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RESEARCH ARTICLE

The cytoplasmic domain of the gamete membrane fusion protein HAP2 targets the protein to the fusion site in *Chlamydomonas* and regulates the fusion reaction

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ABSTRACT

Cell-cell fusion between gametes is a defining step during development of eukaryotes, yet we know little about the cellular and molecular mechanisms of the gamete membrane fusion reaction. HAP2 is the sole gamete-specific protein in any system that is broadly conserved and shown by gene disruption to be essential for gamete fusion. The wide evolutionary distribution of HAP2 (also known as GCS1) indicates it was present in the last eukaryotic common ancestor and, therefore, dissecting its molecular properties should provide new insights into fundamental features of fertilization. HAP2 acts at a step after membrane adhesion, presumably directly in the merger of the lipid bilayers. Here, we use the unicellular alga *Chlamydomonas* to characterize contributions of key regions of HAP2 to protein location and function. We report that mutation of three strongly conserved residues in the ectodomain has no effect on targeting or fusion, although short deletions that include those residues block surface expression and fusion. Furthermore, HAP2 lacking a 237-residue segment of the cytoplasmic region is expressed at the cell surface, but fails to localize at the apical membrane patch specialized for fusion and fails to rescue fusion. Finally, we provide evidence that the ancient HAP2 contained a juxta-membrane, multi-cysteine motif in its cytoplasmic region, and that mutation of a cysteine dyad in this motif preserves protein localization, but substantially impairs HAP2 fusion activity. Thus, the ectodomain of HAP2 is essential for its surface expression, and the cytoplasmic region targets HAP2 to the site of fusion and regulates the fusion reaction.

KEY WORDS: HAP2, GCS1, Gamete membrane fusion reaction, Cell-cell fusion, *Chlamydomonas*

INTRODUCTION

Although gamete fusion during fertilization is a fundamental event in eukaryotes and has been long studied in many model organisms, we know little about the molecules or the cellular and molecular mechanisms that underlie the membrane fusion reaction (Evans, 2012; Inoue et al., 2013). The protein Izumo is the only sperm-specific gene shown by gene disruption to be essential for gamete fusion in a vertebrate (Inoue et al., 2005, 2013; Ellerman et al., 2009; Evans, 2012). And recent work has shown that Izumo and its newly discovered receptor Juno on the oocyte are likely required for membrane adhesion, but not for the subsequent merger of the lipid bilayers (Bianchi et al., 2014). Several *C. elegans* genes are known to be essential for fertilization, but whether they function directly in the membrane fusion reaction is unknown because of the difficulty of experimentally defining distinct steps in gamete interactions (Singson et al., 2008). To date, the sole, broadly conserved protein shown by gene disruption to be essential for the gamete membrane fusion reaction in any system, including all model organisms, is the membrane protein HAP2 (also called GCS1), which is present in plants and protists, and in some multicellular animals (but notably absent in vertebrates) (Mori et al., 2006; von Besser et al., 2006; Liu et al., 2008, 2010; Hirai et al., 2008; Goodman and McFadden, 2008; Cole et al., 2014; Steele and Dana, 2009; Ebchuqin et al., 2014; Kawai-Toyooka et al., 2014).

Early reports on HAP2 in *Arabidopsis* concluded that it was required for gamete attachment (Mori et al., 2006), but by exploiting the ease of experimentally identifying and quantifying the location and properties of HAP2 at individual steps in gamete fusion in the green alga *Chlamydomonas* and the malaria pathogen *Plasmodium* (Sinden, 1983), we showed that HAP2 is required late in the membrane fusion reaction, after species-specific membrane adhesion (Liu et al., 2008; Wong and Johnson, 2010). *Chlamydomonas hap2 minus* gamete mutants undergo tight membrane adhesion with wild-type *plus* gametes at the sites specialized for gamete fusion, but fusion is abrogated (Fig. 1). In addition to defining the step in gamete fusion that requires HAP2, these studies were the first to establish in any system that membrane adhesion and membrane merger per se during the gamete membrane fusion reaction are carried out by distinct membrane proteins. The adhesion receptor on the *Chlamydomonas minus* mating structure is still unknown, but *plus* gametes use the species-limited FUS1 protein for membrane adhesion (Misamore et al., 2003; reviewed by Snell and Goodenough, 2009). *Plasmodium* male gametes use the P48/45 protein for membrane adhesion (van Dijk et al., 2001). Thus, adhesion depends on species-limited proteins, whereas merger uses broadly conserved HAP2. This new concept in fertilization has since been confirmed in *Arabidopsis* and *Tetrahymena* (Sprunck et al., 2012; Cole et al., 2014; Mori et al., 2014; Dresselhaus and Snell, 2014).

