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# Ascidian Sperm Glycosylphosphatidylinositol-anchored CRISP-like Protein as a Binding Partner for an Allorecognizable Sperm Receptor on the Vitelline Coat<sup>\*[5]</sup>

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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 HrUrafin in a low density detergent-insoluble membrane fraction is a physiological binding partner for HrVC70. We found that HrVC70 specifically interacts with HrUrafin, which had been separated by SDS-PAGE and transferred onto a nitrocellulose membrane. HrUrafin has an N-linked sugar chain, essential for binding to HrVC70. *HrUrafin* 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-HrUrafin antibody, which neutralizes the interaction between HrUrafin and HrVC70, potently inhibited fertilization and allorecognizable sperm-binding to HrVC70-agarose. However, no significant difference in the binding ability of HrUrafin 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 HrUrafin on the sperm surface and HrVC70 on the vitelline coat, but that HrUrafin *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)<sup>3</sup>-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

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[5] The on-line version of this article (available at <http://www.jbc.org/>) contains supplemental Fig. S1.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB276042 (*HrUrafin*) and AB276041 (*HrUrafin-L*).

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<sup>3</sup> The abbreviations used are: VC, vitelline coat; CRISP, cysteine-rich secretory protein; EGF, epidermal growth factor; EPPS, N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid; GPI, glycosylphosphatidylinositol; HrTTSP-1, *Halocynthia roretzi* type II transmembrane serine protease-1; LD-DIM, low-density detergent-insoluble membrane; PI-PLC, phosphatidylinositol-specific phospholipase C; PR, plant pathogenesis-related 1; SCP, sperm-coating glycoprotein; ESI-MS, electrospray ionization mass spectrometry; RACE, rapid amplification of cDNA ends; PBS, phosphate-buffered saline.

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to propose that a putative allorecognition 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 allorecognition protein, which is responsible for self-sterility.

To gain insights on the molecular mechanism of allorecognition, 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-associated 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  $-20^{\circ}\text{C}$  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  $\mu\text{g}/\text{ml}$  leupeptin), and homogenized with a Teflon homogenizer at 2,000 rpm, 10 times. The homogenate was filtered through a nylon mesh (50  $\mu\text{m}$ ), and the VC on the mesh

was washed with 0.2 $\times$  artificial seawater 5–10 times, followed by centrifugation at  $7,000 \times g$  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 HCl and incubated at  $13^{\circ}\text{C}$  for 15 min. After centrifugation ( $15,000 \times g$  for 15 min), the supernatant fraction, which contains almost exclusively HrVC70, was neutralized with 50 mM Tris/HCl (pH 8.0) and stored frozen until use.

The LD-DIM fraction from *H. roretzi* sperm was prepared as described previously for sea urchin sperm (18, 19). Briefly, intact sperm suspensions were mixed with an equal volume of homogenizing buffer (10 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu\text{g}/\text{ml}$  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 ( $7,000 \times g$  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 ( $200,000 \times g$ , 18 h,  $4^{\circ}\text{C}$ ), 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  $\lambda$ -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 HrUrabin were carried out as described previously (14), using a digoxigenin-labeled cDNA probe corresponding from Pro<sup>19</sup> to Leu<sup>287</sup> 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 HrUrabins 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.

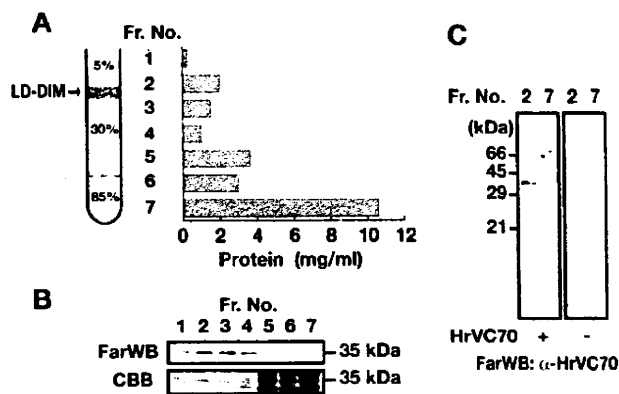
An *N*-linked glycosylation site on each HrUrabin species was predicted from their amino acid sequences. To examine whether a carbohydrate chain is linked to the consensus Asn residue of HrUrabins, the LD-DIM fraction was incubated with 5 units of PNGase F (Sigma) in 50 mM phosphate buffer (pH 8.0) for 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 HrUrabins, 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

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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 (lower panel). The gel was blotted onto a nitrocellulose membrane and subjected to Far Western blotting (FarWB), in which the membrane was incubated with HrVC70 and the HrVC70-interacting proteins were detected by anti-HrVC70 antibody and ECL detection kit (upper panel). 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.

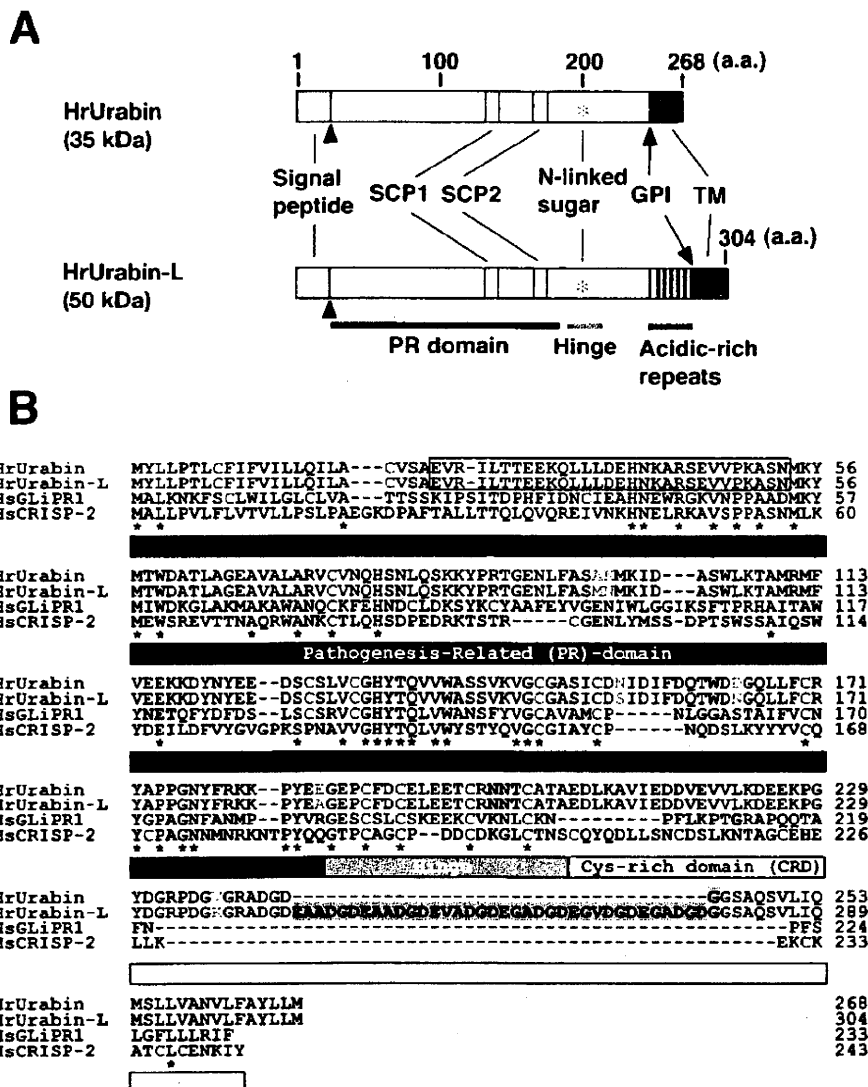
digested with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) according to a procedure described previously (20). Trypsin-digested fragments were subjected to high performance liquid chromatography with a C18 reverse phase column at a constant flow rate of 200 nl/min. After applying the sample 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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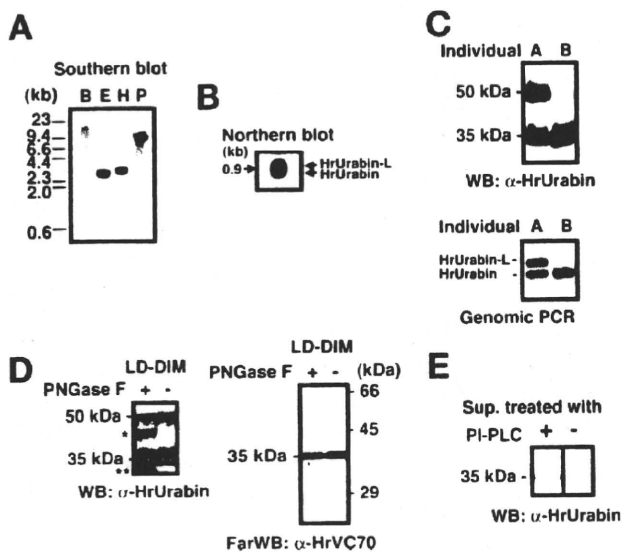
**FIGURE 2. Deduced amino acid sequences of HrUrabrin and HrUrabrin-L.** A, schematic representation of HrUrabrin proteins. Light gray box, signal peptide; arrowhead, predicted cleaved site of the signal peptide; yellow box, SCP motif; blue asterisks, potential N-linked glycosylation site; graded gray boxes, acidic-rich repeat; dark gray box, a cleavable transmembrane domain. Abbreviations: GPI, glycosylphosphatidylinositol-anchorage attachment site; PR, PR (plant pathogenesis related-1) domain conserved in CRISPs; SCP, sperm coating glycoprotein motif, which is comprised with two separate (SCP1 and 2) sequence signatures; TM, transmembrane domain. B, a sequence alignment of HrUrabrin and HrUrabrin-L with human GLIPR1 and CRISP-2. An open box in the alignment, initially determined N-terminal sequence of the 35-kDa HrUrabrin; yellow highlight, SCP motif; blue highlight, potential N-linked glycosylation site; red highlight, acidic-rich repeats; green highlight, GPI-anchor attachment site. Note that HrUrabrin, HrUrabrin-L, and HsGLiPR1 lack C-terminal CRD. Non-conserved residues between HrUrabrin and HrUrabrin-L, which can be used to distinguish respective peptide fragments by mass spectrometric analysis, are indicated by blue letters, whereas Cys residues are indicated by red letters. Asterisks indicate identical amino acids among these proteins.

