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Authors: Matsumoto, Midori, Hirata, Junko, Hirohashi, Noritaka, and Hoshi, Motonori

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Sperm-Egg Binding Mediated by Sperm α-L-Fucosidase in the Ascidian, *Halocynthia roretzi*

Midori Matsumoto*, Junko Hirata, Noritaka Hirohashi† and Motonori Hoshi

Center for Life Science and Technology, Graduate School of Science and Technology, Keio University, 3-14-1, Hiyoshi Kouhoku Yokohama, 223-8522 Japan

ABSTRACT—Spermatozoa bind to the vitelline coat in the ascidians and many other animals. The binding of sperm in *Halocynthia roretzi* is mediated by a sperm α-L-fucosidase and complementary-L-fucosyl residues of glycoproteins in the vitelline coat. cDNA clones for α-L-fucosidase were isolated from growing testis mRNA. It contained a 1398 bp full-length cDNA insert (HrFuc'ase) that encoded the 466 amino acid residues of *H. roretzi* sperm α-L-fucosidase. A putative signal peptide of 21 amino acid residues proceeded the sequence for the mature protein (M.W. 52.4 kDa). The coding sequence for HrFuc'ase showed 47.7% sequence identity to the human liver fucosidase sequence. The polyclonal antibody was prepared against a lacZ-HrFuc'ase fusion protein expressed in *E. coli*. The antibody crossed to a 54 kDa protein in sperm on western blotting and inhibited fertilization in a dose dependent manner. These data suggest that sperm-egg binding is mediated by the sperm α-L-fucosidase, HrFuc'ase in the ascidian, *H. roretzi*.

Key words: ascidian, fertilization, sperm-egg binding, sugar chain, fucosidase

INTRODUCTION

Specific binding of spermatozoa to the egg coat is a necessary step for fertilization in various animals. Gamete recognition and adhesion are mediated by sperm surface carbohydrate-binding proteins such as lectins, glycosyltransferases and glycosidases, that have a high affinity and specificity for complex glycoconjugates in the extracellular coat of eggs (Hoshi *et al*., 1985; Macek and Shur, 1988; Macek, *et al*., 1991). The ligands for these receptors are specific oligosaccharide chains bound to large glycoconjugates, as in the vitelline envelop of sea urchin eggs (Ruiz-Brovo and Lennarz, 1986; Hirohashi and Lennarz, 1998), or on more conventional complex-type glycoproteins, as in the murine (Bleil and Wasserman, 1980) and porcine (Sacco *et al*., 1989) zona pellucidae. Such specificity could result from entirely distinct classes of species-specifi gamete receptors, or rather from subtle sperm surface receptors (Shur, 1989). In the ascidian, *Ciona intestinalis,* glycoproteins of the vitelline coat have the capacity to accept sperm (DeSantis *et al*., 1983; DeSantis, R. and Pinto M. R., 1987). Although the structures responsible for these capacities are yet to be elucidated, α-L-fucosyl residues have a key role in sperm binding (Hoshi *et al*., 1985). Similarly, in *Halocynthia roretzi,* α-L-fucosyl residues of acidic saccharide chains in glycoproteins appear to be essential for sperm receptor activity of the vitelline coat. In *Phallucia mammillata* and several ascidian species, N-acetyl-glucosamine residues have a similar function (Honneger, 1982). In *H. roretzi,* sperm binding to the vitelline coat was blocked by synthetic fucosidase substrates (p-nitrophenyl or 4-methylumbelliferyl α and β fucosides) which presumably acted as competitive fucosidase inhibitors for physiological substrates and /or ligands. (Hirohashi and Hoshi, 1993). α-L-Fucosidase activity in *C. intestinalis* and *H. roretzi* sperm has an optimal pH at 4.0. The spermatozoa bound to the vitelline coat in normal sea water (pH 8.2) detached when they were in acidic seawater (pH 4.0). In addition, a fucosylamine affinity column adsorbed α-L-fucosidase in the sperm extract appreciably in normal sea water at pH 8.2, though than at pH 4.0. From these data, it was suggested that the enzyme was able to form a complex with substrates, like lectins, in normal sea water (Hoshi *et al*., 1985; Hirohashi and Hoshi, 1993).

