Sex might have first evolved in the common ancestor of all eukaryotes ~2 billion years ago, because sex and sex-related genes are widespread within the eukaryotes (1). However, little is known about sex in the ancient common eukaryotic ancestor. Isogamy is primitive sexual reproduction involving gametes of identical size and motility. Oogamy appears to have evolved from isogamy in nearly all lineages of multicellular organisms (2), and it manifests as small motile gametes (sperm) in males and large immotile gametes (eggs) in females (3). Therefore, sexual dimorphism in male and female gametes and sex-specific gamete interaction factors likely result from parallel and multiple evolutions. However, recent studies of GENERATIVE CELL SPECIFIC 1/HAPLESS 2 (GCS1/HAP2) suggest that a common mechanism for gamete fusion exists across various eukaryotic lineages (4). GCS1/HAP2 is a gamete-specific transmembrane protein that is essential for fertilization and is widely conserved in diverse eukaryotic lineages, indicating that GCS1/HAP2 was present in the common ancestor of eukaryotes (4). Although GCS1/HAP2 genes are present in the nuclear genome of both sexes, exclusive and/or functional GCS1/HAP2 expression generally resides in the male or male homologue in flowering plants, green algae, malaria parasites, and metazoan species (5–8). Since various separate lineages of sexually reproducing eukaryotes lack GCS1/HAP2 in their genomes, this gene seems to have been lost, and alternative male gamete fusogens, such as the immunoglobulin superfamily protein IZUMO1 in mammals (9), have evolved to replace the function of GCS1/HAP2 in these organisms (4).

Functional studies of GCS1/HAP2 have been based on oogamous organisms, in which fundamental differences in molecular mechanisms of GCS1/HAP2 between the sexes appear apparent due to the differences in size and motility between egg and sperm (5,8), or on the unicellular volvocine alga Chlamydomonas reinhardtii, which produces morphologically distinct mating-type plus and minus isogametes (7). Interestingly, GCS1/HAP2 expression in Chlamydomonas reinhardtii is not exclusive to minus gametes; slight upregulation of GCS1/HAP2 is also detected in plus gametes after gamete differentiation (5), although the plus GCS1/HAP2 expression is not functional (7). However, details of sex-specific regulation of this gene remain unresolved.

To explore the dynamics of GCS1, we focused on the morphologically indistinguishable plus and minus isogametes in the colonial volvocine alga Gonium pectorale. The isogamous system in G. pectorale offers a model system for focusing on the mating-type specificity of GCS1 (Fig. 1). In C. reinhardtii, plus gametes bear an ~3-μm-long tubular mating structure (TMS) or fertilization tubule filled with actin filaments, whereas the mating structure of minus gametes is dome-shaped without actin accumulation (10–13). GCS1/HAP2 in C. reinhardtii is localized at the minus mating structure and mediates membrane fusion with plus gametes (7). In contrast, plus and minus gametes in G. pectorale are morphologically identical, bearing TMS (bilateral TMS) (14,15) (Fig.1A). We recently established a novel experimental system for examining the activated, TMS-bearing G. pectorale gametes induced in each sex (15) (Fig.1B). Moreover, in volvocine algae, mating-type minus in isogamous species is homologous to male sex in anisogamous/oogamous species based on the presence of the sex-determining minus dominance (MID) genes in male genomes (16–20) (Fig.1A). Therefore, the simple isogamous system

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**Sex-Specific Posttranslational Regulation of the Gamete Fusogen GCS1 in the Isogamous Volvocine Alga Gonium pectorale**

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Male and female, generally defined based on differences in gamete size and motility, likely have multiple independent origins, appearing to have evolved from isogamous organisms in various eukaryotic lineages. Recent studies of the gamete fusogen GCS1/HAP2 indicate that this protein is deeply conserved across eukaryotes, and its exclusive and/or functional expression generally resides in males in male homologues. However, little is known regarding the conserved or primitive molecular traits of males and females within eukaryotes. Here, using morphologically indistinguishable isogametes of the colonial volvocine Gonium pectorale, we demonstrated that GCS1 is differently regulated between the sexes. G. pectorale GCS1 molecules in one sex (homologous to male) are transported from the gamete cytoplasm to the protruded fusion site, whereas those of the other sex (females) are quickly degraded within the cytoplasm upon gamete activation. This molecular trait difference might be conserved across various eukaryotic lineages and may represent male and female prototypes originating from a common eukaryotic ancestor.
in *G. pectorale* enables us to dissect fundamental differences in GCS1 behavior between the sexes in association with the male-female dichotomy.

