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Evidence for participation of GCS1 in fertilization of the starlet sea anemone *Nematostella vectensis*: Implication of a common mechanism of sperm–egg fusion in plants and animals

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ABSTRACT

It has been reported that GCS1 (Generative Cell Specific 1) is a transmembrane protein that is exclusively expressed in sperm cells and is essential for gamete fusion in flowering plants. The *GCS1* gene is present not only in angiosperms but also in unicellular organisms and animals, implying the occurrence of a common or ancestral mechanism of GCS1-mediated gamete fusion. In order to elucidate the common mechanism, we investigated the role of GCS1 in animal fertilization using a sea anemone (Cnidaria), *Nematostella vectensis*. Although the existence of the *GCS1* gene in *N. vectensis* has been reported, the expression of GCS1 in sperm and the role of GCS1 in fertilization are not known. In this study, we showed that the *GCS1* gene is expressed in the testis and that GCS1 protein exists in sperm by *in situ* hybridization and proteomic analysis, respectively. Then we made four peptide antibodies against the N-terminal extracellular region of NvGCS1. These antibodies specifically reacted to NvGCS1 among sperm proteins on the basis of Western analysis and potently inhibited fertilization in a concentration-dependent manner. These results indicate that sperm GCS1 plays a pivotal role in fertilization, most probably in sperm–egg fusion, in a starlet sea anemone, suggesting a common gamete-fusion mechanism shared by eukaryotic organisms.

1. Introduction

Fertilization, which is an essential process for generating a genetic diversity in the next generation consists of a series of processes, including the process of sperm attraction to an egg in a species-specific manner, the process of sperm binding to and penetration of the egg coat, and the process of sperm–egg membrane fusion. Gamete fusion is an intriguing issue since sperm can exclusively fuse with the egg of the same species but not with sperm or somatic cells. Therefore, plasma membranes of sperm and eggs appear to be highly specialized for sperm–egg membrane fusion.

In mammals, several candidate proteins responsible for gamete fusion have been proposed [1–3]. CD9 is the most promising candidate egg-side factor responsible for gamete fusion since the eggs of *CD9* gene-knockout mice are infertile [4]. However, it was recently revealed that a CD9-containing vesicle, referred to as an exosome, is secreted from the egg to the perivitelline space during fertilization and fuses with the sperm membrane [5]. As a result, egg-derived CD9-containing sperm acquires a potency to fuse with eggs, even with CD9-deficient eggs [5]. These findings suggest that CD9 is indirectly responsible for gamete membrane fusion. In addition to egg-side factors, several sperm-side factors have also been proposed. Among them, sperm Izumo1 is a promising candidate for gamete fusion since the Izumo1 gene has been reported to be essential for mouse gamete fusion [6]. It was also shown that the formation of a helical dimer at the N-terminal region of Izumo1 appears to be required for its function in gamete fusion [7]. Recently, a glycophosphatidylinositol (GPI)-anchored protein, designated Juno, was identified as an Izumo1 receptor expressed in eggs [8]. Since inhibition of Juno causes serious gamete fusion blocking, the Izumo1–Juno binding most likely functions in a critical fertilization process. However, this binding is involved in

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gamete attachment but not in membrane fusion, because cultured somatic cells expressing Izumo1 are not able to fuse with either eggs or Juno-expressing cells [7,8].

Generative Cell Specific1 (GCS1), also referred to as HAPLESS2 (HAP2) [9,10], is a male gamete-specific transmembrane protein that was first identified in the lily (*Lilium longiflorum*) and was found to be a gamete fusion factor [9–11]. Interestingly, this gene is present not only in angiosperms but also in animals such as cnidarians and insects and in unicellular organisms such as *Plasmodium* and *Chlamydomonas* [9,10,12–14]. These findings suggest that GCS1 plays a central or ancestral role in gamete fusion, which is governed by a shared mechanism in eukaryotes [11]. Functional domains of GCS1 were also investigated in *Arabidopsis* by a complementation assay using modified GCS1 constructs in which a GFP cDNA was inserted to disrupt characteristic domains [15]. The results showed that the C-terminal domain of GCS1 is not essential for gamete fusion but that the highly conserved N-terminal region is indispensable for gamete fusion [15], although there is another report showing that the C-terminal region of GCS1 may also participate in gamete fusion [16]. Importance of the N-terminal region and unnecessariness of the C-terminal region were also clarified in *Plasmodium* [15]. These findings indicate that at least the N-terminal extracellular region of GCS1 is required for gamete fusion. The HAP2/GCS1 domain is highly conserved in GCS1-possessing eukaryotes, suggesting the importance of this region in gamete fusion [15].

