Although ascidians are hermaphroditic, many species including *Halocynthia roretzi* are self-sterile. We previously reported that a vitelline coat polymorphic protein HrVC70, consisting of 12 EGF (epidermal growth factor)-like repeats, is a candidate allorecognition protein in *H. roretzi*, because the isolated HrVC70 shows higher affinity to nonself-sperm than to self-sperm. Here, we show that a sperm 35-kDa glycosylphosphatidylinositol-anchored CRISP (cysteine-rich secretory protein)-like protein HrUrabin in a low density detergent-insoluble membrane fraction is a physiological binding partner for HrVC70. We found that HrVC70 specifically interacts with HrUrabin, which had been separated by SDS-PAGE and transferred onto a nitrocellulose membrane. HrUrabin has an N-linked sugar chain, essential for binding to HrVC70. HrUrabin mRNA is expressed in the testis but not in the ovary, and the protein appears to be localized on the surface of sperm head and tail. Anti-HrUrabin antibody, which neutralizes the interaction between HrUrabin and HrVC70, potently inhibited fertilization and allorecognizable sperm-binding to HrVC70 agarose. However, no significant difference in the binding ability of HrUrabin to HrVC70 was observed in autologous and allogeneic combinations by Far Western analyses. These results indicate that sperm-egg binding in *H. roretzi* is mediated by the molecular interaction between HrUrabin on the sperm surface and HrVC70 on the vitelline coat, but that HrUrabin *per se* is unlikely to be a direct allorecognition protein.

Ascidians, the invertebrate chordates, are hermaphroditic animals releasing gametes nearly simultaneously during the spawning season. Most ascidians show self-sterility or preference for “non-autologous” (allogeneic) fertilization (1, 2), which appears to be beneficial for the achievement of genetic diversity in the next generation. However, the mechanism of self-sterility during ascidian fertilization is a long-standing enigma. In the early part of the 20th century, Morgan (3–6) studied this problem using the solitary ascidian *Ciona intestinalis*, and found that a barrier against self-fertilization is abolished by the treatment of eggs with weak acid (citrus juice) or protease (pancreatic extract) (3). Because the vitelline coat (VC) (3)-free naked eggs are self-fertile, he proposed that a self/nonself-recognition molecule must reside on the VC or test cells. Later, it was found that only nonself-sperm tightly and specifically bound to the VC of glycerinated eggs in *C. intestinalis* (7). From these results, it is currently believed that a certain allorecognition molecule responsible for self-sterility must reside on the VC.

We have been studying the mechanism of self-sterility using another solitary ascidian, *Halocynthia roretzi*, because a large quantity of readily fertilizable gametes can be easily obtained from this species, which is aqua-cultured in Japan for human consumption (8). Although *H. roretzi* exhibits much more strict self-sterility than *C. intestinalis*, the mode of self-sterility in both species seems to be similar. In *H. roretzi*, the occurrence of a self-fertilization barrier on the VC, but not on the follicle cells, was clearly demonstrated by experiments using “mosaic eggs,” whose follicle cells were replaced by those of different individuals (9). As in *C. intestinalis*, mature oocytes but not immature oocytes are self-sterile in *H. roretzi*, and the self-sterility is lost by treatment with acidic seawater (9). Furthermore, the acquisition of self-sterility during oocyte maturation was blocked by trypsin inhibitors, whereas exogenously added trypsin stimulates the acquisition of self-sterility (10, 11). These results led us
to propose that a putative allorcognition protein might be expressed in immature oocytes as a precursor and that the active form might be generated by a trypsin-like protease, resulting in its attachment to the VC during oocyte maturation. It is also inferred that this putative molecule might be easily extracted from the VC by mildly acidic conditions. 

To test this possibility, we compared the components of the VCs from immature oocytes with those from mature oocytes of *H. roretzi* by SDS-PAGE. We found that a 70-kDa VC protein HrVC70, which had been identified as a sperm receptor consisting of 12 epidermal growth factor (EGF)-like repeats (12), appears to be attached to the VC during oocyte maturation and that this protein is easily and almost specifically extracted from the insoluble VC by 1–10 mM HCl (pH 2–3) (13). HrVC70 is converted by a trypsin-like protease from its precursor HrVC120, which contains 13 EGF repeats, a zona pellucida domain, and a transmembrane helix (13). Difference in the binding ability of sperm to the VC between autologous and allogeneic gamete combinations is not obvious in *H. roretzi* (10). However, we found that nonself-sperm shows a significantly higher affinity to HrVC70-immobilized agarose beads than self-sperm (13). In addition, fertilization was more strongly inhibited by the pretreatment of sperm with nonself-HrVC70 than self-HrVC70 (13). Finally, HrVC70 appeared to be a highly polymorphic protein, showing no identical sequence among 10 individuals tested (13). From these results, together with the fact that even a single amino acid substitution is sufficient to affect the molecular recognition between EGF-like repeat-containing molecules such as Notch, Delta, and Serrate (14, 15), we proposed that HrVC70 is a promising candidate for the allorcognition protein, which is responsible for self-sterility.