In this study, we identified a full-length *G. pectorale* GCS1 (*GpGCS1*) coding sequence from our ongoing nuclear genome project. Using an antibody against the *GpGCS1* protein, we precisely analyzed the expression and localization pattern of the protein and discovered that sex-specific, stepwise regulation localizes *GpGCS1* at the surface of the TMS in a *minus*-specific manner. These regulatory mechanisms are considered to be the biological basis for the sex-specific action of GCS1/HAP2 at the cellular level and may be conserved among various eukaryotic lineages.

**MATERIALS AND METHODS**

**Gonium strains, culture conditions, and gamete induction.** Strains K41 (mating-type *plus*) and K34 (mating-type *minus*) of *Gonium pectorale* [15] were used for the analyses of the *GpGCS1* gene expression and the experiments with the anti-*GpGCS1* antibody. For identification of the *GpGCS1* gene, another *minus* strain (K3–F3–4) [21] was used. In order to strengthen the sex-dependent expression of *GpGCS1*, we also examined

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**FIG 1 Illustration of the advantages of *Gonium pectorale* for use in elucidating the mating-type specification of GCS1/HAP2.** (A) Simplified diagram of phylogenetic relationships of selected genera in the volvocine lineage. The stepwise transition from isogamy to anisogamy/oogamy is considered to be associated with the evolution of multicellularity [31–33]. The *minus*- and male-specific presence of the *minus* dominance (*MID*) genes in isogamous (*Chlamydomonas reinhardtii* [16] and *G. pectorale* [18]), anisogamous (*Eudorina* sp. [20] and *Pleodorina starri* [17]), and oogamous (*Volvox carteri* [19]) species are also indicated. (B) Sexual reproduction and separately induced gamete activation in *G. pectorale*. Vegetative colonies of *plus* and *minus* strains differentiate as gametes by long-term cultivation and mixing of the sexes, inducing gamete activation that leads cell walls to shed and tubular mating structures (TMSs) to emerge. The activated gametes fuse to form a zygote that undergoes meiosis to produce genetically diverse progenies. Alternatively, gamete activation can be induced without mixing of the sexes by treatment with db-cAMP and IBMX [15]. (C to E) Differential interference contrast image of a 16-celled vegetative colony of a *plus* strain (C) and phase-contrast images of separately activated gametes of *plus* (D) and *minus* (E) strains of *G. pectorale*. TMSs (filled arrowheads) emerge in activated gametes. Scale bars, 10 μm.
four wild strains: two parental strains of K41 and K34, Kaneoko4 (matting-type plus) and Kaneoko3 (matting-type minus) (15), and two other strains of G. pectorale (originating from the same soil sample as Kaneoko4 and Kaneoko3 [22]), Kaneoko8 (matting-type plus) and Kaneoko2 (matting-type minus). Culture conditions were described previously (21), and continuous aeration was supplied. Vegetative cells were harvested from 3-day-old cultures. To obtain gametes, 10 to 15 ml of a 3- to 4-week-old culture of each strain (~6 × 10^5 cells) was concentrated to 1.0 to 1.5 ml by centrifugation, transferred to a 3.5-cm petri dish, and cultured at 25°C for 3 to 5 h. For gamete activation, cells were treated for 1 to 3 h with 15 mM db-cAMP ( dibutyryl cyclic AMP) and 1 mM IBMX (3-isobutyl-1-methyloxanthine) as described previously (15).