It would be intriguing to investigate the common mechanism of gamete fusion shared by eukaryotic organisms. In other words, the plant fertilization mechanism might be clarified by studying the animal fertilization mechanism from the angle of GCS1-mediated gamete fusion. Cnidarians are useful animals for fertilization studies, particularly for studies on gamete fusion, since an egg has no proteinaceous egg coat surrounding the egg such as a vitelline coat, which is a formidable barrier against gamete fusion, and no sperm acrosome. Therefore, the first step in fertilization is the event of gamete interaction and fusion in cnidarians. It has been shown that the HAP2/GCS1 gene is present in cnidarians, including *Hydra* and *Nematostella* [12–14]. *Nematostella vectensis* is a useful animal model for fertilization since a genome sequence database is available and also since it is easy to culture and maintain this animal in the laboratory. Although the existence of the GCS1 gene in *N. vectensis* has been suggested as a gene model by the genome database, it is not known whether the GCS1 gene and protein are in fact expressed in the testis and sperm, respectively, and also whether sperm GCS1 protein is involved in fertilization.

In this study, we investigated whether the GCS1 gene is expressed in the testis and whether the protein is present in sperm. We also examined for the first time whether GCS1 is responsible for fertilization using specific antibodies.

2. Materials and methods

2.1. Animal culture and induction of spawning

Adults of a starlet sea anemone, *N. vectensis*, were cultured in 1/3 artificial seawater (ASW) at 18 °C in the dark and were fed five times a week with freshly hatched brine shrimp, *Artemia salina* [17]. Induction of spawning was carried out as described previously [18].

2.2. Fertilization assay

Sperm were preincubated for 10 min in 1/3 ASW with peptide antibodies, Anti-GCS1a, Anti-GCS1b, Anti-GCS1c or Anti-GCS1d IgG, which had been purified by protein A-Sepharose chromatography. Then a small volume of an egg mass (50–100 eggs) coated by the jelly was added to the thus-preincubated sperm suspension (1 ml) in a 24-well multidish that had been coated with 1% BSA. After incubation for 1 h at 25 °C, eggs or zygotes were dispersed from the jelly by treating with 4% cysteine in 1/3 ASW. As control experiments, sperm were preincubated with preimmune IgGs, Pre-GCS1a, Pre-GCS1b, Pre-GCS1c or Pre-GCS1d, and subjected to fertilization experiments as described above. To score the percent of fertilization, the numbers of fertilized eggs (4-cell stage) and unfertilized eggs were counted at 1.5 h post fertilization as described previously [19,20].

2.3. Cloning

Complementary DNA was obtained from the testis of *N. vectensis* by using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies) according to the manufacturer's protocol. NvGCS1 cDNA was amplified using an AccuTaq™ LA DNA polymerase, and cDNAs encoding the N-terminal extracellular region and a signal sequence of NvGCS1 and encoding the C-terminal extracellular region in NvGCS1 were cloned into *Xho* I and *Eco* RI sites in pBlueScript SK$^+$. The full-length ORF was cloned into pCR™2.1-TOPO by using a TOPO-TA cloning kit (Life Technologies). The primers used for amplification of coding sequences were as follows: primer pairs used for amplification of full-length cDNA of NvGCS1 were 5′-ATGGGAAGAGGTCAAATTTATTATG-3′ and 5′-TCATTTTTGTTTAGGTTTGCGC-3′, primer pairs used for amplification of the N-terminal extracellular region in NvGCS1 were 5′-GGCTCGAGATGGGAAGAGGTCAAATTATTA-3′ and 5′-GCGGATCC-TCACCCGGACTCCATGAAGTTA-3′, and primer pairs used for amplification of the C-terminal extracellular region in NvGCS1 were 5′-GGCTCGAGCGATGGCGCATGCGAAAGCCTG-3′ and 5′-GCGGATCC-TCATTTTTGTTTAGGTTTGCG-3′.

2.4. Amino acid sequence alignment and domain predictions

Deduced amino acid sequences of NvGCS1 (*N. vectensis*), HvGCS1 (*Hydra vulgaris*), CrGCS1 (*Chlamydomonas reinhardtii*), and AtGCS1 (*Arabidopsis thaliana*) were searched for by a BLASTP search of NCBI, and the sequences were aligned using Clustal X. Signal sequences, HAP2–GCS1, and transmembrane domains were predicted by SignalP 4.1, BLAST of NCBI, and TMHMM Server v. 2.0, respectively. N-glycosylation sites were predicted by NetNGlyc 1.0.