To gain insights on the molecular mechanism of allorcognition, we have explored a sperm-borne binding partner for HrVC70 by several approaches. We previously reported that a type II transmembrane protease, HrTTSP-1, is a candidate sperm-derived HrVC70-interacting protein as revealed by yeast two-hybrid screening baited with HrVC70 (16). In the present report, we carried out Far Western blot analysis using HrVC70 as a probe, and identified a 35-kDa sperm membrane-associate glycosylphosphatidylinositol (GPI)-anchored protein as a physiological binding partner for HrVC70.

**EXPERIMENTAL PROCEDURES**

**Biological Materials and Preparations of the VC and Low-density Detergent-insoluble Membrane (LD-DIM) Fraction—**

Spawning of *H. roretzi*, collected near Mutsu Bay in northern Japan, was induced by controlling the seawater temperature and light conditions as described previously (17). Sperm and eggs were collected individually and used for fertilization experiments or stored at −20 °C until use. The VC of *H. roretzi* eggs was prepared as described previously (12). Briefly, frozen-thawed eggs were mixed with an equal volume of homogenizing buffer (20 mM EPPS (pH 8.0), 460 mM NaCl, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin), and homogenized with a Teflon homogenizer at 2,000 rpm, 10 times. The homogenate was filtered through a nylon mesh (50 µm), and the VC on the mesh was washed with 0.2× artificial seawater 5–10 times, followed by centrifugation at 7,000 × g for 5 min. The VC (precipitate) was further washed twice with MilliQ water.

HrVC70 was isolated from the VC as follows (12). The VC was mixed with 2–3 volumes of 10 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin. After standing for 20 min on ice, the sperm suspension was disrupted with a Teflon homogenizer at 2,000 rpm, 10 times, and subjected to low-speed centrifugation (7,000 × g for 5 min). One ml of the resulting supernatant was mixed with an equal volume of 85% (w/v) sucrose and transferred into an ultracentrifuge tube, which was gently overlaid by 2 ml of 30% sucrose, and 1 ml of 5% sucrose in this order. After centrifugation (200,000 × g, 18 h, 4 °C), the LD-DIM fraction was obtained in a boundary between the 5 and 30% sucrose layers. Protein concentration in each fraction separated by ultracentrifugation was determined using the ABC kit (Bio-Rad).

**Molecular Biological Techniques—**

Degenerate primers were designed based on the determined N-terminal 30 amino acid residues (forward-1 primer, 5′-GA(A/G)GTN(A/C)GIAT(A/C/T)(C/T)TIACIAC-3′; forward-nested primer, 5′-GA(A/G)GTN-(A/C)GIAT(A/C/T)(C/T)TIACIACIGA(A/G)GA(A/G)AA(A/G)CA-3′; I, inosine), and 3′-RACE PCR was performed on gonad cDNA from a reproductively mature animal prepared with the SMART RACE cDNA amplification kit (Clontech). Nearly entire cDNA fragments were obtained by screening a *H. roretzi* gonad λ-ZAP cDNA library (Stratagene) probed with the 3′-RACE product. The 5′-terminus of the mRNA was determined by 5′-RACE.

Northern blotting and genomic Southern blotting of Hr-Urabin were carried out as described previously (14), using a digoxigenin-labeled cDNA probe corresponding to Pro8257 in *HrUrabin-L*. Whole mount *in situ* hybridization was performed as described previously (16), using a digoxigenin-labeled RNA probe including the entire cDNA sequence of HrUrabin-L.