## RESULTS

**Identification of a Binding Partner (HrUrabrin) 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 (EVRLTTEKQLLLEHNKARSEVVPKASNMKY), 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 *HrUrabrin* (*Halocynthia roretzi* unique raft-derived binding protein for HrVC70) and *HrUrabrin-L* (a long isoform). Schematic representations (A) and deduced amino acid sequences (B) of HrUrabrin and HrUrabrin-L are depicted in Fig. 2. These *HrUrabrin* and *HrUrabrin-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 HrUrabrin and HrUrabrin-L contain a signal peptide, a PR (plant pathogenesis-related 1) domain, which is well conserved in the CRISP (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 HrUrabrin-L), which is followed by a GPI-anchor attachment site and a cleavable transmembrane domain (Fig. 2). Unlike HrVC70, both HrUrabrin and HrUrabrin-L were not so polymorphic among different individuals (data not shown). The molecular masses of unmodified HrUrabrin and HrUrabrin-L were esti-



**FIGURE 3. Genomic Southern, Northern, Western, and Far Western blotting of HrUrabin and HrUrabin-L, both of which are GPI-anchored proteins 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, suggesting 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 treatment decreases the molecular sizes of both HrUrabin (left), but only intact HrUrabin, 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.

mated to be 30,008 and 33,362 Da, respectively. On the other hand, the molecular masses of the mature forms, lacking a signal peptide and a C-terminal portion of GPI-anchor attachment site, and containing a GPI-anchor (~2 kDa), were estimated to be 27 and 30 kDa, respectively.

**Expression and Localization of HrUrabin**—Southern blotting probed with an HrUrabin cDNA, which reacts with both isoforms, using the genomic DNAs digested with several restriction enzymes gave a single band in each lane (Fig. 3A), suggesting that the copy number of these genes seems to be single or very low. To determine whether these genes are independent, we isolated several genomic clones containing the HrUrabin-derived sequence by screening a  $\lambda$  phage genomic library. We found that several clones contained both genes (data not shown). This indicates that HrUrabin and HrUrabin-L are two distinct gene copies rather than splice variants of a single gene, being located adjacently within ~20-kbp in the genome of *H. roretzi*.

Northern blotting showed that about 0.9-kb HrUrabin mRNAs are expressed 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-

thione S-transferase fusion protein, revealed the patch-like localization of HrUrabin on the surface of the sperm head and tail (Fig. 4, C and D).

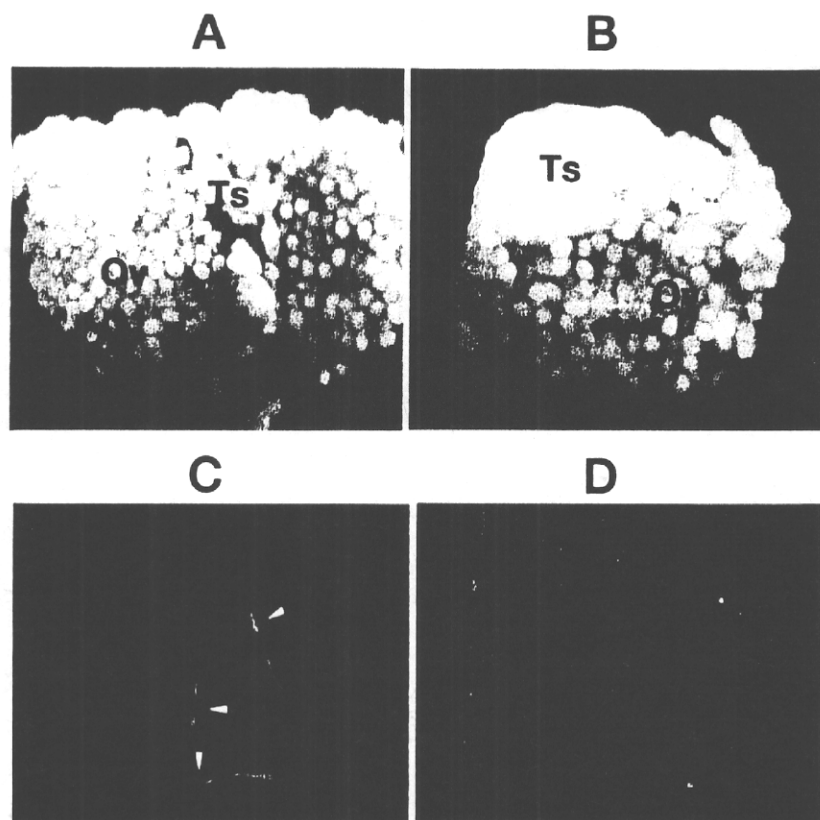
**HrUrabin and HrUrabin-L Genes Encode the 35- and 50-kDa Proteins, Respectively**—Anti-HrUrabin antibody reacted not only to the original 35-kDa protein but also to a novel 50-kDa protein by Western blotting (Fig. 3C, upper panel, left lane). To identify this novel protein, we first determined the N-terminal sequence. The main sequence of its N-terminal five residues was identical to that of 35-kDa HrUrabin, suggesting that the 50-kDa protein is not a cross-reacted unrelated protein but an isoform of HrUrabin. We next analyzed the amino acid compositions of both isoforms. The amino acid composition of 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 Val<sup>237</sup> and Lys<sup>237</sup> were found to be characteristic to HrUrabin and HrUrabin-L, respectively, because a Val<sup>237</sup>-containing peptide (number 24') and a Lys<sup>237</sup>-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 HrUrabin failed to bind to HrVC70 in Far Western analysis (compare left and right panels in Fig. 3D). Neither intact nor deglycosylated 50-kDa HrUrabin-L was capable of binding to HrVC70 (Fig. 3D).

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**FIGURE 4.** *In situ* hybridization of HrUrabin in gonad and immunocytochemistry of HrUrabin in sperm. *A* and *B*, a gonad specimen containing testis and ovary was hybridized with antisense (*A*) and sense (*B*) probes for HrUrabin mRNA. HrUrabin mRNA is expressed in testis but not in ovary. *C* and *D*, immunocytochemistry of *H. roretzi* sperm with anti-HrUrabin (*C*) and control (*D*) antibodies. HrUrabin proteins showed patch-like localization on the surface throughout the sperm as revealed by Alexa Fluor 568-conjugated goat anti-rabbit IgG antibody used as a secondary antibody. Nucleus was stained with 4',6-diamino-2-phenylindole. Arrowhead, sperm head region.

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 allorecognizable 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 1), was observed, but not in the case of unmodified 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).<sup>4</sup> Anti-HrUrabin

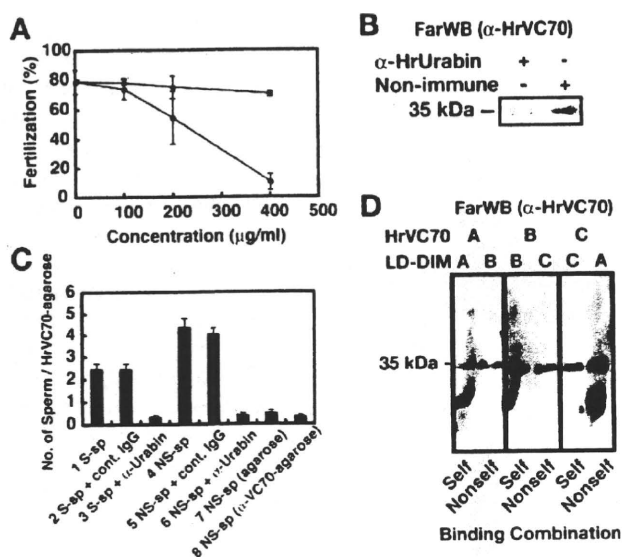
antibody potently inhibited the higher affinity of nonself-sperm to HrVC70 (Fig. 5C, comparison between lanes 4 and 6), whereas control antibody showed no significant inhibition (comparison between lanes 4 and 5). The binding of self-sperm to HrVC70 was also strongly inhibited by anti-HrUrabin antibody (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 allorecognition 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

<sup>4</sup> H. Sawada, unpublished observation.



**FIGURE 5. Involvement of sperm HrUrabin in fertilization and allerecognizable 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-sp) or nonself-sperm (NS-sp) suspension, which had been incubated with control (cont. IgG) or anti-HrUrabin ( $\alpha$ -Urabin) 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 ( $\alpha$ -VC70-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.

to as HrUrabin, in the LD-DIM fraction is a binding partner for HrVC70 on the VC of the eggs in *H. roretzi*. Our previous yeast two-hybrid screening failed to identify HrUrabin (16). A possible reason for this is that a carbohydrate moiety, which is essential for the binding between HrVC70 and HrUrabin (Fig. 3D), may not be properly attached to HrUrabin in yeast cells. On the other hand, HrTTSP-1, which was identified as an HrVC70-interacting protein by yeast two-hybrid screening (16), was not detected by Far Western analysis. Ionic detergent (0.1% SDS), which was used for solubilization of HrVC70, might impair the interaction between HrUrabin and HrTTSP-1 in Far Western analysis. Our present results do not rule out the possibility that HrTTSP-1 is involved in fertilization. Actually, a certain processing product of HrTTSP-1 was found to occur in sperm exudates (a supernatant of activated sperm by alkaline seawater), implying its participation in fertilization.<sup>5</sup> Therefore, it seems to be plausible that both HrUrabin and HrTTSP-1 are involved in gamete interaction. But, taking into account the inhibitory effects of anti-HrUrabin antibody on fertilization and allerecognizable sperm binding to HrVC70-agarose, it should be

<sup>5</sup> H. Hyodo, N. Yokota, and H. Sawada, unpublished data.

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emphasized that at least HrUrabin plays a key role in allerecognizable sperm binding to the VC during fertilization.