2.5. In situ hybridization

Probes for *in situ* hybridization were produced by using a DIG RNA Labeling Kit (SP6/T7) (Roche) according to the manufacturer's protocol. PCR products containing T7 and Ap6 promoter sequences were used as templates for producing DIG-labeled probes. The testis was fixed in ASW containing 4% paraformaldehyde for 12 h at 4 °C, followed by washing 5 times with phosphate buffered saline (PBS) to remove residual fixative. After washing twice with PBS containing 0.1% Tween 20 (PBST), the specimen was incubated in PBST containing 10 μg/ml proteinase K for 10 min at room temperature, followed by further incubation for 40 min at 37 °C. After washing with PBST twice, the specimen was fixed again with PBST containing 4% paraformaldehyde for 1 h. Endogenous alkaline phosphatase was inactivated by incubating with 0.1 M triethanolamine containing 1% anhydrous acetic acid for 10 min at room temperature. After washing three times with PBST, the specimen was incubated in the presence of DIG-labeled probes for 2 days at 50 °C with gentle agitation. After hybridization, the specimen was washed with 2× SSC twice and the residual probes were digested with 20 mg/ml RNase A. After successive washing with 2× SSC, 0.5× SSC and PBST at 60 °C, the samples were incubated

in blocking solution at room temperature for 1 h. Following incubation with alkaline phosphatase-conjugated anti-DIG antibody for 1 h, the samples were washed with PBST 3 times at room temperature and the DIG-labeled probe was detected by BCIP/NBP solutions. After staining, the samples were embedded in paraffin, followed by sectioning with a microtome, and each specimen was observed under a microscope.

2.6. LC–MS/MS analysis

Sperm and eggs were suspended in 2× SDS sample buffer and homogenized with a Teflon homogenizer. After incubation for 5 min at 95 °C, the samples were subjected to SDS–PAGE using a 5–20% gradient gel. After electrophoresis, protein bands were stained with Coomassie Brilliant Blue R-250 and sliced into 30 sections. Proteins in each gel piece were reduced and S-alkylated with monoiodoacetamide, digested with trypsin, and subjected to LC–MS/MS analysis as described previously [21,22].

2.7. Preparation of antibodies

Peptide antibodies were produced by Sigma Aldrich Co. Japan. Briefly, four antigenic peptides designated as GCS1a, GCS1b, GCS1c and GCS1d, which correspond to the regions of amino acid residues 151–168, 298–314, 317–334 and 334–351, respectively, were used for immunization of rabbits. IgG was prepared as follows and used for Western blotting and fertilization experiments. Each antiserum or preimmune serum was applied to a protein A-Sepharose column that had been equilibrated with 0.1 M Tris–HCl (pH 7.4) containing 0.15 M NaCl. After washing with equilibration buffer, IgG was eluted with 0.1 M glycine–HCl (pH 3.0) followed by neutralization with 1 M Tris–HCl (pH 8.0) in eluted fractions. IgG concentration was estimated by measuring A280.

2.8. Western blotting

Sperm was suspended in 1 ml extraction solution (1/3 ASW containing 20 mM Tris–HCl (pH 7.4), 1 mM phenylmethanesulphonylfluoride (Sigma), 1× protease inhibitor cocktail (Sigma), and 0.1% Triton X-100) and homogenized with a Teflon homogenizer. The homogenate was centrifuged and the resulting supernatant was mixed with an equal volume of 2× SDS–PAGE sample buffer and then denatured at 95 °C for 5 min. Each sample was subjected to SDS–PAGE using a 5–20% gradient gel (e-PAGEL, ATTO). After electrophoresis, the gel was transferred to a nitrocellulose membrane and subjected to Western blotting using four peptide antibodies (Anti-GCS1a, Anti-GCS1b, Anti-GCS1c and Anti-GCS1d) as primary antibodies. Preimmune antibodies, referred to as Pre-GCS1a, Pre-GCS1b, Pre-GCS1c and Pre-GCS1d, were used as controls. HRP-conjugated anti-rabbit IgG donkey antibody (GE Healthcare) was used as a secondary antibody. Reacted bands were visualized using an ECL kit according to the manufacturer's protocol.