**Immunological Procedures and Far Western Analysis—**

To develop an anti-HrVC70 polyclonal antibody, the HrVC70 band purified from the VC by SDS-PAGE, was used as an antigen. As to HrUrabin, thioredoxin-tagged fusion protein, including the entire sequence of the processed form of HrUrabin-L, was bacterially expressed using the PET Trx Fusion System 32 (Novagen). The rabbit antisera raised against HrVC70 and HrUrabin were prepared by Hokudo Ltd. Anti-HrVC70 antibody specifically reacted to HrVC70 among the VC components, and showed no cross-reactivity to any sperm membrane proteins on the basis of Western blotting under the conditions tested.
Far Western analysis was carried out as described below. Each fraction separated by discontinuous sucrose density gradient centrifugation was subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, which was incubated for 2 h at room temperature in 3× PBS containing 1% blocking reagent (Roche Applied Science). The membrane was further incubated overnight at room temperature with 0.02% (v/v) of HrVC70 in 3× PBS containing 0.1% SDS and 3% blocking reagent. HrVC70-interacting protein band(s) were detected by anti-HrVC70 rabbit antibody using the ECL system (Amersham Biosciences).

Inhibitory ability of anti-HrUrabin antibody on this interaction was examined by adding anti-HrUrabin antibody during incubation with HrVC70. To distinguish two rabbit antibodies (anti-HrVC70 and anti-HrUrabin), biotin-labeled anti-HrVC70 antibody and horseradish peroxidase-conjugated streptavidin (Calbiochem) were used to detect the HrVC70-interacting protein(s) on a membrane.

For immunocytochemistry, spermatozoa supported on the aminopropyltriethoxysilane-coated slide glass (Matsunami Glass) were fixed with 4% paraformaldehyde in 1× PBS for 1 h at room temperature. After several washes with 1× PBS, the specimen was reacted with 1/1000 diluted anti-HrUrabin antiserum in 1× PBS containing 1% bovine serum albumin for 1 h at room temperature, then washed several times, followed by incubation with 2 μg/ml of Alexa Fluor 568 goat anti-rabbit IgG antibody (Invitrogen) and 5 μg/ml of 4,6-diamino-2-phenylindole (Sigma) in 1× PBS for 1 h at room temperature. After washing with 1× PBS, the specimen was observed under an inverted fluorescent microscope (Nikon).

**Enzymatic Elimination Procedures of GPI Anchor and Sugar Chains**—To examine whether HrUrabin s are sperm-surface GPI-anchored proteins, intact sperm was incubated with 1 unit of phosphatidylinositol-specific phospholipase C (PI-PLC, Sigma) for 1 h at 37 °C, followed by centrifugation at 15,000 × g for 10 min. The obtained supernatant was subjected to Western analysis using the anti-HrUrabin antibody.

An N-linked glycosylation site on each HrUrabin species was predicted from their amino acid sequences. To examine whether a carbohydrate chain is linked to the consensus Asn residue of HrUrabin, the LD-DIM fraction was incubated with 5 units of PNGase F (Sigma) in 50 mM phosphate buffer (pH 8.0) for 2 h at 37 °C. The reacted sample was subjected to SDS-PAGE, followed by Western and Far Western analyses.

**Amino Acid Sequences and Compositions, and LC/MS/MS Analyses**—For determination of amino acid sequences of HrUrabin s, the LD-DIM fraction was subjected to SDS-PAGE in the presence of thioglycolate, followed by electrophoretic transfer onto a polyvinylidene difluoride membrane. The membrane was cut off and subjected to protein sequence analysis.

For analyses of amino acid compositions, the 35- and 50-kDa HrUrabin bands in LD-DIM fraction, which were separated by SDS-PAGE, were excised, extracted, and subjected to acid hydrolysis (6 N HCl in in vacuo, 24 h). The hydrolysate, thus prepared, was subjected to amino acid analysis using a JEOL JLC/500V amino acid analyzer.

The 35- and 50-kDa protein bands were subjected to reduced carboxymethylation with monoiodoacetic acid, and then digested with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) according to a procedure described previously (20). Trypsin-digested fragments were subjected to high performance liquid chromatography with a C18 reverse phase column at a constant flow rate of 200 nl/min. After applying the sample to the column (0.05 × 150 mm), which had been equilibrated with 0.1% formic acid containing 2% acetonitrile, the column was washed with equilibration buffer and eluted by a linear gradient of acetonitrile from 2 to 50% in 60 min. The eluate was automatically subjected to an ESI-MS/MS with an LTQ (Thermo, Fisher Scientific).

**Fertilization and Beads Binding Experiments**—To avoid any nonspecific inhibitory effect on fertilization caused by serum ingredients, purified anti-HrUrabin polyclonal antibody was used for the following experiments. Antibody purification was carried out using Protein A-Sepharose beads (Sigma).

For the fertilization experiments, sperm were preincubated with anti-HrUrabin antibody or non-immune control antibody for 30 min at 13 °C. Into the sperm suspension (100 μl), a small volume of egg suspension was added and incubated at 13 °C. Fertilization ratio was determined by examining the expansion of perivitelline space, 30–60 min after insemination.