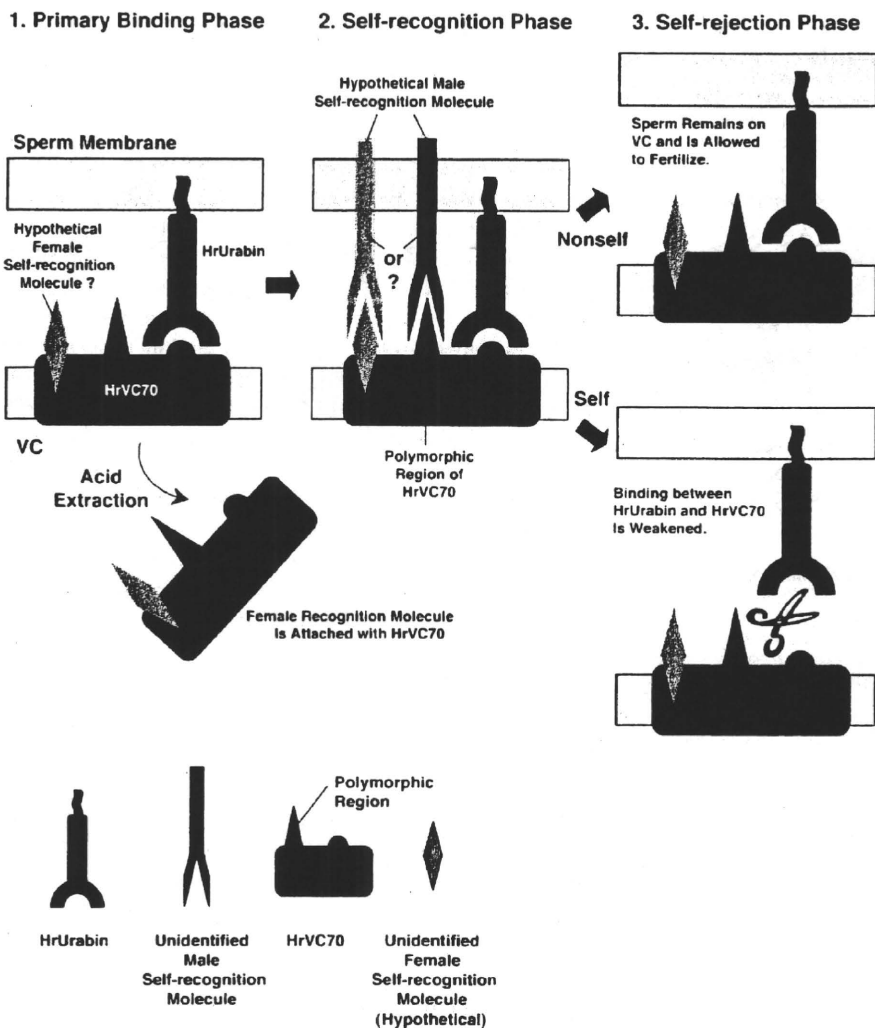
**Functions of HrUrabin and Other CRISP Members in Fertilization**—Physiological functions of CRISP family proteins are poorly understood. One of the most characterized CRISPs is an epididymal glycoprotein CRISP-1 (DE), which is associated with the sperm surface and is predicted to play a role in gamete 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.

**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/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 ( $\Delta$ L2) region (Ser<sup>23</sup>–Asn<sup>96</sup>) 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 ( $\Delta$ L2) antibody, but not anti-L2 (Ser<sup>97</sup>–Lys<sup>129</sup>) antibody, reacted to 50-kDa HrUrabin-L, whereas anti-L1( $\Delta$ L2) 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( $\Delta$ L2) antibody in the cases of



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**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).

HrUrabin-L-missing individuals.<sup>6</sup> These results suggest that 50-kDa HrUrabin-L is a molecular species covalently linked with L1( $\Delta$ 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 all-recognition-linked lysin system" during ascidian fertilization.

<sup>6</sup> M. Akasaka, Y. Harada, and H. Sawada, unpublished data.

*Self/Nonself Recognition Mechanisms during Ascidian Fertilization*—Molecular mechanisms of self-incompatibility in higher plants have been studied well in various species. In Brassicaceae, it is controlled by *S*-locus, which has multiple alleles, and each allele encodes male and female recognition molecules, which enables an allele-specific interaction (28). The self/nonself discrimination between the male and female recognition molecules takes place on the basis of recognition (and then rejection) of "self" in these species (1, 28). We recently revealed the molecular mechanism of the self-incompatibility system in the ascidian *C. intestinalis* (29). In *C. intestinalis*, two unlinked loci are involved in its self-sterility, both of which encode a pair of polycystin-1-like receptors on the sperm surface (s-Themis-A and -B) and fibrinogen-like ligand on the VC (v-Themis-A and -B). Our genetic analysis revealed that the system in *C. intestinalis* is based on the recognition of self-ligands (v-Themis) by their respective receptors (s-Themis). Because the VC of *C. intestinalis* eggs exhibits higher affinity to nonself-sperm than to self-sperm, self-sperm must be detached from the VC when the recognition molecule on the sperm surface finds the self-ligand on the VC. This self-incompatibility system is very similar to those of higher plants, in which pollen tube elongation is blocked when it is recognized as self in the stigma.

Here, we propose a working hypothesis on the self-sterility in *H. roretzi* (Fig. 6). In this hypothesis, the self-incompatible fertilization process consists of three phases. In the first phase (primary binding phase), both self- and nonself-sperm equally bind to the VC. Because anti-HrUrabin antibody abolished the basal binding of self-sperm to HrVC70 beads, the interaction between HrUrabin and HrVC70 seems to be essential for the primary gamete interaction. After this phase, a self-recognition process between a male-recognition molecule on the sperm surface and a female-recognition molecule on the VC may take place (self-recognition phase). When the male side molecule recognizes the self-ligand on the VC, the primary binding ability of sperm must be decreased (self-rejection phase). The interaction

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  $\alpha$ -1-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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# N-Glycosylation of Laminin-332 Regulates Its Biological Functions

## A NOVEL FUNCTION OF THE BISECTING GlcNAc\*

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Laminin-332 (Lm332) is a large heterotrimeric glycoprotein that has been identified as a scattering factor, a regulator of cancer invasion as well as a prominent basement membrane component of the skin. Past studies have identified the functional domains of Lm332 and revealed the relationships between its activities and the processing of its subunits. However, there is little information available concerning the effects of *N*-glycosylation on Lm332 activities. In some cancer cells, an increase of  $\beta$ 1,6-GlcNAc catalyzed by *N*-acetylglucosaminyltransferase V (GnT-V) is related to the promotion of cancer cell motility. By contrast, bisecting GlcNAc catalyzed by *N*-acetylglucosaminyltransferase III (GnT-III) suppresses the further processing with branching enzymes, such as GnT-V, and the elongation of *N*-glycans. To examine the effects of those *N*-glycosylations to Lm332 on its activities, we purified Lm332s from the conditioned media of GnT-III- and GnT-V-overexpressing MKN45 cells. Lectin blotting and mass spectrometry analyses revealed that *N*-glycans containing the bisecting GlcNAc and  $\beta$ 1,6-GlcNAc structures were strongly expressed on Lm332 purified from GnT-III-overexpressing (GnT-III-Lm332) and GnT-V-overexpressing (GnT-V-Lm332) cells, respectively. Interestingly, the cell adhesion activity of GnT-III-Lm332 was apparently decreased compared with those of control Lm332 and GnT-V-Lm332. In addition, the introduction of bisecting GlcNAc to Lm332 resulted in a decrease in its cell scattering and migration activities. The weakened activities were most likely derived from the impaired  $\alpha$ 3 $\beta$ 1 integrin clustering and resultant focal adhesion formation. Taken together, our results clearly demonstrate for the first time that *N*-glycosylation may regulate the biological function of Lm332. This finding could introduce a new therapeutic strategy for cancer.

Laminins (Lms)<sup>2</sup> are large heterotrimeric glycoproteins that are prominent components of basement membranes and are involved in important biological roles, including tissue development and cell differentiation, survival, adhesion, and migration (1). Lms are heavily glycosylated molecules. It has been reported that between 13 and 30% of the total molecular weight of Lms is *N*-linked glycosylated (2). Laminin-111 (Lm111; previously known as laminin-1) is easily purified from mouse Engelbreth-Horm-Swarm tumor and has been intensively investigated for its carbohydrate structures. In comparison with unglycosylated Lm111, which is purified from cell lysates of tunicamycin-treated cells, glycosylation of Lm111 was shown to affect cell spreading and neurite outgrowth activities but not cell adhesion activity or heterotrimer assembly (3, 4). However, tunicamycin extensively inhibited the secretion of laminin into cell culture medium (3, 5).

Laminin-332 (Lm332; previously known as laminin-5) is composed of  $\alpha$ 3,  $\beta$ 3, and  $\gamma$ 2 chains. Lm332 is expressed in the skin and other stratified squamous epithelial tissues, where it associates with hemidesmosomes via integrin  $\alpha$ 6 $\beta$ 4. Genetic absence of Lm332 causes a severe and lethal skin blistering disease, Herlitz's junctional epidermolysis bullosa (6, 7). *In vitro* Lm332 promotes cell motility and scattering through the association of C-terminal globular (G) domains with integrin  $\alpha$ 3 $\beta$ 1 (8), which is thought to be a key factor during wound healing (9, 10) and cancer metastasis (11, 12). Proteolytic cleavage of the Lm332  $\alpha$ 3 and  $\gamma$ 2 subunits (13, 14) affects both cell adhesion and migration (15, 16). Few studies have assessed the functional significance of *N*-glycosylation of Lm332 subunits.

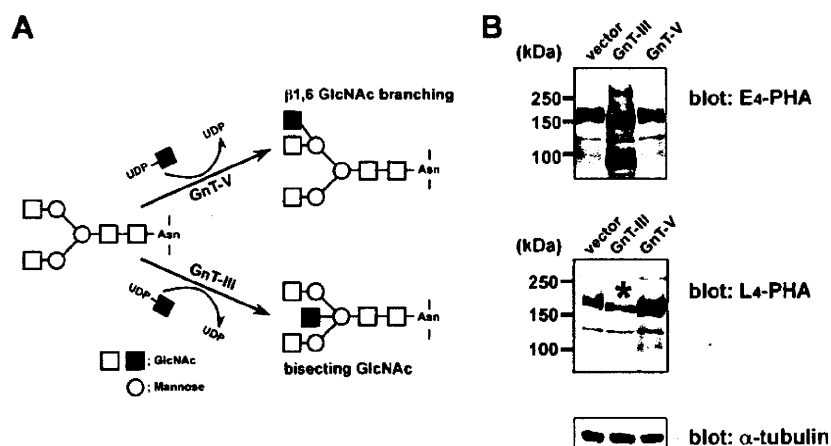
*N*-Acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of the  $\beta$ 1,6-linked GlcNAc branch and defines this subset of *N*-glycans (17, 18) (Fig. 1A). In some cancers, an increase of  $\beta$ 1,6-GlcNAc is related to cancer metastasis. This is

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<sup>2</sup> The abbreviations used are: Lm, laminin; BRL, buffalo rat liver; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; ECM, extracellular matrix; E<sub>4</sub>-PHA, erythroagglutinating phytohemagglutinin; ELISA, enzyme-linked immunosorbent assay; GnT-III, *N*-acetylglucosaminyltransferase III; GnT-V, *N*-acetylglucosaminyltransferase V; LC, liquid chromatography; L<sub>4</sub>-PHA, leucoagglutinating phytohemagglutinin; Lmxyz, laminin-xyz; MS, mass spectrometry; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