3. Results and discussion

3.1. cDNA cloning of NvGCS1

In order to examine whether GCS1 is expressed in the testis of *N. vectensis* and also to determine the nucleotide sequence, mRNA was prepared from the testis of *N. vectensis* and RT-PCR was carried out as described in Section 2. The nucleotide sequence of the isolated cDNA was identical to the sequence of a gene model of NvGCS1. The NvGCS1 gene is predicted to encode 853 amino acids comprising an N-terminal signal sequence (amino acid residues, 1–22), an N-terminal extracellular region (23–601), two transmembrane domains (602–624 and 682–704), an intracellular region (625–681) and a C-terminal extracellular region (705–853) (Fig. 1A and B). There is a highly conserved domain referred to as the HAP2/GCS1 domain (red box in Fig. 1) in the N-terminal extracellular region of GCS1 in eukaryotic organisms, including *Nematostella*, *Hydra*, *Chlamydomonas*, and *Arabidopsis* (Fig. 1). Since the HAP2/GCS1 domain is highly conserved, this domain is thought to be critical for the function of GCS1 [9,12].

The deduced amino acid sequence showed that NvGCS1 contains two transmembrane domains and that the C-terminal region appears to be exposed to the sperm outer surface. In most organisms, GCS1 possesses a single transmembrane domain near the C-terminus, but it is known that *Chlamydomonas* GCS1 contains two transmembrane domains [9]. Therefore, it is not surprising that *Nematostella* GCS1 contains two transmembrane domains at the C-terminal side.

3.2. Expression of NvGCS1

First, we examined whether NvGCS1 is transcribed in the testis, as previously reported for *Hydra* GCS1, by *in situ* hybridization using two nucleotide probes corresponding to the N-terminal and C-terminal extracellular domains. As a result, positive signals were detected with antisense probes of both domains, whereas neither sense probe showed any signals (Fig. 2). Stronger signals were detected in the seminal duct contents, suggesting that GCS1 is exclusively expressed in sperm.

To confirm this, we investigated whether GCS1 protein is localized in sperm by LC–MS/MS analysis. Sperm preparation was homogenized and subjected to SDS–PAGE. After electrophoresis, the gel was stained, cut into 30 pieces, S-alkylated, digested with trypsin, and subjected to LC–MS/MS analysis (Fig. 3). Although five trypsin-digested fragments of NvGCS1 were identified by the Mascot search engine (Matrix Science), two peptides having ion scores higher than 40 are shown in Fig. 3B as confident data and the identified peptide regions are shown in Fig. 3C. NvGCS1 protein appears to exist as a high-molecular-weight species, since these peptides were identified in gel slices Nos. 2 and 3, the molecular mass of which is estimated to be more than 100 kDa. Since the mature protein of NvGCS1 contains five potential N-glycosylation sites (Fig. 3C), the increase in apparent molecular weight may be explained by a posttranslational modification such as N-linked glycosylation. One potential N-glycosylation site (Asn-529 in NvGCS1) appears to be conserved in cnidarians (*Nematotella* and *Hydra*) (see Fig. 1). Although expression of GCS1 mRNA was reported in *Hydra* [15], it should be emphasized that this is the first report showing the occurrence of GCS1 protein in animal sperm. In addition, it was revealed that the majority of GCS1 protein must exist as a high-molecular-weight species rather than as a 94-kDa simple protein.

3.3. Participation of NvGCS1 in fertilization as revealed by antibodies

Previous studies in which GFP was inserted into various regions of *Arabidopsis* GCS1 showed that the N-terminal domain is essential for gamete fusion but that the C-terminal domain is not necessary for GCS1 function. In the N-terminal extracellular region, the HAP2/GCS1 domain appears to be particularly important for gamete fusion. Therefore, we made rabbit antibodies against four peptides in the N-terminal extracellular region (Fig. 1). Four antigenic peptide regions were selected by considering the easiness in chemical syntheses, high immunogenicity, and no cross-reactivity using a genome database: peptides designated as GCS1a, GCS1b, GCS1c and GCS1d. Two peptides, GCS1a and GCS1b, are located at the N-terminal side of the HAP2/GCS1 domain, and two peptides,