The lysosamal α-L-fucosidase [EC.3.2.1.51] is responsible for hydrolysing the α-1, 6-linked fucose joined to the reducing-end N-acetyl glucosamine of the carbohydrate moieties. The mature enzyme from rat liver has a subunit of approximately 55 kDa and exists as tetramer in the native state (Opheim D.J. and Touster O., 1977). cDNA sequence information for the enzyme is available in human, rat and *Dictiosterium* (Ficher and Aronson, 1989; White *et al*., 1985,

* Corresponding author: Tel. +81-45-566-1774;
FAX. +81-45-566-1448.
E-mail: mmatsumo@chem.keio.ac.jp

† Present address: SCRIPPS Institution of Oceanography, 9500 Gilman Dr. La Jolla, CA, 92093-0202

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Muller-Taubenbergrt *et al*., 1989) and it is known that the gene contains one α-L-fucosidase motif composed of 12 amino acids.

Here, we report the sequence for *H. roretzi* sperm α-L-fucosidase gene (HrFuc'ase) cloned by using sequence information obtained for the three α-L-fucosidases (Ficher and Aronson, 1989; White *et al.,* 1985; Muller-Taubenbergrt *et al*., 1989).

MATERIALS AND METHODS

Materials

Ascidians, *Halocynthia roretzi,* were collected from the Gulf of Onagawa, Japan.

Restriction enzymes and modified enzymes were obtained from Takara and Toyobo.

Isolation of HrFuc'ase cDNA

Total RNA was prepared from growing testes by the AGPC (acid guanidine phenol chloroform) method (Sambrook *et al*., 1989) and poly(A)-rich RNA was purified with oligoTex beads (Roche Japan). First-strand cDNA synthesis was performed at 37°C for 1 h using Superscript II (GIBCO-BRL) with a random hexamer (Takara). The degenerate primers deduced from conserved region in α-L-fucosidase of *Dictiosterium*, rat and human were sense primer F1 (5'-GGNGCNAARTAYGTNGTNYTNAC-3') and antisense primer F2 (5'-CCCCANCKRTCATTNACNAYNAC-3') (Ficher and Aronson, 1989, White *et al.,* 1985, Muller-Taubenbergrt *et al*., 1989). The PCR conditions were 30 cycles of 94°C for 30 sec., 48°C for 30 sec., 74°C for 30 sec.

The full-length of HrFuc'ase was amplified with the design of 5' RACE (Rapid amplification of 5'-cDNA ends) and 3'RACE (Rapid Amplification of 3'-cDNA Ends) primers, as well as appropriate nested primers. The primers were: 5'RACE primer; 5'-TGTCGACT-GCGTTCCAGTTC-3', 5' nested primer, 5'-CAATTAGTAAATCCT-TCGTGA-3'. On 3'RACE, poly(A)$^+$ RNA was made from growing testes and used to synthesize single strand cDNA with the dT_{17}-adaptor primer, 5'GACTCGAGTCGACATCGAT_{17}3'. The synthesis of the second strand and the further PCR reactions were carried out with the adapter primer; 5'GACTCGAGTCGACATCGA3' and 3'RACE primer F3; 5'GATTACTTCAGTTCTCGTGC-3', 3'RACE nested primer F4; 5'GCTTGGTTATACAATGATAGC-3'. On 5'RACE, poly (A)$^+$ RNA was made from growing testes and used to synthesize single strand cDNA with the 5'RACE primer; F5 (5'CAATTAGTAAATCCTTCGTGA-3'). This cDNA was then used as a template in a PCR reaction using the 5'primer homopolymeric dA tails added to the 3'end of the ss-cDNA using terminal deoxyribonucleotide transferase at 37°C, for 30 min. The synthesis of the second strand and additional PCR experiments were carried out with the dT_{17}-adapter primer, the adapter primer and F5.

DNA sequencing

The PCR products were cloned into pGEM-T vector (Promega) and sequenced with M13 forward and reverse primers using SequiTherm DNA Polymerase (Epicentre Technologies) and the automated DNA sequencer, LICOR model 4000L (ALOKA). The internal sequence was obtained by nested deletion using a Takara deletion Kit.