## GnT-III Down-regulates Activities of Laminin-332



**FIGURE 1. Characterization of MKN45 transfectants overexpressing GnT-III or GnT-V.** A, biosynthesis pathways of the bisecting GlcNAc and  $\beta$ 1,6-GlcNAc branching structures by GnT-III and GnT-V, respectively. Square, N-acetylglucosamine; circle, mannose. B, 20  $\mu$ g of cell lysates from vector, GnT-III, and GnT-V MKN45 transfectants were run on 7.5% SDS-polyacrylamide gels and then blotted to nitrocellulose membranes. Blotted proteins were probed with biotinylated E<sub>4</sub>-PHA lectin (top) and L<sub>4</sub>-PHA lectin (middle). E<sub>4</sub>-PHA and L<sub>4</sub>-PHA lectin blotting revealed that GnT-III and GnT-V transfectants were significantly modified with bisecting GlcNAc and  $\beta$ 1,6-linked GlcNAc, respectively. An asterisk indicates reduced L<sub>4</sub>-PHA staining in GnT-III transfectants.  $\alpha$ -tubulin was used as a loaded control.

supported by several reports, including GnT-V overexpression in cancer (19, 20) and GnT-V-deficient mouse studies (21). By contrast, bisecting GlcNAc catalyzed by N-acetylglucosaminyltransferase III (GnT-III) (Fig. 1A) suppresses further processing with branching enzymes, such as GnT-V, and elongation of N-glycans (22, 23), resulting in down-regulating cancer metastasis (24). In addition, GnT-III modification of  $\alpha$ 3 $\beta$ 1 integrin inhibits cell migration promoted by GnT-V on the Lm332 substrate (25).

In the present study, to investigate the effect of N-glycosylation on Lm332 function, we focused on GnT-III- and GnT-V-mediated N-glycosylation of Lm332. Therefore, we purified Lm332s from the conditioned media of GnT-III- and GnT-V-overexpressing MKN45 cells. The analysis of lectin blotting and mass spectrometry indicated that Lm332 could be modified by either GnT-III or GnT-V. As a result, GnT-III modification of Lm332 caused a decrease in its keratinocyte cell adhesion and migration activities. Our findings demonstrate a novel regulatory mechanism of Lm332 activities brought on by N-glycosylation.

## EXPERIMENTAL PROCEDURES

**Antibodies and Reagents**—Mouse monoclonal antibodies against the N-terminal regions of the human laminin  $\alpha$ 3 chain (Lsac3) and the  $\gamma$ 2 chain (D4B5) were a generous gift from Dr. Kaoru Miyazaki (Yokohama City University, Yokohama, Japan). Mouse monoclonal antibody against the laminin  $\alpha$ 3 chain (2B10) was prepared as previously described (26). Monoclonal antibodies against the human laminin  $\beta$ 3 chain (kalinin B1) and paxillin were purchased from Transduction Laboratories (Lexington, KY). Control mouse and rat IgG and function-blocking anti-integrin  $\alpha$ 3 (P1B5) and  $\alpha$ 6 (GoH3) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa Fluor 488 goat anti-mouse IgG was obtained from Invitrogen. Biotinylated leucoagglutinating phytohemagglutinin (L<sub>4</sub>-PHA) and biotinylated erythroagglutinating phytohemagglutinin (E<sub>4</sub>-PHA) were

from Seikagaku Biobusiness Corp. (Tokyo, Japan). A monoclonal antibody against  $\alpha$ -tubulin was purchased from Sigma.

**Cell Culture**—The human gastric cancer cell line MKN45 was cultured in RPMI 1640 medium (Nacalai Tesque, Japan). MKN45 transfectants were described previously (27). The Buffalo rat liver-derived epithelial cell line BRL was a gift from Dr. Kaoru Miyazaki (Yokohama City University, Yokohama, Japan) and was maintained in Dulbecco's modified Eagle's medium. Those media were supplemented with 10% fetal calf serum, penicillin, and streptomycin sulfate. Keratinocytes isolated from patients with junctional epidermolysis bullosa lacking Lm332 were a generous gift from Dr. M. Peter Marinkovich (Stanford University). Keratinocytes were grown in 50%

defined keratinocyte medium (Invitrogen) and 50% medium 154 (Cascade Biologics, Portland, OR) containing penicillin and streptomycin sulfate.

**Preparation of Conditioned Medium and Purification of Laminin-332**—For purification of Lm332, the serum-free CM from MKN45 transfectants were collected every 2 or 3 days. The collected media were centrifuged at 1,000 rpm for 10 min. Finally its supernatant was collected and used as a source for purification of Lm332. The protein containing the supernatant was precipitated by 80% saturated ammonium sulfate. The precipitate was dissolved in and dialyzed against a gelatin column buffer (20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.1% CHAPS, 0.005% Brij 35) overnight at 4 °C. Then samples were centrifuged at 19,000 rpm for 30 min at 4 °C to remove the undissolved proteins. The precleared solution was passed through a gelatin column, and then its flow-through was directly applied to an  $\alpha$ 3 antibody (2B10) column. After washing with the antibody column buffer (20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 0.1% CHAPS, 0.005% Brij 35), followed by MilliQ water, binding proteins were eluted by 0.05% trifluoroacetic acid (v/v) and immediately neutralized with Tris-HCl (pH 8.0) containing 0.005% Brij 35 and 0.1% CHAPS.

**Preparation of Cell Lysate**—For preparing cell lysate, cells were washed with cold PBS twice and then lysed with lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA) containing protease inhibitor mixture (Nacalai Tesque). After incubation for 10 min on ice, cell lysates were cleared by centrifugation at 15,000 rpm for 10 min at 4 °C, and its supernatants were used in the following samples. The protein concentration was determined using a protein assay kit (Nacalai Tesque).

**SDS-PAGE, Immunoblotting, and Lectin Blotting**—SDS-PAGE was performed either on 6% gels or on 4.0–7.5% gradient gels under reducing or nonreducing conditions. Separated proteins were stained with Coomassie Brilliant Blue (CBB). ImageJ software was used for densitometric analysis of protein bands. For immunoblotting analyses, proteins resolved by SDS-PAGE were

## GnT-III Down-regulates Activities of Laminin-332

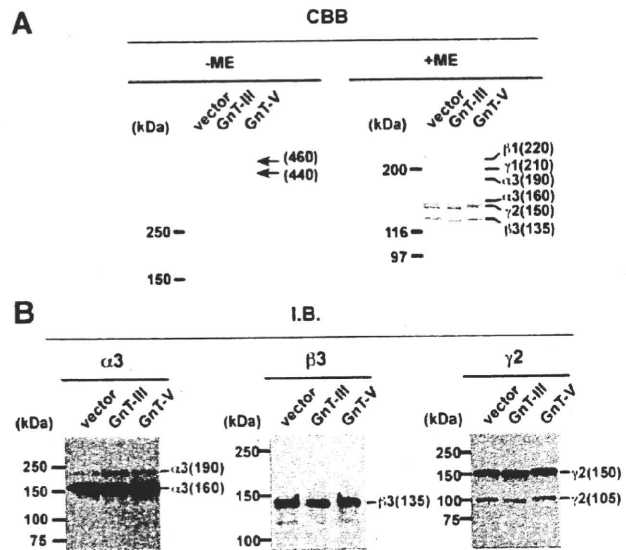
transferred to nitrocellulose membranes. The blots were probed with each specific antibody or biotinylated E<sub>4</sub>-PHA and L<sub>4</sub>-PHA. Immunoreactive bands were detected using an ECL detection kit (GE Healthcare) and a Vectastain ABC kit (Vector Laboratories).

**Analysis of N-Glycan Structures by Liquid Chromatography/Multiple-stage Mass Spectrometry (LC/MS<sup>n</sup>)**—Purified Lm332 was applied to SDS-PAGE using a 4–7.5% gradient gel under reducing conditions and visualized by CBB staining. The gel bands corresponding to  $\alpha$ 3,  $\beta$ 3, and  $\gamma$ 2 subunits were excised from the gel and then cut into pieces, respectively. The gel pieces were destained with 25 mM NH<sub>4</sub>HCO<sub>3</sub> containing 30% acetonitrile and then dehydrated with 100% acetonitrile. The proteins in the gel were reduced and carboxymethylated by incubation with dithiothreitol and sodium monoiodoacetate (25). N-Glycans were released by the treatment with peptide N-glycanase F, and extracted from the gel pieces, as reported (28). The oligosaccharides were reduced with sodium borohydride and desalted. LC/MS<sup>n</sup> was performed using a quadrupole linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer (Finnigan LTQ-FT<sup>TM</sup>; Thermo Fisher Scientific Corp., San Jose, CA) connected to a nanoflow LC system (NanoFrontier nLC; Hitachi High-Technologies Corp., Japan). The eluents were 5 mM ammonium acetate containing 2% acetonitrile, pH 9.6 (pump A), and 5 mM ammonium acetate containing 80% acetonitrile, pH 9.6 (pump B). The borohydride-reduced N-glycans were separated on a Hypercarb column (0.075 × 150 mm; Thermo Fisher Scientific Corp.) with a linear gradient of 5–35% of pump B in 110 min. A single mass scan with Fourier transform ( $m/z$  450–2,000) followed by data-dependent MS/MS for the most intense ions was performed in both positive and negative ion modes as previously reported (29).

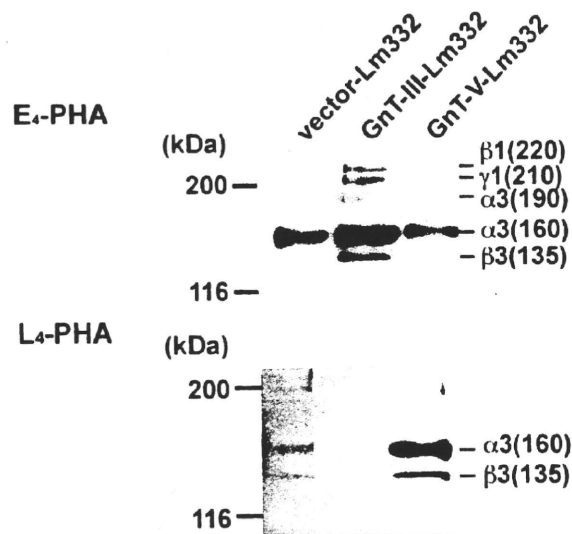
**Cell Adhesion Assay**—Cell adhesion assay was performed as described previously (8). Briefly, each well of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Costar, Cambridge, MA) was coated with a substrate protein and then blocked with 1% bovine serum albumin (BSA).  $2 \times 10^4$  cells in supplement-free keratinocyte growth medium were inoculated per well of 96-well plates. After nonadherent cells were removed, adherent cells were fixed with 25% (w/v) glutaraldehyde and stained with 0.5% crystal violet (w/v) in 20% (v/v) methanol for 10 min. The well was measured for absorbance at 590 nm using a microplate reader. For inhibition assay, the cell suspension was incubated with function-blocking anti-integrin antibodies or with the control IgG for 20 min at room temperature before inoculation.