Identification of the GpGCS1 gene. We performed a BLAST search against the preliminary genome assembly database of a G. pectorale minus strain (K3–F3–4), with Chlamydomonas reinhardtii GCS1/HAP2 (accession number EF397563) as the query, and retrieved several sequences with significant homology; we based the design of GpGCS1-specific primers on these sequences. To identify the cDNA sequence of GpGCS1, polyadenylated mRNAs from minus (K3–F3–4) gametes were isolated using Dynabeads oligo(dT) 25 (Life Technologies, Carlsbad, CA), reverse transcribed with Superscript III reverse transcriptase (Life Technologies), and amplified with KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan) and the GpGCS1-specific primers. We performed rapid amplification of 3’ cDNA ends (3’ RACE) using the GeneRacer kit (Life Technologies). The PCR products were directly sequenced using an ABI PRISM 3100 Genetic Analyzer (Life Technologies) using a BigDye Terminator cycle sequencing ready reaction kit, v. 3.1 (Life Technologies). To determine the genomic sequence, a bacterial artificial chromosome (BAC) clone covering the GpGCS1 locus from G. pectorale Kaneoko3 (clone 016G17; available from Clemson University Genome Institute [CUGI], Clemson, SC) was subjected to shotgun sequencing using ABI 3730xl capillary DNA sequencers (Life Technologies). The cDNA sequence from the K3–F3–4 gametes was confirmed to be completely identical to the exon sequence obtained from the BAC clone sequencing.

Amino acid sequence alignment and domain predictions. The alignment of deduced amino acid sequences of G. pectorale and C. reinhardtii GCS1/HAP2 was constructed using MUSCLE (http://www.ebi.ac.uk /Tools/muscle/) and shaded by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Identity between GpGCS1 and CrGCS1/HAP2 was calculated from pairwise distances computed by MEGAS (23). All positions containing gaps and missing data were eliminated. Signal sequences, HAP2-GCS1, and transmembrane glycosylation sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), respectively. Putative N-glycosylation sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) with a potential score of >0.5.

Phylogenetic analysis of GCS1/HAP2. For GCS1/HAP2 phylogeny, deduced amino acid sequences of GpGCS1 and GCS1 homologs of Volvox carteri (XP_002952384.1 [hypothetical protein VOLCODRAFT_10570] and XP_002952883.1 [hypothetical protein VOLCODRAFT_105707]) and Coccomyxa subellipsoidea (EIE26501.1 [hypothetical protein COCSUDRAFT_39583]) were aligned with the data matrix of reference genes were aligned with the data matrix of reference

Antibody production. The 5’ region of the GpGCS1 cDNA sequence (1,738 bp) was amplified using high-fidelity KOD-Plus DNA polymerase (Toyobo) and GpGCS1-specific primers (5’ - ATAGCAAGCCCCGCAAGC GACACTC-3’ and 5’ - CGGCGTCGTACAGCCTACGCAAC-3’) and then was cloned into pCR Blunt-TOPO vector (Life Technologies). The Sp6-SpI fragment (corresponding to amino acids 28 to 356) of the insert was cloned into the expression vector pQE-82L (Qiagen, Hilden, Germany) and transformed into Escherichia coli Rosetta competent cells (Merck Millipore, Billerica, MA) to prepare recombinant partial GpGCS1 protein. The recombinant proteins were extracted from bacterial cells using an xTractor buffer kit (Clontech, Mountain View, CA) and purified using a HisTrap HP column with a His buffer kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer’s instructions. The purified protein was subjected to SDS-PAGE, and gel slices containing the ~30-kDa recombinant protein were injected into a rabbit to obtain polyclonal antisera (TK Craft, Gunma, Japan). From the antisera, IgG was purified using a MAbTrap kit (GE Healthcare) and used as an anti-GpGCS1 antibody.

Immuno blot analysis. Cells from each condition were harvested by centrifugation, resuspended in SDS sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 100 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 1% protease inhibitor cocktail [P9599; Sigma-Aldrich, St. Louis, MO], Complete protease inhibitor cocktail tablets [Roche Diagnostics, Mannheim, Germany], and 0.02% bromophenol blue) and incubated at 70°C for 10 min. After centrifugation, the supernatants were loaded and separated on a 7.5% Mini-PROTEAN TGX precast gel (Bio-Rad) and blotted onto a Hybond-P membrane (GE Healthcare). The blot was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) at 4°C and incubated overnight with the anti-GpGCS1 antibody (1:5,000 dilution) at 4°C. The blots were then incubated with an anti-IgG antibody conjugated to horseradish peroxidase (1:100,000 dilution; Jackson ImmunoResearch, West Grove, PA), and protein bands were detected with Amersham ECL prime Western blotting detection reagent (GE Healthcare). For Coomassie brilliant blue (CBB) staining, SDS-PAGE gels were stained with EzStain AQua (ATTO, Tokyo, Japan). Images were recorded using a ChemiDoc XRS system with Quantity One software and level adjusted with Adobe Photoshop CS6.