For bead-binding experiments, isolated HrVC70 from the VC was conjugated to the Protein A-Sepharose beads via the anti-HrVC70 antibody as described previously (13). Self- or nonself-sperm suspensions in artificial seawater, which had been incubated for 30 min at 13 °C with control or anti-HrUrabin antibodies at a concentration of 2 mg/ml, was added into a small volume of HrVC70 beads. After the incubation and gentle washes with seawater, sperm were cross-linked onto the beads with 2% paraformaldehyde, and stained with 4,6-diamino-2-phenylindole. The number of sperm bound to a single agarose bead (50–100 beads were examined for each batch) was counted under a light or fluorescent microscope.

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**FIGURE 1. Detection of a sperm membrane-associated binding partner for HrVC70.** A, preparation of sperm LD-DIM fraction. The LD-DIM fraction was obtained in a boundary between the 5 and 30% sucrose layers. B, each fraction was subjected to SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. C, fractions 2 (LD-DIM fraction) and 7 were blotted onto a nitrocellulose membrane and subjected to the above Far Western analysis in the presence (left blot) or absence (right blot) of incubation with HrVC70.
RESULTS

Identification of a Binding Partner (HrUrabin) for HrVC70

In sea urchin sperm, most surface proteins that are involved in gamete interaction are known to reside on a LD-DIM fraction or lipid raft fraction (18, 19). According to the protocol for sea urchin sperm (18, 19), we prepared the LD-DIM fraction from H. roretzi sperm (Fig. 1A). These fractions were subjected to SDS-PAGE and transferred to a nitrocellulose membrane, followed by incubation with HrVC70. HrVC70-interacting proteins on the membrane were detected by an anti-HrVC70 antibody. This Far Western analysis revealed that HrVC70 specifically recognizes a 35-kDa protein from the LD-DIM fraction (Fig. 1, B and C, fraction 2) but not other membrane proteins mainly fractionated in fraction 7. The interaction between these proteins appeared to be enhanced in artificial seawater or high-ionic strength buffer (3× PBS) rather than in 1× PBS (data not shown), suggesting that the interaction is achieved under physiological seawater conditions.

To identify this protein, the 35-kDa protein on a polyvinylidene difluoride membrane was cut out and subjected to protein sequence analysis. On the basis of the determined N-terminal 30 residues (EVRILTTEEKQLLLDEHNKARS-EVVPKAXN), two degenerate primers were synthesized, and 3′-RACE and 5′-RACE were carried out. A cDNA library was screened with the obtained RACE clone, yielding two similar but not identical cDNA clones, which encoded the same sequence to the N-terminal sequence of the 35-kDa protein. We designated these genes Hr-Urabin (Halocynthia roretzi unique raft-derived binding protein for HrVC70) and HrUrabin-L (a long isoform). Schematic representations (A) and deduced amino acid sequences (B) of HrUrabin and HrUrabin-L are depicted in Fig. 2. These HrUrabin and HrUrabin-L are novel genes, showing a similarity to HrTT-1 (21), which was characterized as a gene exclusively expressed in the tail tip of tailbud embryos of H. roretzi. Both HrUrabin and HrUrabin-L contain a signal peptide, a PR (plant pathogenesis-related-1) domain conserved in CRISPs (Cys-rich secretory protein) family with two sperm-coating glycoprotein (SCP) motifs, and a single N-glycosylation site, (and six acidic-rich repeats at the C-terminal side only in HrUrabin-L), which is followed by a GPI-anchor attachment site and a cleavable transmembrane domain (Fig. 2). Unlike HrVC70, both HrUrabin and HrUrabin-L were not so polymorphic among different individuals (data not shown). The molecular masses of unmodified HrUrabin and HrUrabin-L were esti-
mated to be 27 and 30 kDa, respectively. isoforms, using the genomic DNAs digested with several single or very low. To determine whether these genes are inde-
suggesting that the copy number of these genes seems to be
results of whole mount
hybridization also revealed that
HrUrabin failed to bind to HrVC70 in Far Western analysis (com-
pare left and right panels in Fig. 3D). Neither intact nor degly-
cosylated 50-kDa HrUrabin-L was capable of binding to HrVC70 (Fig. 3D).