**Cell Spreading Assay**—For measurement of the cell-spreading area, keratinocytes on purified Lm332 substrates were incubated in keratinocyte growth medium. After 1 h, at least 100 cells were photographed, and their cell spreading areas were measured using Axio Vision software (Carl Zeiss, Germany).

**Cell Scattering Assay**—A scattering assay was done as reported previously (10). Briefly, 500  $\mu$ l of cell suspension ( $2 \times 10^4$  cells in Dulbecco's modified Eagle's medium plus 1% (v/v) fetal calf serum) were inoculated per well of 24-well plates. Test samples were directly added into the culture medium and incubated at 37 °C. After 40 h, cells were fixed with 25% (w/v) glutaraldehyde and stained with 0.5% crystal violet (w/v) in 20% (v/v) methanol for 10 min. The scattered single cells were



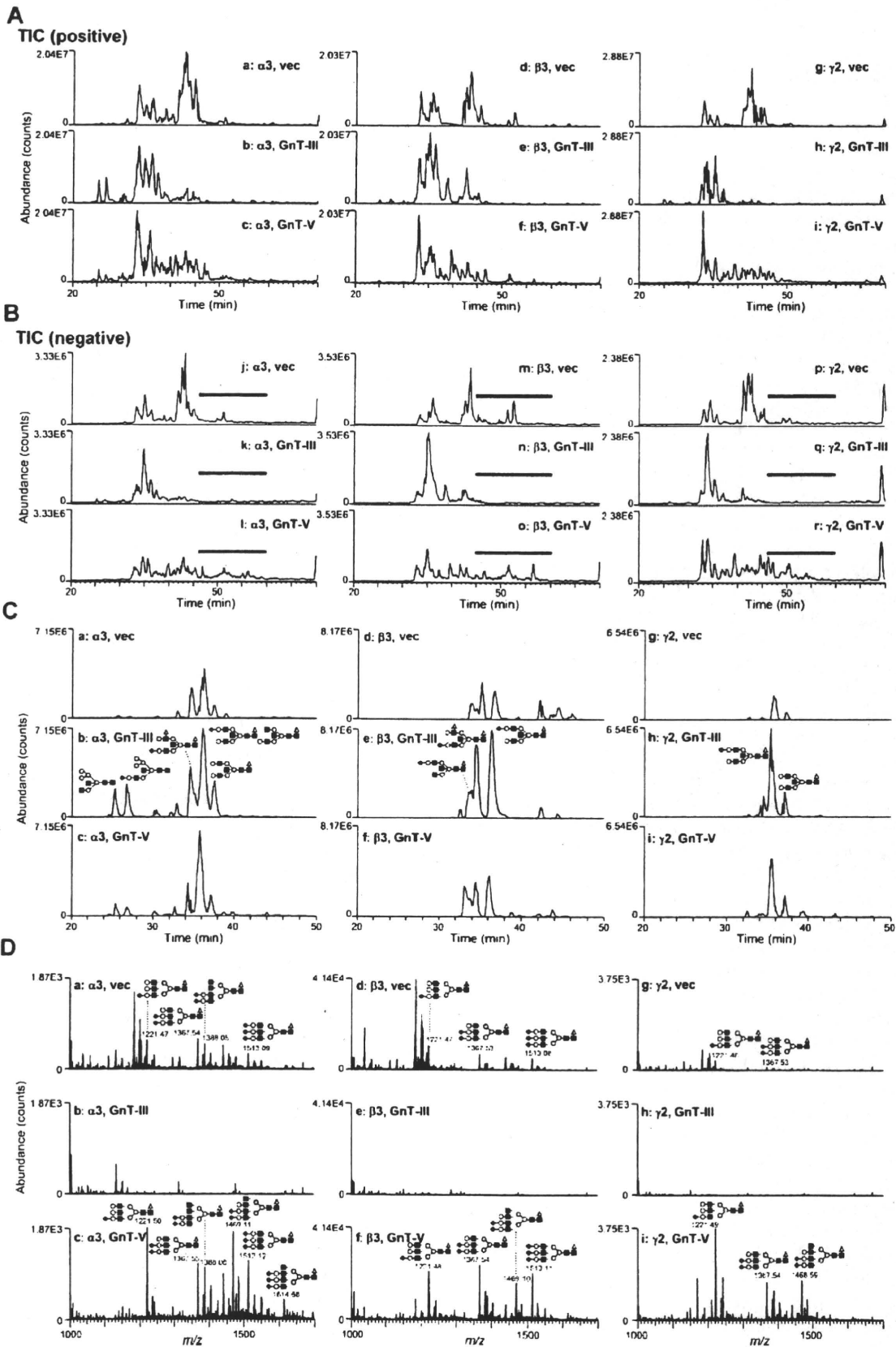
**FIGURE 2. Characterization of laminin-332 purified from conditioned media of vector-, GnT-III-, or GnT-V-MKN45 transfectants.** Purified laminin-332s, vector-Lm332 (*vector*), GnT-III-Lm332 (*GnT-III*), and GnT-V-Lm332 (*GnT-V*), were run on 4–7.5% gradient gels and 6% gels under nonreducing (–ME) and reducing conditions (+ME), respectively. After SDS-PAGE, separated proteins were stained with CBB (A; CBB) or transferred to the nitrocellulose membranes. Blotted proteins were probed with monoclonal antibodies against laminin  $\alpha$ 3 (Ls $\alpha$ 3),  $\beta$ 3 (kalinin B1), and  $\gamma$ 2 (D4B5) chain (B; I.B.). Ordinate indicates molecular sizes in kDa of marker proteins and laminin chains.



**FIGURE 3. Lectin blotting analysis of purified laminin-332s.** 100 ng of laminin-332s (*vector*-, *GnT-III*-, and *GnT-V*-Lm332) was separated on 6% SDS-PAGE gel and then blotted onto the nitrocellulose membranes. Blotted proteins were probed with E<sub>4</sub>-PHA (top) and L<sub>4</sub>-PHA (bottom) lectin. Ordinate indicates molecular sizes in kDa of marker proteins and laminin chains.

counted, and the degree of cell scattering was expressed as the percentage of single cells in each field. At least 300 cells were counted in each field.

**Cell Migration Assay**—A glass bottom dish (Asahi Techno Glass, Japan) was precoated with purified Lm332 and then blocked with 1% BSA for 1 h at 37 °C. 200  $\mu$ l of Lm332-null keratinocyte cell suspension ( $4 \times 10^4$  cells/ml) in growth medium were inoculated into each Lm332-precoated glass bottom dish. After incuba-



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tion for 1 h at 37 °C, cell movement was monitored using time lapse video equipment (Carl Zeiss) for 8 h.

**ELISA**—The ELISA was as follows. The wells of a 96-well plate were coated with test proteins and then blocked with 1.2% BSA at room temperature for 1 h. The wells were washed with PBS containing 0.05% Tween 20 (washing buffer) three times and then incubated with primary antibody for 1 h at room temperature. Furthermore, the wells were washed with washing buffer three times and then incubated with secondary antibody coupled with biotin for 45 min at room temperature. Similarly, the wells were washed three times and incubated with alkaline phosphatase conjugated with avidin D for 45 min at room temperature. After five washes with washing buffer, the bound antibodies were quantified by their absorbance at 405 nm after incubation with *p*-nitrophenylphosphate disodium salt in 100 mM diethanolamine (pH 9.8) containing 0.24 mM MgCl<sub>2</sub>.

**Immunofluorescence Microscopy**—A glass bottom dish (Asahi Techno Glass) was precoated with purified Lm332 and then blocked with 1% BSA for 1 h at 37 °C. 200 μl of the cell suspension (2 × 10<sup>5</sup> cells/ml) in growth medium were inoculated into each Lm332-precoated glass bottom dish. After incubation for 1 h, the cells were washed with PBS and then fixed with 4% (w/v) paraformaldehyde in PBS for 10 min. For permeabilization, the cells were treated with 0.2% (v/v) Triton X-100 in PBS. The fixed cells were blocked with 2% BSA in PBS for 1 h before staining with appropriate primary and secondary antibodies. Fluorescence images were obtained using a fluorescence microscope (Olympus, Tokyo) equipped with ×100/1.35 UPlan-Apochromat oil immersion objectives.

**Statistical Analysis**—Data are presented as mean ± S.D. Student's *t* test, with Microsoft Excel, was used to compare the two groups.

## RESULTS

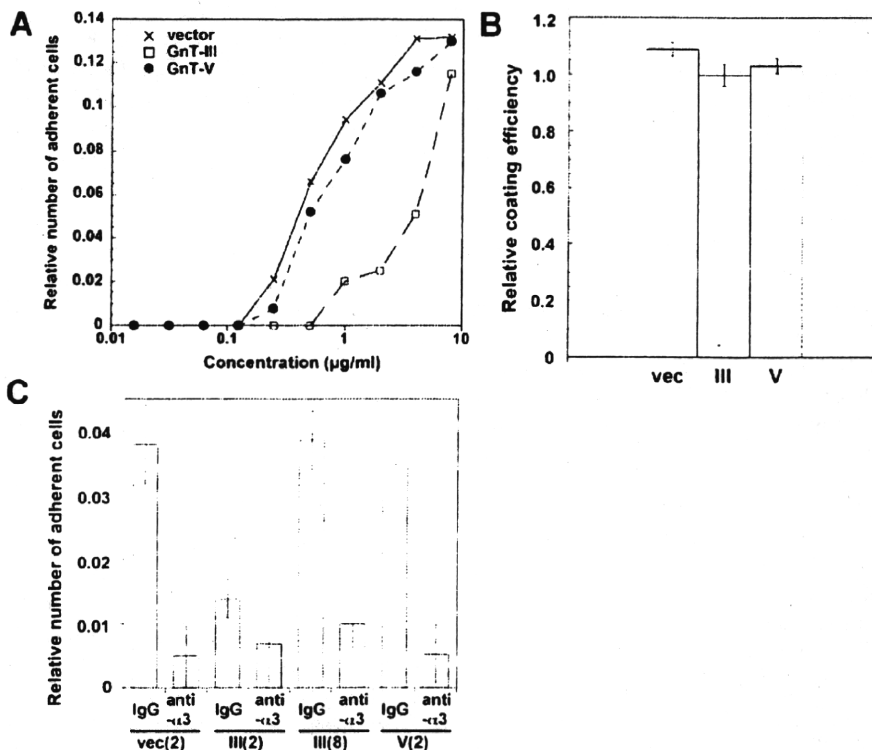
**Characterization of GnT-III and GnT-V Transfectants**—As a source for purification of Lm332 modified by GnT-III- or GnT-V-catalyzed glycosylation, we prepared GnT-III- and GnT-V-overexpressing MKN45 transfectants and also a vector-only transfectant to be used as a negative control as previously described (27). To check the changes in oligosaccharide structures for each transfectant, lectin blotting was performed against the cell lysates from those transfectants using E<sub>4</sub>-PHA, which preferentially binds to bisecting GlcNAc residues in *N*-glycans or L<sub>4</sub>-PHA, which preferentially binds to β1,6-branched GlcNAc residues (30). The blotting results showed that the GnT-III transfectant increased bisecting GlcNAc compared with the other two (Fig. 1B, E<sub>4</sub>-PHA) but decreased GnT-V products (Fig. 1B, L<sub>4</sub>-PHA), supporting the notion that GnT-III antagonizes the action of GnT-V for the modification