The genetically demonstrated conservation of a role for HAP2 at the membrane merger step of fertilization in *Chlamydomonas*, *Plasmodium*, *Tetrahymena* and *Arabidopsis* suggests that the last eukaryotic common ancestor of protists and higher plants likely used HAP2 for gamete fusion. Furthermore, the properties and broad conservation of HAP2 in choanoflagellates and other protists, and in sponges, cnidarians and some bilaterian animals (including *Drosophila*) further indicates that understanding the molecular properties of HAP2 could offer new insights into fundamental mechanisms of gamete fusion.

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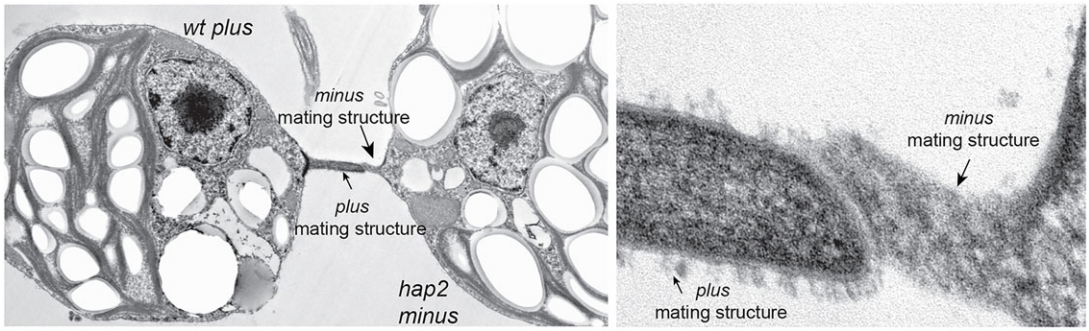

Fig. 1. *hap2* mutant phenotype. (Left) Transmission EM of a *minus hap2* gamete (cell on right) whose activated mating structure is binding to the activated mating structure of a wild-type *plus* gamete. (Right) A higher magnification view of the interaction between the two mating structures.

Work in *Arabidopsis* and *Plasmodium* has begun such a molecular dissection (Wong et al., 2010; Mori et al., 2010). A truncated form of *Plasmodium berghei* HAP2 composed of just the ectodomain and the transmembrane domain (TMD) rescues ookinete (zygote) formation in the *P. berghei hap2* mutant (Mori et al., 2010). By contrast, truncated HAP2 containing the ectodomain plus TMD in *Arabidopsis* failed to support seed formation (used as an indicator of gamete fusion), indicating that the cytoplasmic region was required at some step either in the delivery of HAP2 to the site of fusion or during fusion per se (Wong et al., 2010). Conflicting results have been reported about the parts of the cytoplasmic domain of the *Arabidopsis* protein that are required for seed formation. One group has shown that histidine-rich regions of the cytoplasmic domain are important, and another group reported that a form of HAP2 containing the ectodomain, the TMD, and a 34-residue cytoplasmic segment adjacent to the TMD followed by GFP was sufficient to rescue seed formation (Wong et al., 2010; Mori et al., 2010).