FIGURE 3. Genomic Southern, Northern, Western, and Far Western blott-
ings of HrUrabin and HrUrabin-L, both of which are GPI-anchored pro-
teins having an N-linked sugar. A, Southern blotting of HrUrabin on
 genomic DNAs digested with BamHI (B), EcoRI (E), HindIII (H), and PstI (P)
 restriction enzymes. B, Northern blotting of HrUrabin in H. roretzi gonad. The
 two bands of HrUrabin and HrUrabin-L were not differentiated under these
 experimental conditions. C, Western blotting (WB) and genomic PCR analysis
 of HrUrabin on two individuals. The B individual showed neither a 50-kDa
 band in the Western blot nor an HrUrabin-L band in the genomic PCR, sug-
gest that HrUrabin-L encodes the 50-kDa protein and that it does not play
 an essential role in fertilization. D, Western (left) and Far Western (right) blots
 of the sperm LD-DIM fraction treated with PNGase-F. The PNGase-F treat-
ment decreases the molecular sizes of both HrUrabin (left), but only intact Hr-
Urabin, retaining an N-linked carbohydrate, shows a binding ability to
HrVC70. E, supernatant of the PI-PLC-treated sperm was subjected to Western
 blotting using anti-HrUrabin antibody. Note that HrUrabin and HrUrabin-L
(data not shown) are GPI-anchored proteins, which are exposed, at least in
part, on the sperm surface.

Northern blotting showed that about 0.9-kb HrUrabin mRNAs are ex pressed in gonad, although two mRNA species were scarcely separated under these conditions (Fig. 3B). The results of whole mount in situ hybridization also revealed that HrUrabin mRNAs are specifically expressed in the testis but not in the ovary (compare Fig. 4, A and B). Immunocytochemistry using a specific antibody, raised in a rabbit against gluta-

Among the 10 protein fragments tested, the 50-kDa protein did not coincide with the number of amino acids deduced from a cDNA of HrUrabin-L or HrUrabin, although the amino acid composition of the 35-kDa protein coincided well with the number of amino acids deduced from a cDNA of HrUrabin sequence (data not shown).

To examine whether or not the 50-kDa protein is encoded by HrUrabin-L, trypsin-digested fragments of the reduced and carboxymethylated 50- and also 35-kDa proteins were separated by reversed phase high performance liquid chromatography, followed by electrospray ionization mass spectrometry (ESI-MS). Each peptide was identified by subsequent tandem mass spectrometry (MS/MS). The results clearly showed that the 50-kDa protein is a gene product of HrUrabin-L (Fig. S1) and also confirmed that the 35-kDa protein is a gene product of HrUrabin. HrUrabin and HrUrabin-L showed amino acid substitutions at 6 positions (blue letters shown in Fig. 2B), among which only Val237 and Lys237 were found to be characteristic to HrUrabin and HrUrabin-L, respectively, because a Val237-containing peptide (number 24’) and a Lys237-containing peptide (numbers L23’) occur only in the 35- and 50-kDa proteins, respectively (see Fig. S1). Another 5 substitutions were polymorphisms commonly observed in HrUrabin and HrUrabin-L, which was revealed by LC/MS/MS analysis.

During the course of this study, we noticed that a few individuals (at a ratio of ~5–10%) expressed neither the 50-kDa protein detected by Western blotting nor the HrUrabin-L gene copy detected by genomic PCR analysis (see Fig. 3C, right lanes). These results agreed with our previous conclusion that the 50-kDa protein is encoded by HrUrabin-L. In addition, this also indicates that HrUrabin-L is not essential for fertilization in H. roretzi.

Posttranslational Modifications of HrUrabin and HrUrabin-L—Because the molecular sizes of HrUrabin and HrUrabin-L, which were estimated by SDS-PAGE, were higher than those deduced from their cDNA sequences, it is likely that these proteins are posttranslationally modified. This is partly due to the existence of an N-linked carbohydrate moiety occurring in both HrUrabin and HrUrabin-L (Fig. 2), because ~3–5-kDa lower molecular mass bands were observed in both proteins under SDS-PAGE after the treatment with PNGase-F (Fig. 3D, left). This N-linked sugar chain is essential for the binding of HrUrabin to HrVC70, because deglycosylated 35-kDa Hr-
Urabin failed to bind to HrVC70 in Far Western analysis (compare left and right panels in Fig. 3D). Neither intact nor degly-
cosylated 50-kDa HrUrabin-L was capable of binding to HrVC70 (Fig. 3D).
Ascidian Sperm Binding Partner for HrVC70

Because both isoforms of HrUrabin contained a GPI-anchor attachment site, we examined the susceptibility of these proteins on the sperm surface to PI-PLC. We found that HrUrabin and HrUrabin-L were partially released from the intact sperm surface by treatment with PI-PLC, which was detected by Western blotting (Fig. 3E for HrUrabin, data not shown for HrUrabin-L). We thus conclude that HrUrabin and HrUrabin-L are GPI-anchored proteins, which are exposed on the sperm cell surface.

