. However, Baba et al. created an acrosin-knockout mouse and showed that acrosin is not essential but is important for fertilization, since a significant (about 30 min) delay during the penetration of sperm through the ZP was observed in this animal [6]. It is currently believed that acrosin is involved, at least in part, in the dispersal of acrosomal contents during acrosome reaction [7] and in the secondary binding of sperm to the ZP [8,9]. However, more detailed studies on the targets of sperm proteases, including acrosin, are necessary to elucidate the roles of sperm proteases in fertilization.

In order to explore the sperm proteases involved in fertilization, we previously examined the effects of protease inhibitors on fertilization using the ascidian *Halocynthia roretzi*, since large quantities of fertilizable gametes are easily obtained in this aquacultured species and also since fertilization experiments using this species are easy compared to experiments using mammals [10]. We found that trypsin and chymotrypsin inhibitors had strong inhibitory effects on fertilization [10–12] and also that the sperm-egg interaction was inhibited by protease inhibitors [13,14]. From these results, we thought that trypsin-like protease(s) and chymotrypsin-like protease, later identified as the proteasome, of sperm play important roles in ascidian fertilization. We therefore purified two trypsin-like proteases, HrAcrosin and HrSpermosin, from sperm of *H. roretzi* [15], and we found that both of these proteases are involved in fertilization [16–18].

To investigate their precise roles in fertilization, we explored the binding partners of these proteases using affinity-beads, and we isolated several VC proteins [19,20]. However, the N-terminal sequences of these proteins showed no significant homology to any protein. In the present study, therefore, we attempted to identify these proteins by cDNA cloning. Unexpectedly, we found that some of these proteins are identical to the C-terminal coding region (CT) and von Willebrand factor type D (vWF-D) domain, which is located at the C-terminal region of vitellogenin. We also noticed that the CT disappears during fertilization. These results suggest that the CT and vWF-D of vitellogenin play a novel role in ascidian fertilization.

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Materials and methods

Animals and gametes. The solitary ascidian, *H. roretzi*, type C, was used in this study. Sperm and eggs were collected from dissected gonads as described previously [10,12].

Molecular cloning. In order to determine a cDNA sequence corresponding to the 30-kDa VC protein, we carried out 3′- and 5′-RACE-PCR experiments using a SMART RACE cDNA Amplification Kit (Clontech) with the following primers:

a (fwd-1): 5′-AARGGNGTNCCIGCIYTICA-3′
b (rev-1): 5′-GCYTCYTCIGTRTTIG-3′
c (fwd-2): 5′-CACGCAGTCCTATTAGAAGCTTGCG-3′
d (fwd-3): 5′-GTCCTATTAGAAGCTTGCGAATCAGTTGCC-3′
e (rev-2): 5′-GGCAACTGATTCGCAAGCTTCTAATAGG-3′
f (rev-3): 5′-TTCGCAAGCTTCTAATAGGACTGCGTG-3′.

The first PCR was carried out using the primers a and b and *H. roretzi* genomic DNA as a template. 3′-RACE and 5′-RACE were performed using the primers c and d and primers e and f, respectively, which were designed from the results of the first PCR, and *H. roretzi* gonad cDNA as a template, as described in [21].

Pulldown assay. The interaction between CUB domain-1 and the 30-kDa VC protein was analyzed by a pulldown assay using the MagneHis Protein Purification System (Promega). The 30-kDa VC protein inserted into the expression vector pCold TF (Takara) was expressed in *Escherichia coli* BL21(DE3). The recombinant His-tag fused 30-kDa VC protein was immobilized on MagneHis Ni-particles by incubating for 1 h at room temperature. The particles were washed with phosphate buffered saline (PBS) and incubated with [^{35}S]-labeled CUB domain-1, which had been synthesized using the TNT T7 Quick Coupled Transcription/Translation System (Promega), in PBS containing 1% bovine serum albumin. After 3 h of incubation at room temperature, the particles were washed with PBS three times followed by incubation in SDS sample buffer. The eluate was analyzed by SDS–PAGE and autoradiography. The intact pCold TF vector was used as a control.

Southern and Northern blot analyses. For Southern blot analysis, genomic DNA was obtained from the gonad using proteinase K-phenol/chloroform/isoamyl alcohol according to the standard procedure [21]. After exhaustive digestion with *Bam*H I, *Eco*R I, *Hin*d III, or *Pst* I, agarose gel electrophoresis was carried out and the DNA fragments were blotted onto a Hybond-N+ membrane (GE Healthcare) [21]. For Northern blot analysis, poly(A)$^{+}$ RNAs were prepared from muscle, intestine, gonad, gill (branchial basket), endostyle and hepatopancreas and then subjected to agarose gel electrophoresis, followed by transfer onto a Hybond-N+ membrane. The membranes were hybridized with the DIG-labeled PCR-amplified DNA encoding the 30-kDa VC protein (622–978 bp in Fig. 1A) as a probe and washed under high stringency conditions. Hybridization was carried out as described in [21]. Signals were detected with a DIG nucleic acid detection kit (Roche Applied Science).