of some target glycoproteins, such as α3β1 integrin (25). On the other hand, GnT-V overexpression had no effect on the GnT-III product level (Fig. 1B, E<sub>4</sub>-PHA) but led to a strong increase of β1,6-branched GlcNAc in MKN45 cells (Fig. 1B, L<sub>4</sub>-PHA). The immunoblotting using an anti-α-tubulin antibody showed that the amount of a loaded protein was almost the same among the three samples (Fig. 1B, α-tubulin).

**Purification and Characterization of Lm332 from MKN45 Transfectants**—To examine whether Lm332 could be modified by GnT-III or GnT-V to regulate its functions, we tried to purify Lm332 from the CM of vector-, GnT-III-, and GnT-V-MKN45 transfectants using a laminin α3 chain antibody (2B10) column. Purified Lm332 was run on 4–7.5% gradient gel and 6% gel for CBB staining and immunoblotting, respectively (Fig. 2). CBB staining under nonreducing conditions revealed two major bands at ~460 and 440 kDa, which correspond to the Lm332 forms with an unprocessed 150-kDa γ2 chain and with a processed 105-kDa γ2 chain, respectively (Fig. 2, CBB, left). SDS-PAGE under reducing conditions separated the Lm subunits. All purified Lm332s contained the three major bands of 160-, 150-, and 135-kDa proteins, which correspond to the processed α3 chain, the unprocessed γ2 chain, and the β3 chain, respectively (Fig. 2, CBB, right). In addition to those bands, there are three extra bands, which were larger than the 160-kDa processed α3 chain band. Mass spectrometry analysis showed that those bands are laminin β1, γ1, and an unprocessed α3 chain (190 kDa) (data not shown). A likely explanation is that this represented laminin-311 (Lm311; laminin-6), which is composed of α3, β1, and γ1 chains. Lm332 purified from GnT-III MKN45 transfectant (GnT-III-Lm332) contained slightly increased amounts of those extra bands rather than the Lm332 from vector MKN45 (vector-Lm332) and from GnT-V MKN45 transfectants (GnT-V-Lm332), suggesting that the processing of the α3 chain, but not the β3 and γ2 chains, could be affected by the addition of bisecting GlcNAc. The quantified band intensity upon CBB staining under reducing conditions showed that the ratio of β3 chain in Lm332 to β1 chain in Lm311 was 10:1 (data not shown). Under nonreducing conditions, it was difficult to find the bands corresponding to Lm332 with an unprocessed laminin α3 chain (490 kDa) and Lm311 (600 kDa). The compositions of the Lm332 chain were also confirmed by immunoblotting using each chain-specific antibody. Consistent with the results shown in CBB staining, GnT-III-Lm332 contained slightly more unprocessed α3 chains (190-kDa) than those of vector-Lm332 and GnT-V-Lm332 (Fig. 2, I.B. α3). By contrast, there were no differences between the 135-kDa β3 chain, the unprocessed 150-kDa γ2 chain, and the processed 105-kDa γ2 chain among three Lm332s (Fig. 2, I.B. β3 and γ2).

**FIGURE 4. Analysis of *N*-glycan structures of purified laminin-332s by LC/MS<sup>n</sup>.** Total ion chromatograms (TICs) obtained by single mass scans of *N*-glycans extracted from the gel separated Lm332 subunits (left, α3; middle, β3; right, γ2) of vec-Lm332 (top, vec), GnT-III-Lm332 (middle, GnT-III), and GnT-V-Lm332 (bottom, GnT-V) in positive (A) and negative (B) ion modes. C, extracted ion chromatograms of representative bisected *N*-glycans acquired by single mass scans. The extracted ion chromatogram of α3 subunit is shown at *m/z* 822.3, 915.9, 996.9, 1048.9, 1,142.4, and 1,215.5 (a, vector; b, GnT-III-Lm332; c, GnT-V-Lm332). The extracted ion chromatogram of β3 subunit is shown at *m/z* 1,061.4, 1,142.4, and 1,215.5 (d, vector; e, GnT-III-Lm332; f, GnT-V-Lm332). The extracted ion chromatogram of γ2 subunit is shown at *m/z* 1,142.4 and 996.9 (g, vector; h, GnT-III-Lm332; i, GnT-V-Lm332). The insets show deduced structures (gray triangle, fucose; open circle, galactose; gray circle, mannose; black square, *N*-acetylglucosamine; black diamond, *N*-acetylneuraminic acid). D, integrated mass spectra (*m/z* 1,000–1,700) acquired at elution positions indicated by boldface lines in total ion chromatograms in negative ion mode (Fig. 4B) (left, α3; middle, β3; right, γ2; top, vector; middle, GnT-III-Lm332; bottom, GnT-V-Lm332). The insets show deduced structure (gray triangle, fucose; open circle, galactose; gray circle, mannose; black square, *N*-acetylglucosamine; black diamond, *N*-acetylneuraminic acid).

**GnT-III Down-regulates Activities of Laminin-332**



**FIGURE 5. Cell adhesion activities of purified laminin-332s.** A, cell adhesion activities of vector-Lm332 (crosses), GnT-III-Lm332 (open squares), and GnT-V-Lm332 (closed circles). The 96-well plates were coated with the indicated concentrations of each Lm332. After 1.2% BSA blocking, Lm332-null keratinocytes suspended in supplement-free keratinocyte medium were plated to each well and incubated for 20 min. Each point represents the mean of triplicate assays. Other experimental conditions are described in "Experimental Procedures." B, 2 µg/ml Lm332s were coated to the 96-well ELISA plates at 4 °C overnight. After blocking those wells with 1.2% BSA, coating efficiency of three kinds of Lm332s was estimated by ELISA using anti-laminin  $\gamma 2$  chain antibody. C, inhibitory effects of function blocking antibody against integrin  $\alpha 3$  (anti- $\alpha 3$ ) or control mouse IgG (IgG) on cell adhesion of Lm332-null keratinocytes to different kinds of Lm332s. The numbers in parentheses indicate the concentrations for Lm332 (µg/ml). Each bar represents the mean  $\pm$  S.D. of triplicate assays.

To establish whether Lm332 was modified by GnT-III or GnT-V, lectin blotting using  $E_4$ -PHA and  $L_4$ -PHA lectin was performed against three purified Lm332s. A comparison of bands corresponding to  $\alpha 3$  and  $\beta 3$  chains among three purified Lm332s indicated that increased GnT-III and GnT-V products presented on GnT-III-Lm332 and GnT-V-Lm332, respectively (Fig. 3). Upon  $E_4$ -PHA lectin blotting against GnT-III-Lm332, bisecting GlcNAc was added to  $\beta 1$  and  $\gamma 1$  chains as well as to the unprocessed  $\alpha 3A$  chain (Fig. 3,  $E_4$ -PHA). To ascertain the *N*-glycan structures on Lm332 subunits directly, we also performed liquid chromatography/multiple-stage mass spectrometry (LC/MS<sup>n</sup>) analyses. Each subunit of Lm332 was separated by SDS-PAGE under reducing conditions, and *N*-glycans in each subunit were released by in-gel digestion with peptide:*N*-glycanase F. The oligosaccharides were extracted from the gel and subjected to LC/MS<sup>n</sup> as described under "Experimental Procedures." Total ion chromatograms were acquired by single mass scans (*m/z* 450–2,000) in positive (Fig. 4A) and negative (Fig. 4B) ion modes, respectively. Structures of *N*-glycans in major peaks were deduced from *m/z* values of protonated molecules acquired by Fourier transform ion cyclotron resonance-MS and fragment ions in MS<sup>n</sup> spectra. Additional bisected *N*-glycans were confirmed based on the fragment ions

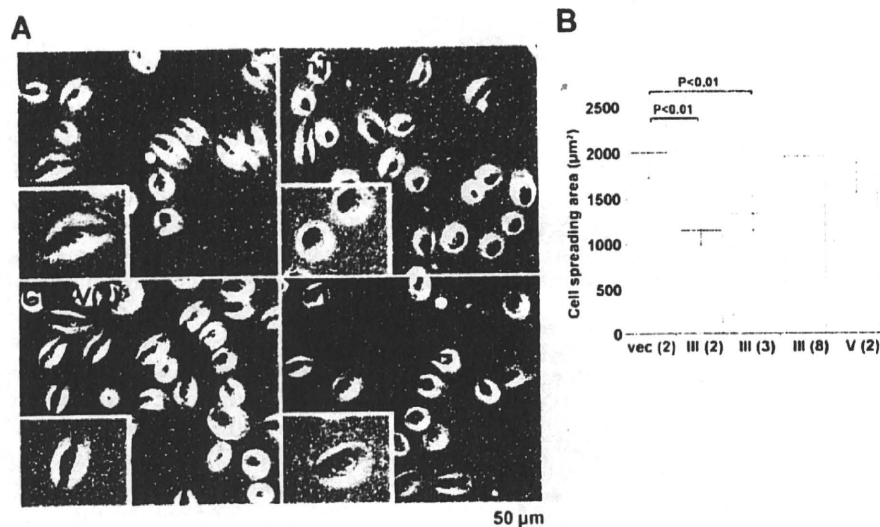
at *m/z* 792 ([HexNAc-Hex-HexNAc-HexNAc-OH + H]<sup>+</sup>) and *m/z* 938 ([HexNAc-Hex-HexNAc-(dHex-)-HexNAc-OH + H]<sup>+</sup>) in MS/MS and MS/MS/MS spectra. The extracted ion chromatograms of representative bisected *N*-glycans were acquired by single mass scans, and the structures in each peak were deduced (Fig. 4C). The peaks of the bisected *N*-glycans from all three subunits of GnT-III-Lm332 (Fig. 4, C, b, e, and h) were more intense than those from both the vec-Lm332 (Fig. 4C, a, d, and g) and GnT-V-Lm332 (Fig. 4C, c, f, and i). We also examined the  $\beta 1,6$ -GlcNAc structures on all subunits of three Lm332s (Fig. 4D). The majority of *N*-glycans in all three subunits of vec-Lm332 (Fig. 4D, a, d, and g) and GnT-V-Lm332 (Fig. 4D, c, f, and i) were sialylated triantennary, whereas few triantennary forms were found in those of GnT-III-Lm332 (Fig. 4D, b, e, and h). These results, taken together, suggest that all subunits of Lm332 were modified by GnT-III and GnT-V, and they also support the notion that introduction of GnT-III inhibits GnT-V products.