Southern Hybridization

Genomic DNA prepared from *H. roretzi* muscle using standard procedures was digested for 6 hr with restriction enzymes (*Eco*RI, *Bam*HI and *Sal*I) and then loaded into 0.6% agarose gel and electrophoresed. Southern hybridization was carried out using a 450 bp PCR fragment with F1 and F2.

Preparation of fusion protein of HrFuc'ase and Antibody preparation

The 330 bp fragment consisting of 152 to 262 amino acid of HrFuc'ase was subcloned in frame into pUR292 expression vector (Rüter and Muller-Hill, 1983) with the correct orientation. *E. coli* F"11recA expressing β-galactosidase (β-gal) fusion protein after IPTG induction was solubilized in SDS-PAGE lysis buffer and extracts were electrophoresed on 5% SDS-PAGE (Laemmli, 1970). After staining with Coomasie Brilliant Blue (CBB; Sigma, St. Louis, MO), a high molecular weight fusion protein was cut from the gels and eluted electrophoretically. Eluted samples were dialyzed against distilled water and freeze dried.

Purified protein was emulsified in Freund's complete adjuvant (Difco, Detroit, MI), and injected subcutaneously into rabbits following a four-fold booster injection of the purified protein in Freund's incomplete adjuvant. The antiserum was characterized by Western blots of the fusion protein.

Preparation of sperm protein

Dry sperm was diluted with sonication buffer containing 10 mM citrate buffer (pH 4.5) and 10 mM NaCl. The sperm suspension was subjected to sonication for 2 min. The resultant suspension was centrifuged at 15,000 g for 20 min and then 100,000 g for 60 min, and the supernatant was collected.

Western blotting

After SDS-PAGE, the gels were stained with CBB to visualize separated proteins. For immunochemical detection of antigens, proteins were electrophoretically transferred using a semi-dry blotting system to Immobilon-P (Millipore Corp. Bedford, MA) PVDF membrane (Towbin *et al*., 1979). Blotted membranes were blocked for 2 hr in Tris-buffered saline (TBS, pH 7.2) containting 5% nonfat dry milk (Difco, Detroit, MI), and incubated overnight with the antiserum at 1/1,000 dilution. After rinsing with TBS containing 0.5% Tween 20, the blots were incubated for 1 hr with an Avidin-Biotin-Complex (ABC) conjugated goat anti-rabbit antibody (Vector Laboratories) at 1/2,000 dilution and positive reactions were detected with Konica Immunostaining HRP-1000 (Konica, Tokyo).

Fertilization assay

Aliquots of sperm suspension (10^6 cells/100 μl) were incubated with antiserum against HrFuc'ase or preimmune serum for 15 min at room temperature. Various dilutions of antiserum and sperm were examined. Then, approximately 100 unfertilized eggs were added and incubated for another 10 min. For inactivation of the sperm, a final concentration of 0.04% SDS was added, incubated for 10 min and washed with sea water. After 1 hr incubation, fertilized eggs were scored.

RESULTS

Identification and sequence of the ascidian α-L-fucosidase cDNA clone

Using one pair of degenerate primers encoding the conserved region of the fucosidase domain [GAKYVVLT and VVVNDRW] (Ficher and Aronson, 1989; White *et al.* 1985; Muller-Taubenbergrt *et al.* 1989), a 450 bp fragment of the ascidian fucosidase homologue, named HrFuc'ase, was isolated with RT-PCR. 5'RACE was used to obtain the 5' portion of the encoding region, and 3'RACE was used to obtain the 3' portion of the encoding. The entire coding sequence based on the 5' fragments obtained with RT-PCR with the

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Fig. 1 Alignment of amino acid sequences of fucosidase homologue of human, rat and *Dictiosterium*. Amino acid identities for representative genes are compared with HrFuc'ase. The signal sequences are indicated with italics and the fucosidase motif is shown boxed.