Immunological procedures. The 30-kDa VC protein inserted into the expression vector pET32a (Novagen) was expressed in *E. coli* BL21(DE3). The recombinant 30-kDa VC protein was extracted and subjected to SDS–PAGE followed by staining with Coomassie Brilliant Blue R-250. The band was excised, cut into small pieces, and suspended in PBS, which was emulsified with Freund complete adjuvant. An ICR mouse was intraperitoneally injected three times at two-week intervals with the emulsion containing 50 μg each of antigen. Antiserum was obtained two weeks after the last booster. Preimmune serum was used as a control. The VC was isolated according to [22] and subjected to SDS–PAGE [23] followed by blotting onto a Hybond-ECL nitrocellulose membrane. Western blots were detected with ECL detecting reagents using anti-30-

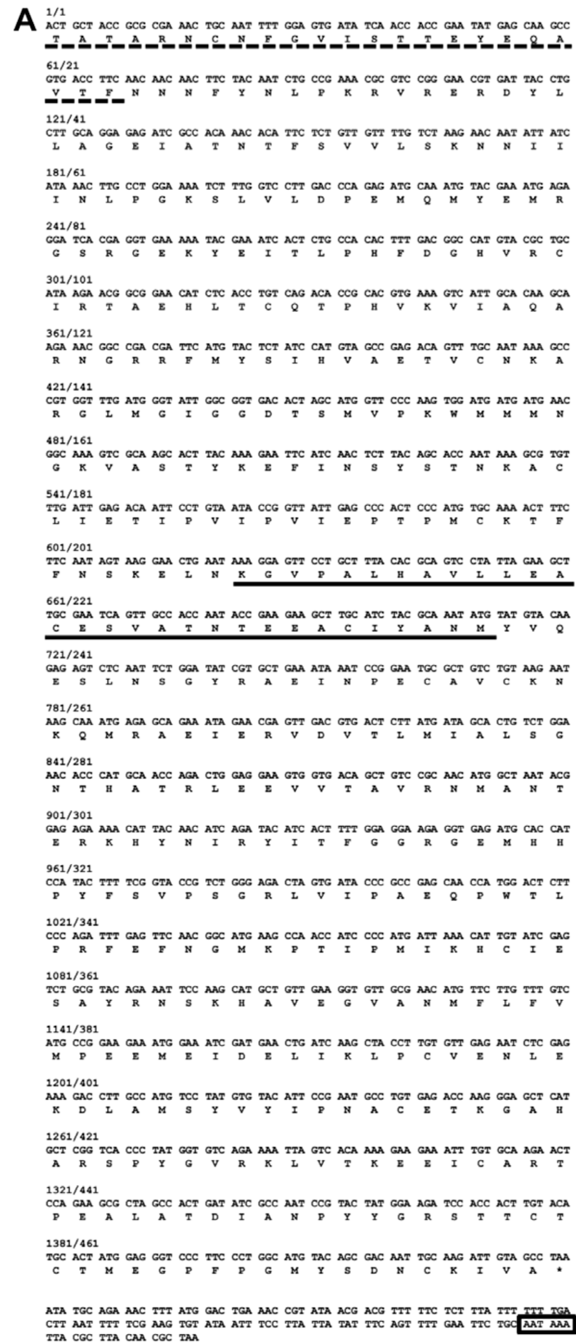

Fig. 1. (A) Nucleotide and deduced amino acid sequences of the 30-kDa VC protein. The underline indicates the N-terminal sequence, which is identical to the sequence of the VC protein isolated as a protein binding to CUB1 peptide of HrProacrosin. Poly(A) addition signal is enclosed by an open square. The upstream region of the 30-kDa VC protein is considered to be a vWF-D domain and corresponds to the 25-kDa VC protein, which is capable of binding to CUB1 peptide and Pro-rich region of HrSpermosin. The N-terminal sequence of the vWF-D domain fragment is indicated by a dotted line. A homology search revealed that the 30-kDa VC protein is a CT of vitellogenin. (B) Pulldown assay of CUB domain-1 with the 30-kDa VC protein. The [^{35}S]-labeled 30-kDa VC protein was pulled down with a recombinant CUB domain-1 fusion protein.

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