The difficulty of experimentally accessing gametes and of determining the cellular location of the protein continues to be a major challenge with dissecting the properties of HAP2 domains in many model organisms. Indeed, only recently was it discovered that *Arabidopsis* HAP2 is absent from the surface of sperm before it encounters the egg, and present solely in intracellular vesicles. The protein is redistributed to the sperm surface only after signals generated by sperm-egg interactions trigger exocytosis of HAP2-containing storage vesicles (Mori et al., 2006, 2010; Liu et al., 2008; Sprunck et al., 2012; Snell, 2013). Thus, the previously described mutated/truncated forms of *Arabidopsis* HAP2 might have failed to rescue fusion in the *hap2* mutant, not because the mutated forms failed to support fusion per se, but because they failed to be trafficked to the appropriate storage vesicles, because they failed to be redistributed appropriately to the cell surface during sperm signaling or because they failed to localize to the site of fusion during interactions with the egg.

In *Chlamydomonas*, HAP2 is constitutively present on the cell surface at a specialized, apically localized membrane patch, the mating structure (Fig. 1), the surface area of which represents less than 0.1% of the surface area of the cell (Liu et al., 2008). The ease of determining HAP2 surface expression, localization and fusion competence in this organism overcomes the limitations of such studies in other systems, because it is straightforward to distinguish mutant forms that fail to localize properly from those that localize properly, but fail to support fusion. Here, we have tested the function of HAP2 domains in protein localization and gamete fusion in *Chlamydomonas*. We find HAP2 without its ectodomain fails even to be expressed at the cell surface and that a segment in the cytoplasmic domain is required to target surface-expressed HAP2 to the fusogenic site. Furthermore, we determined that the presence of multiple cysteines just below the TMD is an ancient feature of the protein, and that disruption of a cysteine dyad in the *Chlamydomonas* multi-cysteine motif has no effect on protein localization, but strongly impairs HAP2 fusion capacity. Thus, the HAP2 ectodomain in *Chlamydomonas* is not an autonomous functional unit in the membrane fusion reaction. Rather, the fusion function of HAP2 is regulated by its cytoplasmic domain, which also contains motifs that target the protein to the site of fusion.

RESULTS

The predicted extracellular region (~600 residues) of HAP2 family members contains 18 widely conserved cysteines, which are present in two regions, C-rich 1 near the N terminus and C-rich 2 near the transmembrane domain (TMD), separated by a less well-conserved region whose length varies across organisms (Liu et al., 2008; Fig. 2A). Several other amino acid residues also are broadly conserved. Thus, the entire extracellular region of HAP2 represents a conserved cysteine rich domain, which includes a previously described, 50-residue conserved region termed the HAP2/GCS1 (H/G) domain (Pfam10699). Compared with the ectodomain, the predicted cytoplasmic region shows lower overall conservation. We uncovered one major exception, however, which is the presence of a multi-cysteine motif near the TMD in HAP2 homologs in members of the major evolutionary groups across kingdoms, including the Excavata, Rhodophyta, Chlorophyta, Viridiplantae, Alveolata, Ameobozoa and Opisthokonta (Fig. 2B). Thus, the ancient HAP2 possessed motifs in both the ectodomain and the cytoplasmic domain that remain in present-day organisms.

The HAP2 ectodomain, including the conserved H/G domain, influences expression at the cell surface

We examined the role of the ectodomain in HAP2 function and properties by expressing in *hap2* cells a transgene, *HAP2-Δ76-627*, whose expression was driven by the endogenous promoter and encoded a truncated protein containing only the signal peptide and the adjacent 55 residue segment, followed by the TMD and the cytoplasmic domain (with a 3× HA tag inserted after residue 702) (Δecto; Fig. 3A). As we showed previously, the wild-type, HA-tagged form of HAP2, which rescues fusion in the *hap2* mutant, is expressed as two forms, and only the upper form is expressed on the cell surface, as evidenced by its trypsin sensitivity in live gametes (Liu et al., 2008; Fig. 3B). *HAP2-Δ76-627* gametes expressed only low