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conserved fucosidase domain of 150 amino acids, 5'RACE and 3'RACE suggested a protein of 466 amino acids. This open reading frame has three putative initiation codons, but we have assigned the initiation codon to the ATG at nucleotide position 261, because the 21-amino acid sequence following this ATG possessed the features characteristic of signal sequences and this ATG was flanked by sequences that fit Kozak's criteria for translation initiation codon (Kozak, 1981). The initiation was followed by an open reading frame of 1398 bp. An inframe stop codon occurred at nucleotide position 1659 and the 3'-untranslated region composed of 377 bp included polyadenylation sites (AATAAA). The deduced amino acid sequences suggested that cleavage of the signal peptide would yield a protein of 445 amino acid with a calculated molecular weight of 52.4 kDa. This signal peptide-like sequence was a typical of the membrane-spanning domain of many membrane proteins. The protein contained one α-L-fucosidase motif composed of 12 amino acids at residues 283-294. The α-L-fucosidase clone was named as HrFuc'ase.

To confirm that the cloned HrFuc'ase can bind to the fucose residue, the molecular modeling was performed using with Quanta/Charmm force files and the Protein Data Base (data not shown). The molecular model shows that it has a putative space for interaction of fucose residue thus HrFuc'ase may bind a fucose residue.

Sequence analysis of H. roretzi α-L-fucosidase

Fig. 1 shows a comparison of the amino acid sequence of HrFuc'ase with other α-L-fucosidases. HrFuc'ase shows amino acid identities related to the human (47.7%), rat (47.4%) and *Dictiosterium* (37.6%) with fucosidase of motifs (Ficher and Aronson, 1989; White *et al.* 1985; Muller-Taubenbergrt *et al.* 1989). The sequence PSKLQTHK-WENC at residues 283–294, corresponded to the consensus sequence of the α-L-fucosidase, PXXLXXXKWEXC.

To confirm the existence of the HrFuc'ase in the ascidian genome, southern blot analysis of the ascidian genomic DNA was performed. Hybridization with HrFuc'ase cDNA gave single bands for DNA digested with *Eco*RI, *Bam*HI and *Sal*I (data not shown), suggesting that HrFuc'ase is encoded by a single gene in the ascidian genome.

Identification of HrFuc'ase on western-blots with polyclonal antibody

To identify the gene product encoded by the HrFuc'ase cDNA, the antiserum was directed against a fusion protein. The resulting antiserum was used for the Western blot analyses of proteins extracted from mature sperm (Fig. 2). The antibody crossed with the 54 kDa band strongly and the 37 kDa band weakly. The molecular size of the band which crossed strongly with the antiserum was most similar to the molecular weight of 52.4 kDa calculated from this cDNA clone. The HrFuc'ase protein was contained more in the preparation of 100,000 × g supernatant than in 15,000 × g supernatant (Fig. 2, lane 1 and lane 2).

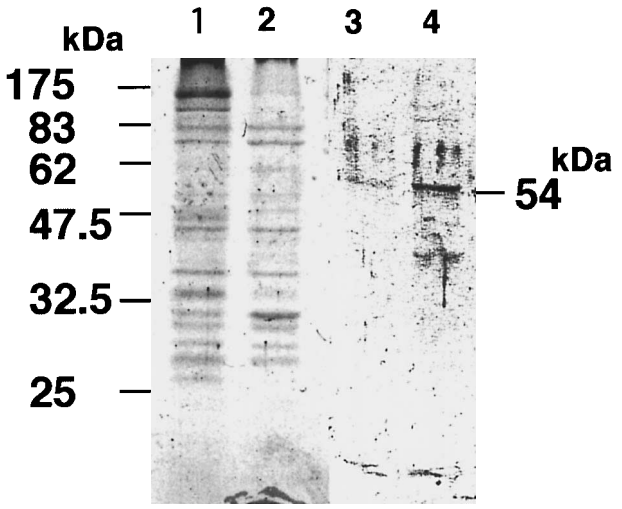


Fig. 2 Detection of HrFuc'ase protein in sperm using HrFuc'ase polyclonal antibody. The CBB staining (lanes 1 and 2) and the Western blot with HrFuc'ase polyclonal antibody (lanes 3 and 4) of SDS-PAGE of sperm membrane are shown. Loaded samples: 15,000 g sup (lanes 1 and 3), 100,000 g sup (lanes 2 and 4).

Inhibition of fertilization with HrFuc'ase antiserum

To elucidate the function of HrFuc'ase further, the effect of HrFuc'ase antserum upon fertilization was examined (Fig. 3). Fertilization was inhibited by HrFuc'ase antibody in a dose-dependent manner.