Roles of HrUrabin in Fertilization and Allorecognition—To investigate the roles of HrUrabin in fertilization, we examined the effects on fertilization of an anti-HrUrabin antibody. As shown in Fig. 5A, anti-HrUrabin antibody but not the control antibody specifically inhibited fertilization in a concentration-dependent manner. The inhibitory effect on fertilization was also observed by anti-HrVC70 antibody (data not shown). In addition, we found that anti-HrUrabin antibody potently inhibited the molecular interaction between HrUrabin and HrVC70 on a nitrocellulose membrane (Fig. 5B). These results indicate that the apparent association of HrVC70 to a 35-kDa protein(s) on a membrane is certainly due to the interaction between HrVC70 and HrUrabin and that the inhibitory effect of anti-HrUrabin antibody on fertilization is caused by blocking the interaction between HrUrabin and HrVC70.

We previously reported that sperm of H. roretzi were able to bind to HrVC70 that is immobilized to protein A-agarose with the aid of anti-HrVC70 antibody (13). The number of nonself-sperm bound to these beads was significantly higher than that of self-sperm bound to the same beads (13). We repeated this experiment using a newly produced anti-HrVC70 antibody, and confirmed that an alloreconizable sperm binding to HrVC70 was reproducibly observed (Fig. 5C, comparison between lanes 1 and 4). Although the number of sperm bound to HrVC70-agarose was variable among individuals (or batches) (data not shown), the binding of nonself-sperm was significantly higher than that of self-sperm in most cases. The binding of self-sperm to HrVC70, which is immobilized to anti-HrVC70-adsorbed Protein A-agarose (lane 7) or anti-HrVC70-adsorbed Protein A-agarose without immobilizing HrVC70 (lane 8). These results coincided with our previous observation that self-sperm also adhere to the VC of intact eggs under physiological conditions (9–11). Anti-HrUrabin antibody potently inhibited the higher affinity of nonself-sperm to HrVC70 (Fig. 5C, comparison between lanes 4 and 6), whereas control antibody showed no significant inhibition (comparison between lanes 4 and 5). The binding of self-sperm to HrVC70 was also strongly inhibited by anti-HrUrabin antibody (Fig. 5C, lane 3) but not by control antibody (lane 2).

As mentioned earlier, HrUrabin and HrUrabin-L are not highly polymorphic. Therefore, we examined whether the binding of HrVC70 to HrUrabin on a nitrocellulose membrane is allospecific or not, by using HrVC70 and sperm LD-DIM fractions, both of which were prepared from the same and different individuals. As shown in Fig. 5D, no significant differences were observed between autologous and allogeneic interactions between HrUrabin and HrVC70 in several combinations. These results suggest that HrUrabin is not a direct allore cognition molecule, although this protein seems to be indispensable for self-incompatible fertilization in H. roretzi.

DISCUSSION

Difference in the Results by Far Western Analysis and Yeast Two-hybrid Screening—The present paper demonstrated that a sperm 35-kDa GPI-anchored CRISP-like glycoprotein, referred

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4 H. Sawada, unpublished observation.
TABLE 4. Involvement of sperm HrUrabin in fertilization and allorecognition of sperm binding to HrvC70. A, the concentration-dependent inhibitory effect of anti-HrUrabin antibody on fertilization. A small volume of egg suspension was added into a sperm suspension, which had been preincubated with anti-HrUrabin antibody (closed circle) or control antibody (closed square). Fertilization ratio was determined on the basis of the expansion of perivitelline space. Error bars indicate the standard deviation (n = 3). B, inhibitory effect of anti-HrUrabin antibody on the binding of HrvC70 to HrUrabin on a membrane. Biotin-labeled anti-HrvC70 antibody was used for the detection. C, inhibitory effect of anti-HrUrabin antibody on the binding of sperm to HrvC70-coated agarose beads. Self-sperm (S-spl) or non-self-sperm (NS-spl) suspension, which had been incubated with control (cont. IgG) or anti-HrUrabin (α-HrUrabin) antibodies, was added into a small volume of suspension of HrvC70-coated agarose beads (no mark). Protein A-beads alone (agarose), or anti-HrvC70-conjugated beads without HrvC70 (α-C70-agarose). The number of sperm bound to a single agarose bead was counted for 50 –100 beads. In the control experiments (lanes 7 and 8), no significant sperm binding above the background level was detected. Error bars indicate the standard error (n = 50). D, interaction between HrvC70 and HrUrabin is not allo-specific. Sperm LD-DIM fractions prepared from individuals A, B, and C, were subjected to Far Western analyses using HrvC70 suspensions prepared from the same individuals.