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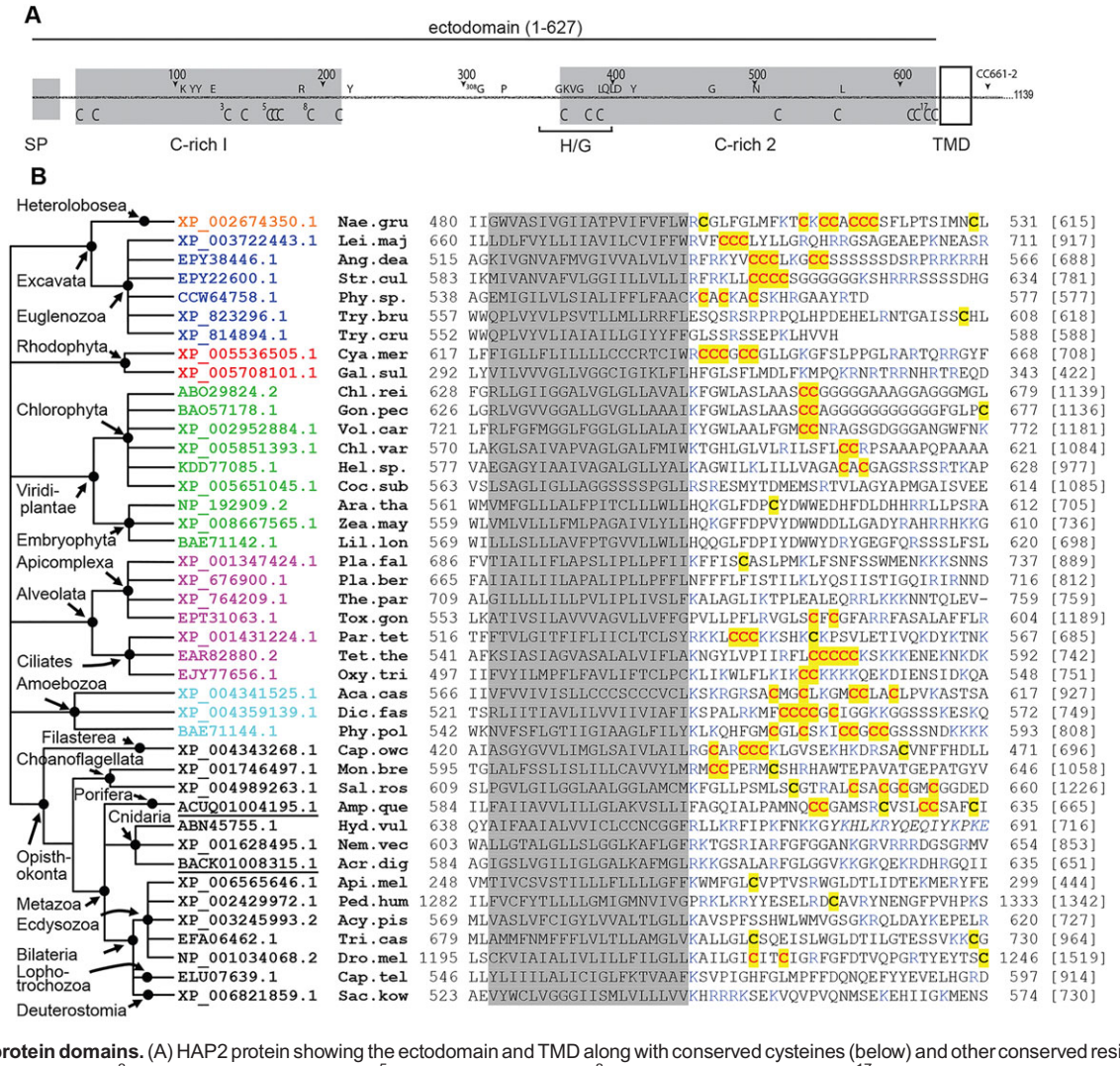