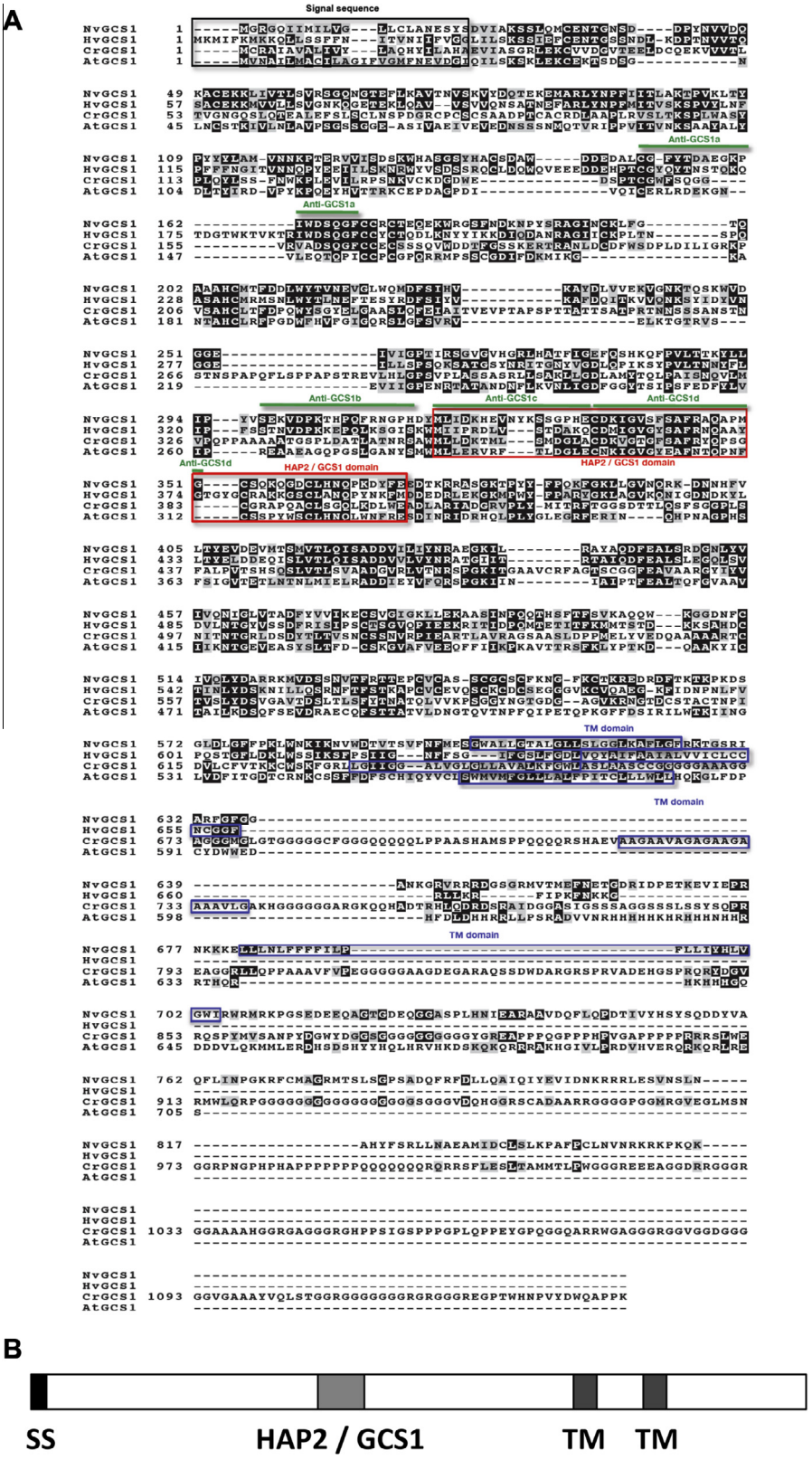

Fig. 1. Deduced amino acid sequence of GCS1 in *Nematostella vectensis*. (A) Deduced amino acid sequences of NvGCSI (accession number, XP_001628495), HvGCS1 (*Hydra vulgaris*; ABN45755), CrGCS1 (*Chlamydomonas reinhardtii*; ABO29824), and AtGCS1 (*Arabidopsis thaliana*; BAE71143) were aligned using Clustal X. Identical and similar residues were highlighted by black and gray, respectively, using BOXSHADE 3.21. NvGCS1 protein consists of 853 amino acids, with an N-terminal signal sequence (SS, amino acid residue numbers, 1–22), N-terminal extracellular region (23–601), two transmembrane domains (602–624 and 682–704), one intracellular region (625–681), and a C-terminal extracellular region (704–853). The HAP2/GCS1 domain, signal sequence, and transmembrane domain are indicated by an open red box, black box, and blue box, respectively. Identical and similar amino acid residues are highlighted by black and gray, respectively. Respective antigenic peptide regions are also indicated. Note that the HAP2/GCS1 domain is conserved in plants, animals, and unicellular organisms. (B) Schematic illustration of NvGCS1. SS, signal sequence; HAP2/GCS1, HAP2/GCS1 domain; TM, transmembrane domain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