Light microscopy. Light microscopy was performed using a BX61 microscope (Olympus, Tokyo, Japan) equipped with differential interference contrast optics and a BX60 microscope (Olympus) equipped with phase-contrast optics. The images were level adjusted with Adobe Photoshop CS6.

Indirect immunofluorescence analysis. Immunostaining was performed as described previously (27), with modifications. In brief, gametes were attached to coverslips precoated with 0.1% polyethyleneimine, fixed with 3.7% formaldehyde-containing solution, and extracted by detergent to remove chlorophyll autofluorescence. After washing and blocking steps, the cells were incubated overnight with the GpGCS1 antibody (1:1,000 dilution) and/or an anti-actin antibody (1:2,000 dilution; clone C4; MP Biomedicals, Morgan, CA) at 4°C. The cells were again washed, blocked, and stained with Alexa Fluor 488 goat anti-rabbit and/or 568 goat anti-mouse IgG (1:1,000 dilution each; Life Technologies) for 1 to 3 h at room temperature.
temperature. The cells were then washed and mounted with ProLong gold or SlowFade gold antifade reagents (Life Technologies). For activated gametes without cell walls, the chlorophyll extraction step and all detergents in the solutions were omitted because they tended to cause bursting of the cells. For unactivated gametes, images were captured using an LSM780 confocal microscope (Carl Zeiss, Oberkochen, Germany) with an oil-immersion lens (magnification, 1000x; numeric aperture, 1.40) and ZEN 2011 software (Carl Zeiss). For activated gametes, images were captured using a BX60 microscope with a UPlanFL N objective (magnification, 100x; objective, 1.3; Olympus). Adobe Photoshop CS6 software was used for level adjustment and preparation of merged images.

Accession numbers. The cDNA and genomic sequence of GpGCS1 have been deposited in the DDBJ database under accession numbers AB915401 and AB915402, respectively. The amino acid alignment of GCS1/HAP2 proteins has been deposited in TreeBASE under accession number S15345 (http://www.treebase.org/treebase-web/home.html).

RESULTS

Identification and characterization of GpGCS1. We performed a BLAST search against the G. pectorale genome assembly with C. reinhardtii GCS1/HAP2 (CrGCS1/HAP2) as a query and found one locus with significant similarity. Based on RT-PCR, genomic PCR, and 3’ RACE analysis, we identified a full-length coding sequence (3,408 bp) and exon-intron structure of the G. pectorale GCS1 (GpGCS1) gene. The GpGCS1 gene comprises 13 exons, and three polyadenylation sites are found in the 3’-untranslated region (Fig. 2A). The GC content of the GpGCS1 coding sequence was high (72.6%), similar to that of CrGCS1/HAP2 (69.4%). Alignment of deduced amino acid sequences of GCS1/HAP2 from G. pectorale (upper) and C. reinhardtii (lower). Identical and similar residues are shaded in black and gray, respectively. Signal sequences, HAP2-GCS1, and transmembrane domains are indicated by boxes. Asterisks within the HAP2-GCS1 domain represent invariant cysteine residues conserved among the GCS1/HAP2 homologs. Open triangles indicate putative N-glycosylation sites. The region corresponding to the antigen used to generate anti-GpGCS1 antibody is also marked.