Fig. 2. HAP2 protein domains. (A) HAP2 protein showing the ectodomain and TMD along with conserved cysteines (below) and other conserved residues (above). ^{308}G is less well conserved; ^{3}C, not present in *Plasmdium*; ^{5}C, not present in plants; ^{8}C, not present in metazoans; ^{17}C, not present in lower metazoans (see alignment file in Liu et al., 2008). The conserved cysteines in the ectodomain, which are in two subregions (C-rich I and C-rich 2), are separated by gaps of varying sizes in different species. The much smaller HAP2-GCS1 (H/G) domain (Pfam 10699) is also shown. (B) Taxonomic categories of HAP2 proteins with predicted transmembrane regions and their C-terminal segments. Each sequence is denoted by its NCBI accession number followed by its species name abbreviation. For the two proteins without NCBI protein records, their accession numbers (underscored) refer to the genome contig DNA sequences. Predicted transmembrane regions have a grey background. Up to 30 residues are shown after the predicted transmembrane regions, with cysteines highlighted with a yellow background and with positively charged residues (lysine and arginine) in blue letters. Cysteines in motifs 'CC', 'CxC' and 'CxxC' are in red letters. Starting and ending residue numbers are shown before and after each sequence, respectively, and the sequence length is shown in brackets. Sequence accession numbers are colored according to their major taxonomic categories as follows: Heterolobosea, orange; Euglenozoa, blue; Rhodophyta (red algae), red; Viridiplantae (green plants), green; Alveolata, magenta; Amoebozoa, cyan; Opisthokonta, black. Species name abbreviations are as follows: Aca.cas, *Acanthamoeba castellanii*; Acr.dig, *Acropora digitifera*; Acy.pis, *Acyrthosiphon pisum*; Amp.que, *Amphimedon queenslandica*; Ang.dea, *Angomonas deanei*; Api.mel, *Apis mellifera*; Ara.tha, *Arabidopsis thaliana*; Cap.tel, *Capitella teleta*; Cap.owc, *Capsaspora owczarzaki*; Chl.rei, *Chlamydomonas reinhardtii*; Chl.var, *Chlorella variabilis*; Coc.sub, *Coccomyxa subellipsoidea*; Cya.mer, *Cyanidioschyzon merolae*; Dic.fas, *Dictyostelium fasciculatum*; Dro.mel, *Drosophila melanogaster*; Gal.sul, *Galdieria sulphuraria*; Gon.pec, *Gonium pectorale*; Hel.sp., *Helicosporidium sp.*; Hyd.vul, *Hydra vulgaris*; Lei.maj, *Leishmania major*; Lil.lon, *Lilium longiflorum*; Mon.bre, *Monosiga brevicollis*; Nae.gru, *Naegleria gruberi*; Nem.vec, *Nematostella vectensis*; Oxy.tri, *Oxytricha trifallax*; Par.tet, *Paramecium tetraurelia*; Ped.hum, *Pediculus humanus*; Phy.pol, *Physarum polycephalum*; Phy.sp., *Phytomonas sp.*; Pla.ber, *Plasmodium berghei*; Pla.fal, *Plasmodium falciparum*; Sac.kow, *Saccoglossus kowalevskii*; Sal.ros, *Salpingoeca rosetta*; Str.cul, *Strigomonas culicis*; Tet.the, *Tetrahymena thermophila*; The.par, *Theileria parva*; Tox.gon, *Toxoplasma gondii*; Tri.cas, *Tribolium castaneum*; Try.bru, *Trypanosoma brucei*; Try.cru, *Trypanosoma cruzi*; Vol.car, *Volvox carteri*; Zea.may, *Zea mays*.

levels of HAP2-Δ76-627 (which appeared as a single band), as shown by immunoblotting (Fig. 3B), and they failed to fuse when mixed with wild-type *plus* gametes. We were unable to detect the protein on the mating structures, whereas a strong signal was seen at the mating structures in cells expressing the wild-type, full-length HAP2-HA (Fig. 3E). The HAP2-Δ76-627 on live cells was resistant to trypsin treatment (Fig. 3B), indicating that the ectodomain is required for HAP2 trafficking to the cell surface.