Effects of anti-HrUrabin antibody on fertilization and allorecognition of sperm binding to HrvC70. Possible Posttranslational Modification in HrUrabin-L. Because the amino acid composition of the 50-kDa protein (50-kDa HrUrabin-L) did not coincide with the number of amino acids deduced from HrUrabin-L cDNA (data not shown), and also because the trypsin-digested fragments of 50-kDa HrUrabin-L showed no peptide-12/L20 by LC/MS analysis (see Fig. S1), yet unknown posttranslational modification may occur in the 50-kDa protein in addition to the N-glycosylation and the attachment of GPI-anchor. In connection with this, it is interesting to note our preliminary observation that a non-catalytic domain, a L1 (ΔL2) region (Ser23–Asn96) of the light chain (L-chain), of a sperm trypsin-like protease HrSpermosin, which is involved in sperm binding to and penetration through the VC during fertilization (27), seems to be tightly or covalently bound to 50-kDa HrUrabin-L. Anti-L1 (ΔL2) antibody, but not anti-L2 (Ser97–Lys129) antibody, reacted to 50-kDa HrUrabin-L, whereas anti-L1(ΔL2) antibody showed no reactivity to the bacterially expressed glutathione S-transferase-HrUrabin-L fusion protein. Furthermore, the 50-kDa band on a membrane was not detected by Western blotting using anti-L1(ΔL2) antibody in the cases of

5 H. Hyodo, N. Yokota, and H. Sawada, unpublished data.

FIGURE 5. Involvement of sperm HrUrabin in fertilization and allorecognition of sperm binding to HrvC70. A, the concentration-dependent inhibitory effect of anti-HrUrabin antibody on fertilization. A small volume of egg suspension was added into a sperm suspension, which had been preincubated with anti-HrUrabin antibody (closed circle) or control antibody (closed square). Fertilization ratio was determined on the basis of the expansion of perivitelline space. Error bars indicate the standard deviation (n = 3). B, inhibitory effect of anti-HrUrabin antibody on the binding of HrvC70 to HrUrabin on a membrane. Biotin-labeled anti-HrvC70 antibody was used for the detection. C, inhibitory effect of anti-HrUrabin antibody on the binding of sperm to HrvC70-coated agarose beads. Self-sperm (S-spl) or non-self-sperm (NS-spl) suspension, which had been incubated with control (cont. IgG) or anti-HrUrabin (α-HrUrabin) antibodies, was added into a small volume of suspension of HrvC70-coated agarose beads (no mark). Protein A-beads alone (agarose), or anti-HrvC70-conjugated beads without HrvC70 (α-C70-agarose). The number of sperm bound to a single agarose bead was counted for 50 –100 beads. In the control experiments (lanes 7 and 8), no significant sperm binding above the background level was detected. Error bars indicate the standard error (n = 50). D, interaction between HrvC70 and HrUrabin is not allo-specific. Sperm LD-DIM fractions prepared from individuals A, B, and C, were subjected to Far Western analyses using HrvC70 suspensions prepared from the same individuals.

Ascidian Sperm Binding Partner for HrvC70

Functions of HrUrabin and Other CRISP Members in Fertilization.—Physiological functions of CRISP family proteins are poorly understood. One of the most characterized CRISP members is an epididymal glycoprotein CRISP-1 (DE), which is associated with the sperm surface and is predicted to play a role in gamete fusion (22). The PR domain is reported to be a binding region of CRISP-1 to the egg surface (23). On the other hand, it is also reported that the PR domain functions as a protease in a specific CRISP member (24). In contrast, our results suggest that HrUrabin plays a pivotal role in the primary binding of sperm to HrvC70, a sperm receptor on the VC, rather than gamete fusion, during fertilization. In macaque, it is reported that an antibody raised against sperm surface protein, which is released by treatment with PI-PLC, revealed the existence of GPI-anchored CRISP on the sperm membrane, possibly participating in fertilization (25). This protein exists in two isoforms with molecular masses of 24 and 48 kDa, similarly to HrUrabin, although they assumed that the 48-kDa isoform is a dimer of the 24-kDa isoform (25). As mentioned above, CRISP members on the sperm surface seem to play diverse roles in fertilization in different species.