FIG 2 Identification of the GCS1 ortholog in Gonium pectorale. (A) Structure of the GpGCS1 gene. Solid and open boxes represent coding and noncoding exon sequences, respectively. Solid lines indicate introns. Polyadenylation sites and the positions of primers used for RT-PCR analysis are also indicated. (B) Alignment of deduced amino acid sequences of GCS1/HAP2 from G. pectorale (upper) and C. reinhardtii (lower). Identical and similar residues are shaded in black and gray, respectively. Signal sequences, HAP2-GCS1, and transmembrane domains are indicated by boxes. Asterisks within the HAP2-GCS1 domain represent invariant cysteine residues conserved among the GCS1/HAP2 homologs. Open triangles indicate putative N-glycosylation sites. The region corresponding to the antigen used to generate anti-GpGCS1 antibody is also marked.
Liu et al. (28) reported that CrGCS1/HAP2 contains nine putative N-glycosylation sites and showed that the protein is actually N-glycosylated. We compared locations of putative N-glycosylation sites between *C. reinhardtii* and *G. pectorale* GCS1/HAP2 and revealed that five of eight potential N-glycosylation sites in GpGCS1 were conserved in CrGCS1/HAP2. All of these sites were N-terminal to the transmembrane domain, suggesting that sugar chains of volvocine GCS1/HAP2 are exposed to extracellular environments. Additionally, the GCS1/HAP2 proteins from these volvocine algae possessed longer sequences (1,136 amino acids in *G. pectorale* and 1,139 amino acids in *C. reinhardtii*) than land plants and malaria parasites (e.g., 705 and 828 amino acids in *Arabidopsis thaliana* and *Plasmodium berghei*, respectively) because of the long, single-amino-acid repeat-containing sequences that were C-terminal to the transmembrane domain (Fig. 2B). For phylogenetic analysis, two loci found in the *Volvox carteri* genome database, version 2.0 (http://www.phytozome.net/volvox), with significant similarity to CrGCS1/HAP2 (locus names Vocar20011213m.g [V. carteri] and Vocar2000015.m.g [V. carteri 2]) (Fig. 3) were included. The phylogenetic analysis showed that the GCS1/HAP2 proteins of volvocine algae (*C. reinhardtii*, *G. pectorale*, and *V. carteri*) form a robust monophyletic group (Fig. 3). These results indicated that the structural features of the GCS1/HAP2 proteins are also conserved in GpGCS1 and that the volvocine GCS1 proteins function by a common mechanism to mediate gamete membrane fusion. Two amoebozoans, *Dictyostelium discoideum* and *Physarum polycephalum*, were unexpectedly positioned in separate lineages in the GCS1/HAP2 tree (Fig. 3). This may represent different patterns of gene evolution in these species. Some of the other relationships between major groups of eukaryotes do not seem to be well supported, possibly due to the limited information of the amino acid sequences used for the phylogenetic analysis.

**Gamete-specific expression of GpGCS1.** We performed semiquantitative RT-PCR analysis using the GpGCS1-specific primers (indicated in Fig. 2A) to determine the expression pattern of the GpGCS1 transcripts. No obvious GpGCS1 expression was detected in vegetative cells of either sex (Fig. 4; also see Fig. S1 in the supplemental material). After gamete induction by long-term cultivation (Fig. 1B), GpGCS1 expression was upregulated in both sexes, although the upregulation was more pronounced in *minus* than in *plus* gametes (Fig. 4; also see Fig. S1) and decreased after mixing of the gametes (Fig. 4), as was also seen in *CrGCS1/HAP2* (5). Interestingly, the expression was enhanced in *minus* gametes after activation of the gametes by treatment with db-cAMP and IBMX (Fig. 1B), while GpGCS1 expression almost disappeared in *plus* gametes (Fig. 4). Similar enhanced expression was also recognized in *GpMID* of *minus* gametes, as reported previously (15) (Fig. 4).

**minus-enhanced expression and plus-specific degradation of GpGCS1.** To analyze GpGCS1 expression at the protein level, we prepared an antibody against the N-terminal region of GpGCS1 (Fig. 2B). By immunoblot analysis using the anti-GpGCS1 antibody, the GpGCS1 signal was detected as a major band at ~140 kDa in *plus* and *minus* gametes, whereas no such signal was observed in vegetative cells (Fig. 5A). The quantity of the protein was substantially greater in *minus* than in *plus* gametes, consistent with the results of RT-PCR (Fig. 4). We next examined GpGCS1 dynamics upon gamete activation. In *plus* gametes, the quantity of GpGCS1 protein decreased rapidly and almost disappeared after incubation with db-cAMP and IBMX for 1 h, whereas it remained intact in *minus* gametes (Fig. 5B). This result suggests that gamete activation not only represses GpGCS1 transcription but also degrades the GpGCS1 protein in a plus-specific manner.