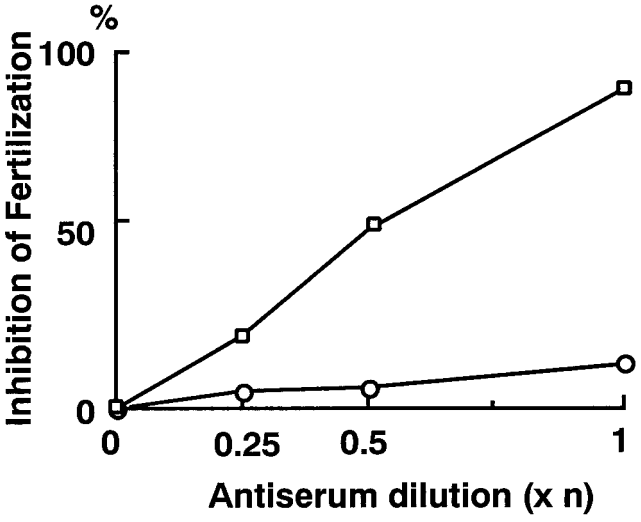


Fig. 3 Inhibition of HrFuc'ase polyclonal antibody on fertilization. Panel shows the effect of the antibody concentration. Control experiments are shown with ○ and the experiments with HrFuc'ase polyclonal antibody are shown with □.

DISCUSSION

Previous studies on egg-sperm binding in the ascidians, *Ciona* and *Phallusida*, employed synthetic substrates for glycosidases (Hoshi *et al.* 1985). It was shown that sperm binding was blocked by the substrate for enzyme p-nitrophe-

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nyl or 4-methylumbelliferyl α-L-fucoside and their β-anomers that act as competitive inhibitors. The spermatozoa bound to the vitelline coat in normal sea water (pH 8.2) detached after incubation at pH 4.0. Because the fucosylamine affinity column adsorbed the activity of α-L-fucoside in the sperm extract appreciably in normal sea water at pH 8.2, though to a lesser extent than pH 4.0, it was suggested that the enzyme was able to form a complex with substrates, like a lectin, in normal seawater.

Here, we have isolated one fucosidase cDNA, HrFuc'ase from *H. roretzi* testes as a candidate for egg-binding protein. If fucosidase is an egg binding protein, it should exist on the sperm plasma membrane and must have a signal sequence to the relrease to the external lamela of the plasma membrane. The cloned HrFuc'ase fulfils these requirements. The sequence PSKLQTHKWENC at residues 283–294, corresponds to the consensus sequence PXX-LXXXKWEXC, known as the α-L-fucosidase motif. Molecular modeling of HrFuc'ase with Quanta/Charmm force files and the Protein Data Base, suggest a space for interaction with fucose residue in the enzyme. The 3D structure model of HrFuc'ase showed that it could bind to the fucose residue and it could act as egg binding protein in normal seawater.

The dose-dependent inhibition of fertilization by antiserum prepared against β-galactosidase-HrFuc'ase fusion protein suggests the presence of the enzyme in the outer surface of sperm and its crucial role for fertilization, presumably for specific-binding to the vitelline envelop. And we showed also that antiserum against HrFuc'ase fusion protein, reacted with the 54 kDa protein of *H. roretzi* sperm by Western blot analysis. The molecular size is very close to the estimated MW of HrFuc'ase polypeptide (52.4 kDa). HrFuc'ase protein was contained in the preparation of 100,000 g supernatant and more than 15,000 g supernatant. This showed that HrFuc'ase protein was not stored in lysosomes or any organelles. Threfore, HrFuc'ase might attach to the sperm membrane weakly.

It is reported that, besides α-L-fucosidase, trypsin-like protease, acrosin and spermosin, and 20S proteasome plays an important role in the sperm binding to the vitelline envelop in *H. roretzi* (Hoshi *et al*., 1981; Sawada *et al.,* 1983; Takizawa *et al.,* 1993; Sawada *et al.,* 1993). Thus, sperm binding to the vitelline coat in *H. roretzi* seems to be mediated by two systems, though nothing known about the relation of the two systems.

Taking the data presented in this paper and our previous findings into account, it is concluded that the cloned HeFuc'ase is the gene for α-L-fucosidase that mediates sperm-binding to the vitelline coat in *H. roretzi*.

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