To examine the function of the conserved H/G domain, we generated HAP2 forms with mutations/deletions in several parts of the domain (Fig. 3C). A chimeric HAP2 (PbCr) made by replacing the *Chlamydomonas* H/G domain with the corresponding *Plasmodium bergheii* domain failed to be expressed at the cell surface or at the mating structure and, thus, failed to rescue fusion (Fig. 3C-E). Similarly, a form of HAP2 in which the conserved DKVG in the H/G domain was deleted (ΔDKVG) was not expressed at the cell surface or mating structure, and hence also failed to rescue

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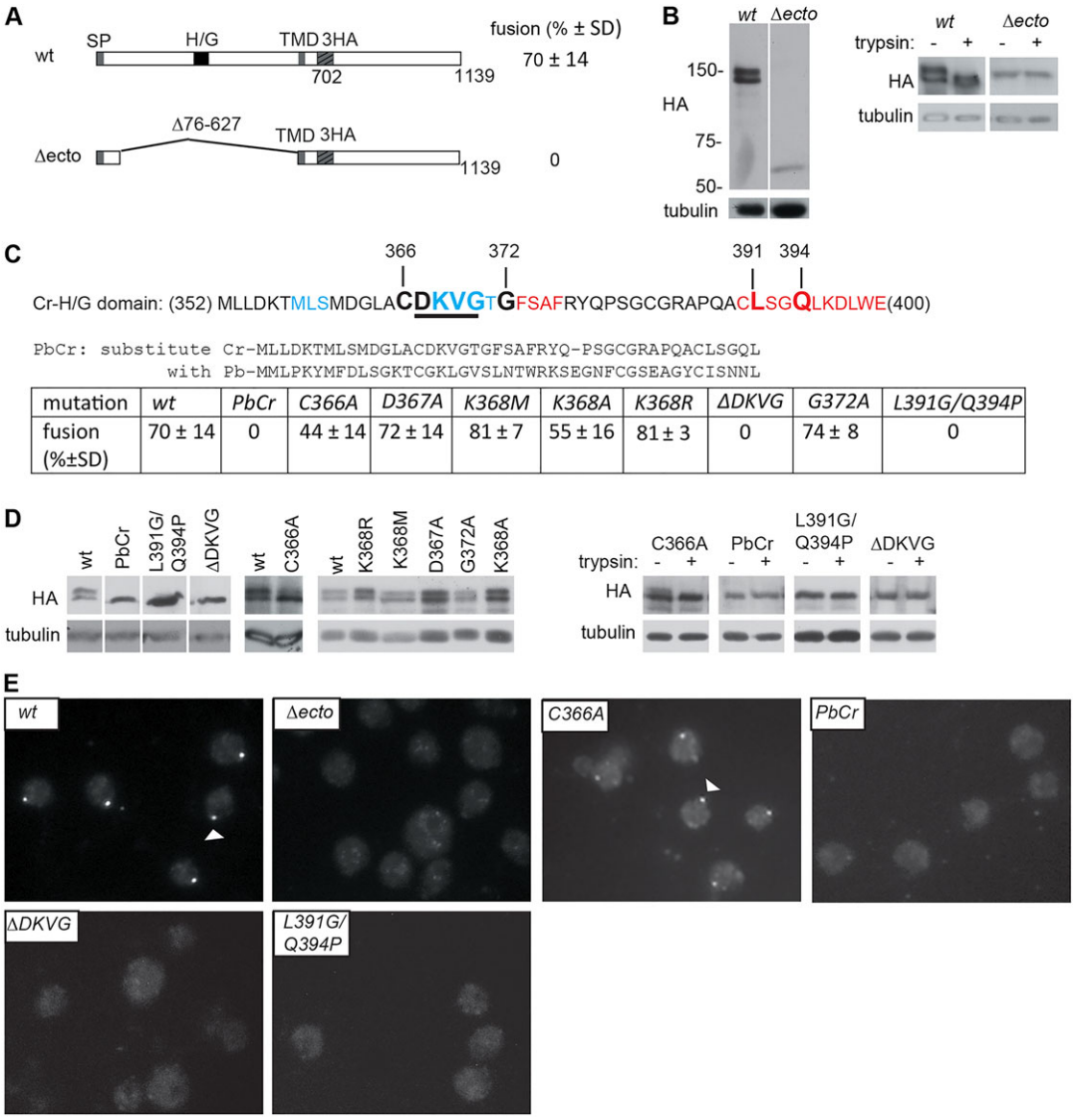