**minus-specific TMS localization of GpGCS1.** We next performed indirect immunofluorescence analysis using the same antibody to investigate the subcellular localization of GpGCS1. In
the unactivated minus gamete, a strong GpGCS1 signal was observed at the anterior end of the cell (Fig. 6A, arrowheads in images on the left), which is the primordial site for TMS formation in G. pectorale (15). Many small punctate signals of GpGCS1 were also observed in the cytoplasm immediately beneath the anterior end, indicating that GpGCS1 is transported to the primordial TMS site by a significant number of membrane vesicles. In the unactivated plus gamete, however, no signal was detected in the anterior end, while punctate signals were observed in the cytoplasm surrounding the nucleus (Fig. 6A, right), suggesting that GpGCS1 in plus gametes was not transported to the primordial TMS but instead remained internal. When the gametes were activated, the GpGCS1 signal was observed along the TMS in minus but not in plus gametes, while accumulation of actin in the TMS was observed in both gametes (15) (Fig. 6B). No obvious GpGCS1 signal was observed in the activated plus gamete, which is consistent with the plus-specific loss of the GpGCS1 protein revealed by the immunoblot analysis (Fig. 5B). In the activated minus gamete, the GpGCS1 signal was observed along the periphery of the actin filaments and was most concentrated at the tip of the TMS (Fig. 6B, left), suggesting that GpGCS1 is localized to the plasma membrane of the minus TMS, especially the TMS tip where the minus TMS first attaches to the plus TMS and membrane fusion appears to be initiated (14).

**DISCUSSION**

In this study, we identified the GCS1 ortholog from the colonial volvocine alga G. pectorale, generated antibody against the GpGCS1 protein, and used this antibody to explore fundamental differences in GCS1 dynamics between two opposite sexes. In C. reinhardtii, a unicellular relative of G. pectorale, upregulation of GCS1/HAP2 gene expression by gamete induction was observed in both sexes (5), but sex-based differences in GCS1 behavior at the protein level were not confirmed. Liu et al. (7, 28) reported that the hemagglutinin (HA)-tagged version of CrHAP2/GCS1 in the transgenic CrGCS1/HAP2-HA minus strain (hap2 mutant with introduced HAP2-HA) is expressed exclusively in gametes as prevention against polygamy, information regarding the GCS1/HAP2 protein in plus gametes prior to fusion has been lacking. In our immunoblotting experiments with GpGCS1 in G. pectorale, only one band was observed to be comparable in size to the lower form of CrGCS1/HAP2-HA (Fig. 5). Given that the putative N-glycosylation sites of GpGCS1 and CrGCS1/HAP2 are not completely conserved (Fig. 2B), the difference in the migration patterns observed between G. pectorale and C. reinhardtii GCS1/HAP2 may be derived from different glycosylation patterns between the species. Our comparison of the GCS1/HAP2 sequence in G. pectorale and C. reinhardtii (Fig. 2B) will also provide useful information for identifying actual N-glycosylation sites, their functions, and other functional domains of GCS1 for future study.

By combining the results of our expression and localization analyses, we propose a stepwise regulation model for minus-specific GpGCS1 localization on the TMS (Fig. 7, steps 1 to 3). Transcription of GpGCS1 is inhibited during the vegetative stage in

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**FIG 4** Semiquantitative RT-PCR of GpGCS1 expression in strains K41 (mating-type plus) and K34 (mating-type minus). Polyadenylated mRNAs from vegetative cells, gametes, and activated gametes of plus and minus strains were isolated and subjected to semiquantitative RT-PCR analysis. Expression levels were also examined at the indicated times after mixing plus and minus gametes. GpMID expression was examined to confirm gamete induction in the minus strain. The loading volume for each lane was normalized to the quantity of EF-like gene (internal control) product.

**FIG 5** Immunoblot analysis of GpGCS1 expression in strains K41 (mating-type plus) and K34 (mating-type minus). (A) Gamete-specific, minus-enhanced expression of GpGCS1. Total protein from each sample was subjected to SDS-PAGE, blotted, and probed with anti-GpGCS1 antibody. CBB staining of a duplicate gel shows equal protein loading in each lane. (B) plus-specific degradation of GpGCS1 in activated gametes. plus or minus gametes from the same culture were harvested before and after incubation with db-cAMP and IBMX for the indicated times and subjected to immunoblot analysis as described for panel A.