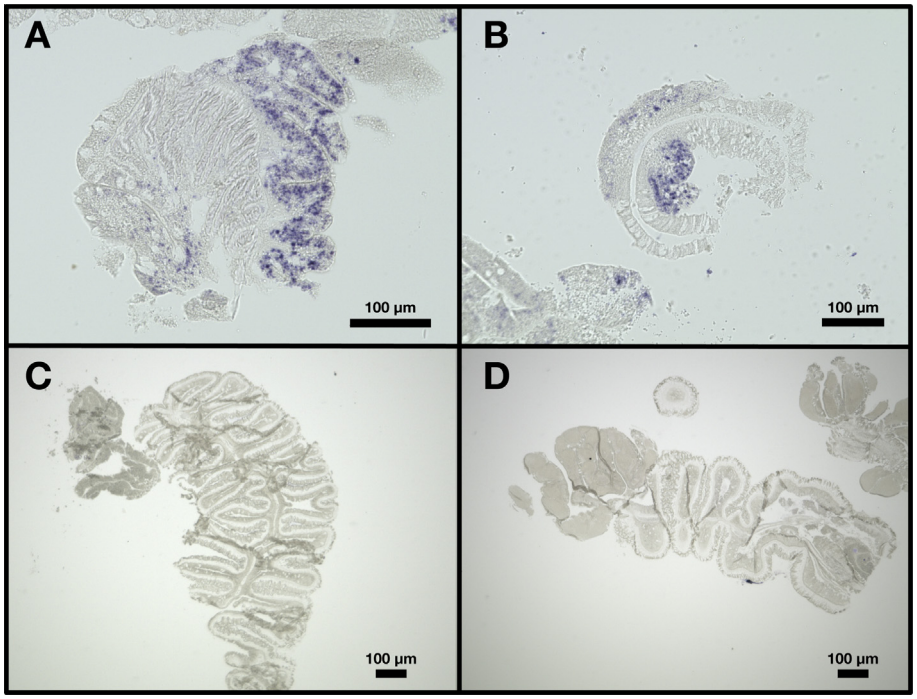

Fig. 2. *In situ* hybridization of NvGCS1. *In situ* hybridization was carried out using antisense (A and B) and sense (C and D) probes for the N-terminal (A and C) and C-terminal (B and D) extracellular regions of NvGCS1. Note that the testes of male adults were stained by antisense probes but not by sense probes.

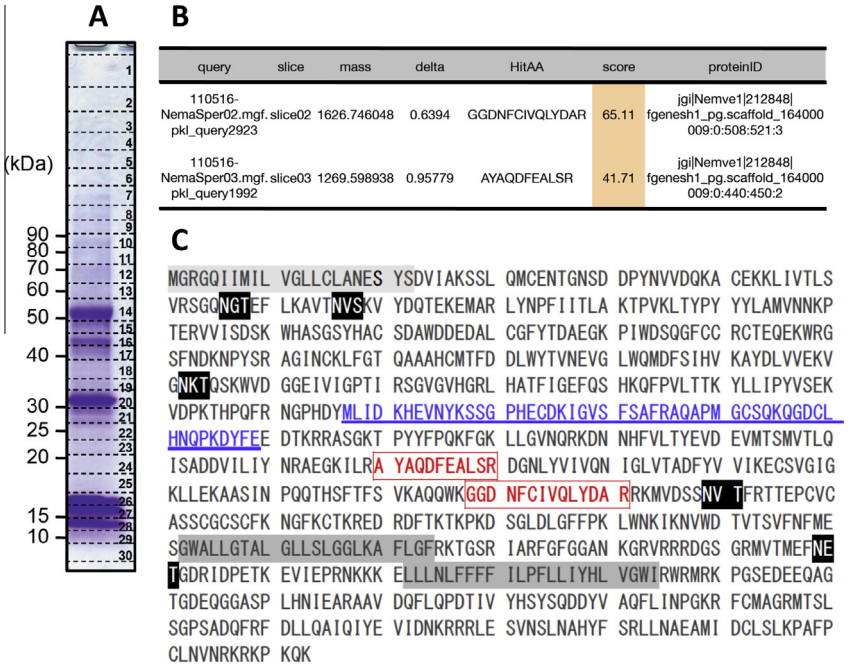

Fig. 3. Presence of GCS1 in sperm as revealed by LC–MS/MS analysis. (A) Sperm of *N. vectensis* was homogenized and subjected to SDS–PAGE using 5–20% gradient gel. After electrophoresis, proteins in a gel were stained with Coomassie Brilliant Blue R-250, and the gel was sliced into 30 pieces. Proteins in each piece were reduced and alkylated with iodoacetamide and then digested with trypsin. The trypsin-digested fragments were subjected to nano-LC, followed by ESI–MS/MS. (B) Two NvGCS1 peptides with an ion score higher than 40 were detected in gel slices Nos. 2 and 3. (C) The trypsin-digested fragments identified by LC–MS/MS are indicated by red box, and the HAP2/GCS1 domain is indicated by blue underline. Five potential N-glycosylation sites, signal sequence, and transmembrane domains are highlighted by black, light gray, and dark gray, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

GCS1c and GCS1d, are located within the HAP2/GCS1 domain. GCS1c and GCS1d were synthesized with the expectation of neutralizing activities toward GCS1 function.