**Comparison of GnT-III- and GnT-V-Lm332-induced Cell Adhesion Activities**—The localization of laminin isoform can be defined by the laminin  $\alpha$  subunit, which is expressed in a tissue-specific distribution. For example, the laminin  $\alpha 2$  chain containing laminin and the laminin  $\alpha 4$  chain containing laminin localizes to the skeletal muscle and blood vessels, respectively. On the other hand, Lm332, which includes the laminin  $\alpha 3$  chain, extensively localizes to the skin and stratified squamous tissues *in vivo*. To understand the biological activities of Lm332, we used the keratinocyte as a model cell, since Lm332 is a major component of keratinocyte extracellular matrix (ECM).

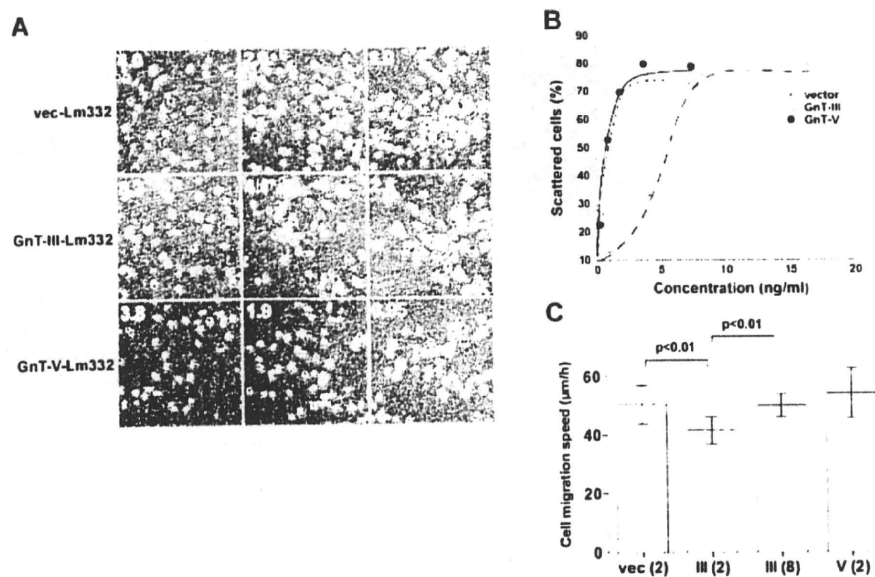
Lm332 promotes keratinocyte or other epithelial cell spreading and adhesion more effectively than other ECM proteins, such as collagen, fibronectin, and Lm111 (31). To examine the effect of GnT-III and GnT-V modification on Lm332 activities, we tried to perform a cell adhesion assay among three Lm332s. Generally, it is difficult to measure the biological functions of other ECMs in Lm332-expressing cells because the endogenous Lm332 strongly affects the experimental results. In order to prevent this, we used purified Lm332 and the Lm332-null keratinocytes established from patients with genetic mutations of the laminin  $\beta 3$  chain. Cell adhesion activity of GnT-V-Lm332 toward Lm332-null keratinocytes was similar to that of vector-Lm332 but was decreased in GnT-III-Lm332 (Fig. 5A). Cells displayed round morphology and formed filopodia around cells



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**FIGURE 6. Effects of *N*-glycosylation on laminin-332-mediated cell spreading.** Lm332-null keratinocytes were plated to the wells, which were precoated with vector-Lm332 (vec), GnT-III-Lm332 (GnT-III), and GnT-V-Lm332 (GnT-V), and then incubated for 1.5 h. *A*, cell morphology of Lm332-null keratinocytes on each substrate. *Inset*, a representative cell morphology. *B*, the cell spreading area was calculated using computer software (AxioVision). The numbers in parentheses are the concentrations for Lm332 ( $\mu\text{g/ml}$ ). Each bar represents the mean  $\pm$  S.D. of triplicate assays.



**FIGURE 7. Cell scattering and migration activities of *N*-glycosylated laminin-332s.** BLR cells were incubated with the indicated concentrations of Lm332s. After 40 h of incubation, cells were fixed and stained with crystal violet for observing cell scattering. *A*, cell morphology of BLR cells in each indicated condition. The numbers on the left are the concentrations for Lm332 (ng/ml) in cell culture medium. The arrowheads indicate the cluster of cells. *B*, scattered cells were counted and indicated as a percentage against total number of the cells. Crosses, vector-Lm332; open squares, GnT-III-Lm332; closed circles, GnT-V-Lm332. *C*, effects of vector-Lm332 (vec), GnT-III-Lm332 (III), and GnT-V-Lm332 (V) on migration of Lm332-null keratinocytes. The migration on each substrate was monitored by time lapse microscopy for 8 h. Each bar represents the mean  $\pm$  S.D. of the migration speeds of nine cells. The numbers in parentheses are the concentrations for Lm332 ( $\mu\text{g/ml}$ ). Other experimental conditions are under "Experimental Procedures."

plated on culture dishes coated with 2  $\mu\text{g/ml}$  GnT-III-Lm332 (Fig. 6A). In contrast, cells cultured on 2  $\mu\text{g/ml}$  vector-Lm332 or GnT-V-Lm332 exhibited well spreading morphology with lamellipodia (Fig. 6A). The effect of 8  $\mu\text{g/ml}$  GnT-III-Lm332 on cell morphology and spreading was similar to those of 2  $\mu\text{g/ml}$  vector-Lm332 and GnT-V-Lm332 (Fig. 6, A and B). To ascer-

tain whether GnT-III modification on Lm332 caused a decrease in its binding to the plates, coating efficiency was checked by ELISA using laminin  $\gamma 2$  chain monoclonal antibody (D4B5). As a result, there were no differences in coating efficiencies among the three Lm332s (Fig. 5B). Lm332 mainly binds integrin  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  as a cell surface receptor (6, 31). To ensure whether *N*-glycosylation affects the integrin binding function of Lm332, an inhibition assay was done using integrin  $\alpha 3$  (P1B5) and  $\alpha 6$  (GoH3) inhibitory antibodies. In all conditions, the anti- $\alpha 3$  antibody treatment completely diminished cell attachment toward the Lm332 substrate (Fig. 5C). Anti- $\alpha 6$  antibody (GoH3) (data not shown) as well as control IgG did not show inhibitory effects on the cell adhesion toward all Lm332 substrates. The possibility still remained that the effects of GnT-III-Lm332 could be due to the small amount of contaminating non-Lm332 subunits. To exclude this possibility, we attempted the cell adhesion assay using immunocaptured Lm332s to the plates with the laminin  $\gamma 2$  antibody (D4B5). A similar result to that in Fig. 5A was obtained in the experiment (data not shown). These results suggest that GnT-III products on Lm332 decreased its cell adhesion and cell spreading activities but had no influence on the integrin usage.

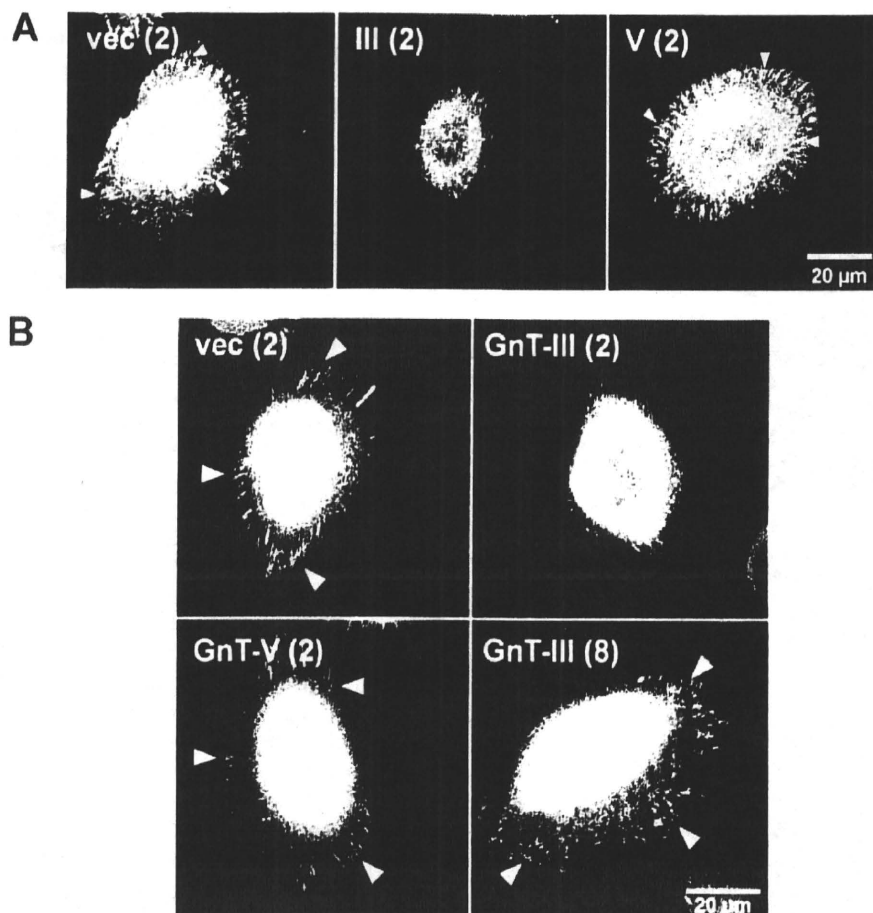
**Effects of GnT-III and GnT-V Modification of Lm332 on Cell Scattering and Migration Activities—**Cell scattering, which represents epithelial cell dissociation, is a well known Lm332 function (10, 11). To test whether GnT-III or GnT-V modification of Lm332 can influence its cell scattering activity, we investigated the scattering morphology using a Buffalo rat liver epithelial cell line (BLR cells), which is sensitive to the scattering activity of Lm332 (10). When the final concentration of vector-Lm332 at 2.0 ng/ml was added to the culture medium of BLR cells, more than 70% of the BLR cells markedly exhibited scattering morphology, as shown in Fig. 7A. GnT-V-Lm332 showed similar scattering activity to vector-Lm332. In contrast, GnT-III-Lm332 at 2.0 ng/ml did not

scattered cells (%)