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both sexes. After culturing for several weeks to induce starvation, the cells differentiate into gametes and GpGCS1 transcription is switched on (Fig. 4). GpGCS1 transcription is specifically upregulated in the minus gametes, resulting in enhanced accumulation of GpGCS1 protein in these gametes (step 1) (Fig. 5A). However, GpGCS1 protein is also expressed at a substantial level in plus gametes (Fig. 5A). After translation, a possible transportation mechanism present only in minus gametes enables GpGCS1 to accumulate at the primordial sites for the TMS, whereas GpGCS1 remains within the cytoplasmic region in plus gametes (step 2) (Fig. 6A). After activation of the plus and minus gametes, intracellular cAMP rapidly increases to trigger cell wall loss and formation of the TMS in both gametes (15). GpGCS1 at the primordial TMS site in minus gametes is transported to the plasma membrane of the active TMS (Fig. 6B). In contrast, GpGCS1 distributed in the cytoplasmic region of plus gametes rapidly degrades upon gamete activation (step 3) (Fig. 5B). These regulatory mechanisms are likely to enable GpGCS1 to be specifically localized at the minus TMS and to function as a minus-specific fusogen in this organism.

Our immunostaining clearly showed that GpGCS1 was distributed differently in unactivated gametes, in the internal cytoplasmic region in plus gametes, and in the anterior primordial TMS site in minus gametes (Fig. 6A). This distributional difference between the sexes presumably serves as a cellular basis for the minus-specific TMS localization and could be determined by the presence or absence of the MID gene, the master regulator for sex determination (16, 18). Since the MID protein is thought to be a transcription factor (16), MID may regulate the expression of the component(s) for (i) membrane trafficking machinery that delivers GCS1 to the primordial TMS or (ii) posttranslational modification (such as N-glycosylation) for distinguishing plus and minus GCS1 molecules to achieve the selective transport of this molecule.

We also showed that the gamete activation signal induced selective degradation of GpGCS1 in plus gametes (Fig. 5B). This selective degradation may be induced by plus-specific signaling, while the MID gene negatively regulates this signaling in minus gametes. Another potential explanation is that the gamete activation signal initiates the protein degradation machinery in the cytoplasm of both plus and minus gametes, but that rapid translocation of GpGCS1 to the fusion site occurs in minus gametes, separating the protein from the degradation machinery and allowing it to persist and function in these gametes. The degradation of GpGCS1 may involve proteolytic mechanisms, such as autophagy and proteasome-dependent pathways. Further cell biological and biochemical analyses will be necessary to assess these possibilities.

In flowering plants, GCS1/HAP2 was detected as a punctate structure in sperm cells and redistributed to the cell surface in response to egg cell-secreted EGG CELL 1 protein (29). In G.
pectorale, punctuated GpGCS1 signals are also observed in the cytoplasm of unactivated gametes of both sexes (Fig. 6A), but the elevation of intracellular cAMP (15) may trigger transport of GCS1 to the fusion site only in minus gametes. Such a transportation mechanism for GCS1 might be conserved in males or male homologues across various eukaryotic lineages (5–8), originating from a possible isogamous common ancestor (1, 4). Our findings provide novel molecular insights into the prototypes of male and female prior to evolution of gamete size and motility, although more information from other isogamous organisms is still needed. The opposite sexes in the common ancestor of eukaryotes might have differed from one another in molecular traits of the fusogen GCS1, as resolved in the present study (Fig. 7). Furthermore, multiple or independent evolution of oogamy from isogamy within the eukaryotes (2) might have been based on such sex-dependent differences in GCS1.

The anisogamous/oogamous colonial volvocine algae may have evolved from an ancestral isogamous colonial algae with a bilateral TMS (30, 31) (Fig. 1A). The present molecular biological results for G. pectorale provide a promising platform for investigating how GCS1 dynamics evolved in each sex in association with the isogamy-oogamy transition. Further analyses using the anisogamous and oogamous volvocine algae will help to answer this interesting question of evolutionary biology.

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