Specificity of anti-GCS1 antibodies was examined by Western blotting. Sperm homogenates were subjected to SDS–PAGE, and the proteins in a gel were transferred to a nitrocellulose membrane, followed by Western blot analysis. The results showed that all of the peptide antibodies reacted to a single protein with a molecular mass of about 110 kDa (Fig. 4), whereas few or no cross-reacted bands were detected among sperm proteins by pre-immune antibodies. These results indicate that the four anti-GCS1 antibodies specifically react to NvGCS1 protein and that

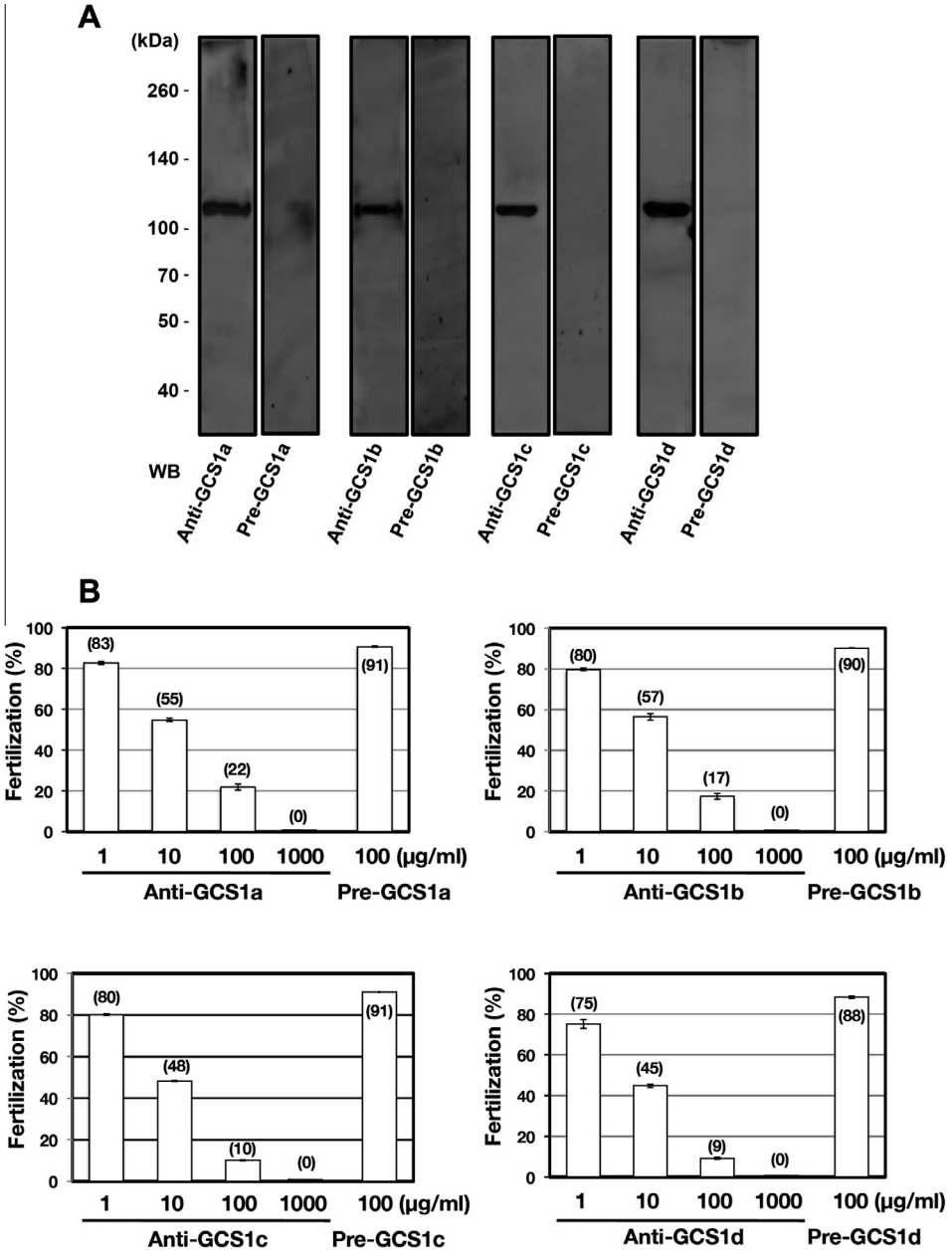

Fig. 4. Participation of GCS1 in fertilization as revealed by antibodies. (A) Sperm was homogenized and subjected to SDS–PAGE and Western blotting. Four peptide antibodies (anti-GCS1a, anti-GCS1b, anti-GCS1c and anti-GCS1d) specifically reacted to a 110-kDa protein, whereas few or no cross-reacted bands were detected by preimmune antibodies. (B) After sperm had been preincubated with anti-GCS1a, anti-GCS1b, anti-GCS1c or anti-GCS1d IgG, a small volume of an egg mass was added to the sperm suspension. Fertilization ratio was determined by counting the numbers of unfertilized eggs and cleaved embryos (4-cell embryos) at 1.5 h after insemination. In each experiment, 50–100 eggs were used, and 6 experiments were carried out. Each value is expressed as the mean ± standard error ($n = 6$). The mean values of fertilization ratios (%) are shown in parentheses. Note that the inhibitory effects of Anti-GCS1c and Anti-GCS1d were significantly stronger than those of Anti-GCS1a and Anti-GCS1b at 100 μg/ml (a versus b, $P = 0.055$; a versus c, $P = 0.00050$; a versus d, $P = 0.00020$; b versus c, $P = 0.0042$; b versus d, $P = 0.0017$; c versus d, $P = 0.064$).

NvGCS1 appears to be modified posttranslationally since the estimated molecular mass of NvGCS1 is significantly larger than the calculated value (94.1 kDa) as previously reported for *Chlamydomonas* GCS1 [23].

Since these antibodies appear to be specific to NvGCS1, we examined the effects of Anti-GCS1 IgG on fertilization. After sperm had been preincubated with anti-GCS1 IgG, an egg mass in a small volume was added to the preincubated sperm suspension. Fertilization ratio was determined by counting unfertilized eggs and cleaved embryos after dissolving the jelly layer, and the results are shown as