Concentration (ng/ml)

Cell migration speed ( $\mu\text{m/h}$ )

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**FIGURE 8. Effects of N-glycosylation on laminin-332-mediated  $\alpha 3 \beta 1$  integrin association and focal contact formation.** For visualizing  $\alpha 3$  integrin (A) and paxillin (B), Lm332-null keratinocytes were plated on the indicated Lm332s and then incubated for 1.5 h. After fixation, cells were stained with appropriate primary and secondary antibodies. The numbers in parentheses are the concentrations for Lm332 ( $\mu\text{g/ml}$ ). The arrowheads indicate  $\alpha 3 \beta 1$  integrin clustering (A) and focal contacts (B).

show scattering activity. The concentration for the half-maximal scattering activity ( $\text{ED}_{50}$ ) was  $\sim 0.5$  ng/ml for vector-Lm332 and GnT-V-Lm332 but 5.0 ng/ml for GnT-III-Lm332 (Fig. 7B). We also compared cell migration activity among three Lm332s. When coated at 2  $\mu\text{g/ml}$ , GnT-III-Lm332 showed decreased cell migration activity compared with vec-Lm332 and GnT-V-Lm332. However, when GnT-III-Lm332 was coated at 8  $\mu\text{g/ml}$  for each plate, the activity was recovered to a level similar to those of 2  $\mu\text{g/ml}$  vec-Lm332 and GnT-V-Lm332 (Fig. 7C). These results strongly support the notion that remodeling of N-glycans plays an important biological role.

**Effects of N-Glycosylation on Lm332-mediated  $\alpha 3 \beta 1$  Integrin Association and Focal Complex Formation**—Upon cell binding to ECM proteins, integrins undergo conformational changes, and their cytoplasmic domains interact through linker molecules, such as paxillin with the actin cytoskeleton and with signaling proteins (32). Furthermore, ECM induces integrin clustering, which can amplify various cell signaling from ECM (33). To clarify the reason why GnT-III-Lm332 showed lower activities than either vec-Lm332 or GnT-V-

Lm332, we examined whether the modification of Lm332 by GnT-III or GnT-V affects the  $\alpha 3 \beta 1$  integrin clustering or focal contact formation using fluorescence microscopy. Integrin  $\alpha 3$  clustering (Fig. 8A) and focal contacts (Fig. 8B, paxillin) were distinctly observed in those cells cultured on 2  $\mu\text{g/ml}$  vec-Lm332 as well as on GnT-V-Lm332 but not those cultured on GnT-III-Lm332. When cells were plated on 8  $\mu\text{g/ml}$  GnT-III-Lm332, the focal contacts were clearly visualized by paxillin staining (Fig. 8B). These results suggest that modification of Lm332 by bisecting GlcNAc reduces the efficiency of integrin  $\alpha 3 \beta 1$  clustering, which may in turn suppress focal contact formation.

**DISCUSSION**

In this study, we intensively investigated the effects of N-glycosylation on the biological functions of Lm332. GnT-III-Lm332 and GnT-V-Lm332 were modified with bisecting GlcNAc and  $\beta 1,6$ -branched GlcNAc, respectively. Our further analyses demonstrated that introduction of bisecting GlcNAc to Lm332 decreased its cell spreading, adhesion, and scattering activities as well as its migration activity. Although three kinds of Lm332s showed no significant differences concerning integrin  $\alpha 3 \beta 1$  binding function, GnT-III-Lm332 decreased  $\alpha 3 \beta 1$  integrin clustering and the resultant focal contact formation.

In some cancer tissues, overexpression of GnT-V was observed by immunohistochemistry (20, 34, 35). Deficient GnT-V expression suppressed tumor growth and metastasis in animal models (21). Furthermore, Patridge *et al.* (36) reported that modification of growth factor receptors, such as epidermal growth factor and transforming growth factor- $\beta$  receptors, with N-glycans using poly-N-acetylglucosamine produced by GnT-V caused preferential receptor binding to galectins, resulting in formation of a lattice that opposes constitutive endocytosis. These data suggest that GnT-V affects the cancer by promoting cell motility and changing the stability for growth factor receptors at the cell surface. Ly6 family member C4.4A can bind to Lm332, and its association of galectin-3 influences Lm332 adhesion (37). In addition, galectin-3-dependent oligomerization potentiates NG2-mediated activation of  $\alpha 3 \beta 1$  integrin (38). Recently, we found that galectin-3 could bind to

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Lm332.<sup>3</sup> It is possible that galectin-3 links GnT-V-modified-Lm332 and  $\alpha 3\beta 1$  integrin as well as growth factor receptors via poly-*N*-acetylglucosamine, resulting in the clustering and activation of the Lm332- $\alpha 3\beta 1$  integrin-growth factor receptor complexes as well as causing resistance to their endocytosis. In contrast, modification of GnT-III suppresses further processing with branching enzymes, such as GnT-V, and elongation of *N*-glycans. Our previous study showed that the increase of GnT-V products on integrin  $\alpha 3$  promoted cell migration on Lm332, whereas introduction of bisecting GlcNAc into the integrin  $\alpha 3$  subunit resulted in a decrease of  $\alpha 3\beta 1$  integrin-mediated cancer cell motility by inhibition of  $\beta 1,6$ -branched GlcNAc modification on the  $\alpha 3$  integrin subunit (25). Lm332, which is a specific substrate for  $\alpha 3\beta 1$  integrin, promotes cell motility during wound healing and tumor progression (11, 12). In contrast to integrin  $\alpha 3\beta 1$ , although introduction of bisecting GlcNAc significantly reduced the activities of Lm332, the modification of GnT-V does not affect the activities of Lm332 compared with the activities of vector-Lm332. It could be argued that intrinsic GnT-V-mediated glycosylation is enough for occupation of functional *N*-glycosylation sites of vector-Lm332 as well as of GnT-V-Lm332. As shown in both Fig. 3 and Fig. 4,  $\beta 1,6$ -branched GlcNAc was expressed on vector-Lm332. Actually, we found that the integrin  $\alpha 5$  subunit has fewer important *N*-glycosylation sites for its biological functions and expression on its cell surface, although it has 14 potential *N*-glycosylation sites (39).

Matsui *et al.* (40) showed that loss of *N*-glycosylation was not necessary for Lm332 assembly and secretion. The present study consistently showed that the modification of either GnT-III or GnT-V had no effect on Lm332 assembly and secretion. However, GnT-III-Lm332 was slightly resistant to the proteolytic processing of the laminin  $\alpha 3$  subunit compared with the vector-Lm332 and GnT-V-Lm332. A slight increase in unprocessed laminin  $\alpha 3$  subunits (190-kDa form) might result in the up-regulation of Lm311 formation, since laminin  $\beta 1$  and  $\gamma 1$  preferentially assembled with the unprocessed  $\alpha 3$  chain rather than with the processed  $\alpha 3$  chain (41). These results may also suggest that *N*-glycosylation affects trimeric formation among laminin subunits *in vivo*. In this study, since purified GnT-III-Lm332 contained a very small amount of the unprocessed  $\alpha 3$  chain, the effect of those Lms in purified GnT-III-Lm332 was thought to be negligible. Moreover, the activities of Lm311 and Lm332 with the unprocessed form of the  $\alpha 3$  chain are not so different from those of Lm332 with the processed  $\alpha 3$  subunit (160-kDa form) (16, 41), compared with the differences between GnT-III-Lm332 and either vector-Lm332 or GnT-V-Lm332. Therefore, the decreased activities of GnT-III-Lm332 were mainly caused by the addition of bisecting GlcNAc to Lm332. In previous studies, Lm311 slightly stimulated cell spreading in the presence of a low concentration of Lm332, whereas Lm311 showed no cell spreading activities by itself (41). Although it is not the case for this study, the clarification of the molecular mechanism underlying the association of GnT-III-Lm332 with Lm311 could be a very interesting theme.

<sup>3</sup> Y. Kariya and J. Gu, unpublished data.

Moreover, since syndecan-1, -2, and -4 can bind to the G4-G5 domain in the unprocessed laminin  $\alpha 3$  chain (42, 43), we cannot definitely exclude the effect of syndecan on Lm332 activities, although the vast majority of Lm332 was processed (without the G4-G5 domain), compared with unprocessed Lm332 (with the G4-G5 domain) in this study. A previous study (44) showed that deglycosylation of recombinant Lm332 containing the heterotrimeric, C-terminal part of the coiled-coil domain and G domains did not affect its binding to integrin  $\alpha 3\beta 1$ . However, we found that the addition of bisecting GlcNAc to Lm332 diminished Lm332-mediated  $\alpha 3\beta 1$  integrin clustering and the subsequent cell adhesion, spreading and scattering as well as the migration induced by Lm332. It is reasonable to assume that galectin-3 can link molecules via poly-*N*-acetylglucosamine, because galectin-3 binding to GnT-III-Lm332 is probably much less than its binding to either vector- or GnT-V-Lm332. Details of the molecular mechanism in play here will require further study.

In the basement membranes of the skin and of other tissues, there are many components, such as type IV collagen, nidogens, proteoglycans, agrin, and laminin-511 (Lm511; laminin-10), that contribute to structure and receptor interactions. Since most ECM proteins are glycoproteins, alteration of carbohydrates could change carbohydrate/carbohydrate or carbohydrate/protein interactions in the basement membrane, which presumably affects functional activities of diverse ECM. To understand how carbohydrate modifications affect the skin and other tissues, therefore, the analysis of carbohydrate functions of individual or combined ECM proteins is quite important. This will be further addressed in future studies.

In conclusion, this study reports for the first time that Lm332 can be modified by both GnT-III and GnT-V, resulting in the regulation of cell spreading, adhesion, scattering, and migration activities induced by Lm332. In addition, *N*-glycosylation of Lm332 can influence the proteolytic processing of laminin subunits and  $\alpha 3\beta 1$  integrin clustering. Given the importance of Lm332 and the dynamic changes in *N*-glycosylation during tumor formation and cancer metastasis, *N*-glycan could represent a new therapeutic target.

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