HrvC70 consists solely of 12 tandem EGF-like repeats. Although the EGF motif is widely distributed in many extracellular proteins involved in intermolecular interactions, little is known about their physiological binding partners. Our present finding revealed for the first time that a CRISP-like glycoprotein is a novel ligand for EGF modules. The ascidian C. intestinalis also has multiple genes encoding both EGF and zona pellucida domains (26), but such proteins are not known as egg-coat proteins in animals other than ascidians. Whether the interaction between CRISP-like glycoprotein and the EGF module is involved in fertilization of C. intestinalis, and also in other protein-protein interactions is an intriguing issue remaining to be studied.
These results suggest that 50-kDa HrUrabin-L is a molecular species covalently linked with L1(H9004/L2) peptide of HrSpermosin. Because the VC-lysin system responsible for sperm penetration of the VC must be activated after sperm recognizes the VC as nonself, covalent linkage between HrUrabin and HrSpermosin may be coupled to accomplish “the allorecognition-linked lysin system” during ascidian fertilization.

FIGURE 6. A working hypothesis on the role of HrUrabin in gamete interaction. 1, primary binding phase: HrUrabin on the sperm lipid rafts binds to HrVC70 on the VC, probably via the non-polymorphic region of HrVC70 (13). 2, self-recognition phase: unidentified sperm-borne self-recognition molecule recognizes polymorphisms of HrVC70 or an unknown hypothetical polymorphic binding partner tightly associated with HrVC70. If the latter possibility is the case, HrVC70-conjugated beads used in the bead-binding experiments may also contain such a female self-recognition molecule. 3, self-rejection phase: if the self-recognition between male and female recognition molecules does not occur, and also if the sperm recognizes HrVC70 (or its complex) as nonself, the sperm-borne lysin system may be activated, allowing the sperm passage through the VC. On the other hand, if the sperm recognizes the HrVC70 (or its complex) as self, a downstream signaling pathway is activated, resulting in the detachment of sperm from the VC. It seems likely that some unidentified proteases or glycosidases are involved in this weakening process: HrVC70 or/and HrUrabin may be a target of such hydrolytic cleavages because their interaction is considered to be a main force of the primary gamete interaction. Although no appreciable difference in the sperm binding to the VC has been observed between autologous and allogeneic gamete combinations, it is plausible that sperm detached from self-HrVC70 may continue to loosely adhere to the VC, due to the adhesive nature of the VC in this species and also a possible interaction between sperm and the VC, which is mediated by sperm fucosidase (30).
between HrVC70 and sperm-surface HrUrabin must be weakened in autologous gamete combination, but the molecular interaction between HrUrabin and HrVC70 would not change on the basis of Far Western analysis. This must be an important factor why HrUrabin shows equivalent affinity for self- and nonself-derived HrVC70 by Far Western analyses. HrUrabin may play a key role in several processes during self-incompatible fertilization, although HrUrabin itself is unlikely to be a direct allorecognition molecule. It is thought that HrUrabin is involved in the primary binding phase, which is essential for the onset of the self-recognition process. It appears to have a crucial role also in the self-rejection phase, because the affinity of sperm surface HrUrabin to HrVC70 must be lowered after the sperm recognizes the self-ligand (HrVC70 or its complex) on the VC. Difference in sperm binding ability to the VC between autologous and allogeneic gamete combinations, which is well known in *C. intestinalis* (7), is not obvious in *H. roretzi* (10). Presumably, sperm may loosely adhere to the VC even after the detachment from self-HrVC70, due to the adhesive nature of the VC in this species. In addition, a sperm α-L-fucosidase-mediated interaction between sperm and the VC (30) may also contribute to this autologous adhesion.

We assume that a putative sperm-borne self-recognition molecule must specifically recognize the autologous HrVC70. However, such a self-recognition protein has not yet been identified in *H. roretzi*. Although the polymorphisms of HrVC70 may provide a molecular basis of allorecognition, another possibility is also considered: a hypothetical molecule tightly associated with HrVC70 may be involved in self-recognition (see Fig. 6). Such a putative molecule might be co-extracted with weak acid and immobilized to agarose beads. Whether homologs of *C. intestinalis* self-incompatibility genes (two pairs of s-/v-Themis) encode sperm- and VC-derived self-recognition molecules in *H. roretzi*, and also whether homologs of HrUrabin and HrVC70 are involved in the self-incompatible fertilization in *C. intestinalis* are very intriguing issues remaining to be solved.

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