percent fertilization in Fig. 4B. All anti-GCS1 IgGs inhibited fertilization in a concentration-dependent manner, whereas control IgG showed no inhibitory effect at 100 μg/ml. Anti-GCS1a, Anti-GCS1b, Anti-GCS1c and Anti-GCS1d IgGs reduced the fertilization ratio to 22% (21.9 ± 1.5 (SE)), 17% (17.3 ± 1.4), 10% (10.3 ± 0.23) and 9% (9.16 ± 0.47), respectively. Inhibitory effects of Anti-GCS1c and Anti-GCS1d were significantly stronger than those of Anti-GCS1a and Anti-GCS1b at 100 μg/ml (Fig. 4B). By the *t*-test, there was a statistically significant difference ($P < 0.01$) in the inhibitory abilities at 100 μg/ml between the antibodies against the HAP2/GCS1 domain (Anti-GCS1c and Anti-GCS1d) and the antibodies against other regions (Anti-GCS1a and Anti-GCS1b), whereas no significant difference ($P > 0.05$) was found between the inhibitory abilities of Anti-GCS1a and Anti-GCS1b or between

those of Anti-GCS1c and Anti-GCS1d. Fertilization was completely blocked by these antibodies at 200 μg/ml (0% fertilization; data not shown in Fig. 4B) and 1000 μg/ml (0% fertilization). These results coincide well with previous results showing that the HAP2/GCS1 domain, which is composed of antigenic peptide regions of GCS1c and GCS1d, is highly conserved among eukaryotes and appears to play a key role in gamete fusion.

In *N. vectensis*, it is not necessary to consider the possibility that these antibodies impaired the process of sperm acrosome reaction or sperm binding to a proteinaceous egg coat such as the vitelline coat. Although we cannot exclude the possibility that these antibodies can affect sperm motility or other sperm functions, it seems most plausible that the anti-GCS1 antibodies tested in this study inhibited sperm–egg membrane fusion during fertilization. In this context, it should be emphasized that this is the first report showing that GCS1 is responsible for gamete fusion also in the animal kingdom.

Our results strongly support the idea that an ancestral and common mechanism of gamete fusion shared by plants, animals and even unicellular organisms plays a pivotal role in sperm–egg membrane fusion. Elucidation of the binding partner and the precise role in membrane fusion or other fertilization processes of GCS1 are intriguing and important issues that remain to be solved.

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