mutation	wt	PbCr	C366A	D367A	K368M	K368A	K368R	ΔDKVG	G372A	L391G/Q394P
fusion (%±SD)	70 ± 14	0	44 ± 14	72 ± 14	81 ± 7	55 ± 16	81 ± 3	0	74 ± 8	0

Fig. 3. The ectodomain including the conserved H/G domain influences HAP2 expression at the cell surface. (A) Diagrams and fusion abilities of wild-type and HAP2-Δecto proteins (see Materials and methods for a description of methods used to determine the percentage of fusion). (B) Left panels, immunoblots of wild-type and *HAP2-Δecto* gametes. Right panels, immunoblots assessing trypsin sensitivity of wild-type and *HAP2-Δecto* gametes. Unlike the upper form of wild-type HAP2, HAP2-Δecto is insensitive to trypsin. The lower panels are loading controls showing tubulin. (C) Modifications of the H/G domain of *Chlamydomonas* HAP2 (Cr-H/G domain) and corresponding fusion abilities. The modified residues in the H/G domain (352-400) are highlighted with larger font or underlined. Blue, predicted β-sheet; red, predicted α-helix. (D) Left panels, immunoblots of gametes with HAP2 modified in the H/G domain. Right panels, immunoblots assessing trypsin sensitivity. Tubulin is shown as a loading control. (E) Anti-HA immunofluorescence images of the indicated gametes. Arrowheads indicate mating structures stained in wild-type *HAP2-HA* gametes and *HAP2-C366A* gametes.

fusion (Fig. 3C-E). Furthermore, HAP2-L391G/Q394P, in which the predicted helical region C390 to E400 would be disrupted (Fig. 3C), also failed to be expressed at the surface or the mating structure and failed to rescue fusion (Fig. 3D,E). To our surprise, HAP2 forms containing mutations of highly conserved residues, including D367A, K368A, K368R, K368M and G372A were properly localized and nearly fully functional (Fig. 3C-D). Notably, the mutation C366A, which would be predicted to disrupt putative disulfide linkages, reduced expression, but the protein retained nearly half the fusion capacity of wild-type HAP2 (Fig. 3C-E). In summary, these data indicate that mutation of several highly conserved residues in the H/G domain failed to disrupt HAP2 localization or function, whereas deletions or mutations predicted to disrupt structure prevented HAP2 expression at the cell surface, and hence blocked rescue of gamete fusion.

The ectodomain and TMD are sufficient for trafficking HAP2 to the cell surface

We tested for roles of the cytoplasmic domain and the TMD in HAP2 properties by use of cells expressing truncated proteins containing the HAP2 signal peptide and just the ectodomain (HAP2-ecto) or the ectodomain and the TMD (HAP2-ecto+TMD) (both followed by a C-terminal HA tag). As shown in Fig. 4A,B, in the cells expressing HAP2-ecto, a single form of HAP2 was detected. It failed to be expressed at the cell surface, as shown by lack of trypsin sensitivity, it was not localized at the mating structure, and it failed to rescue fusion (Fig. 4A-E). We anticipated that because of its missing TMD and cytoplasmic region, it might be secreted, but we were unable to detect HAP2-ecto in the culture medium, even after 3 h of gamete activation brought about by mixing with wild-type *plus* gametes (Liu et al., 2010; Fig. 4D).

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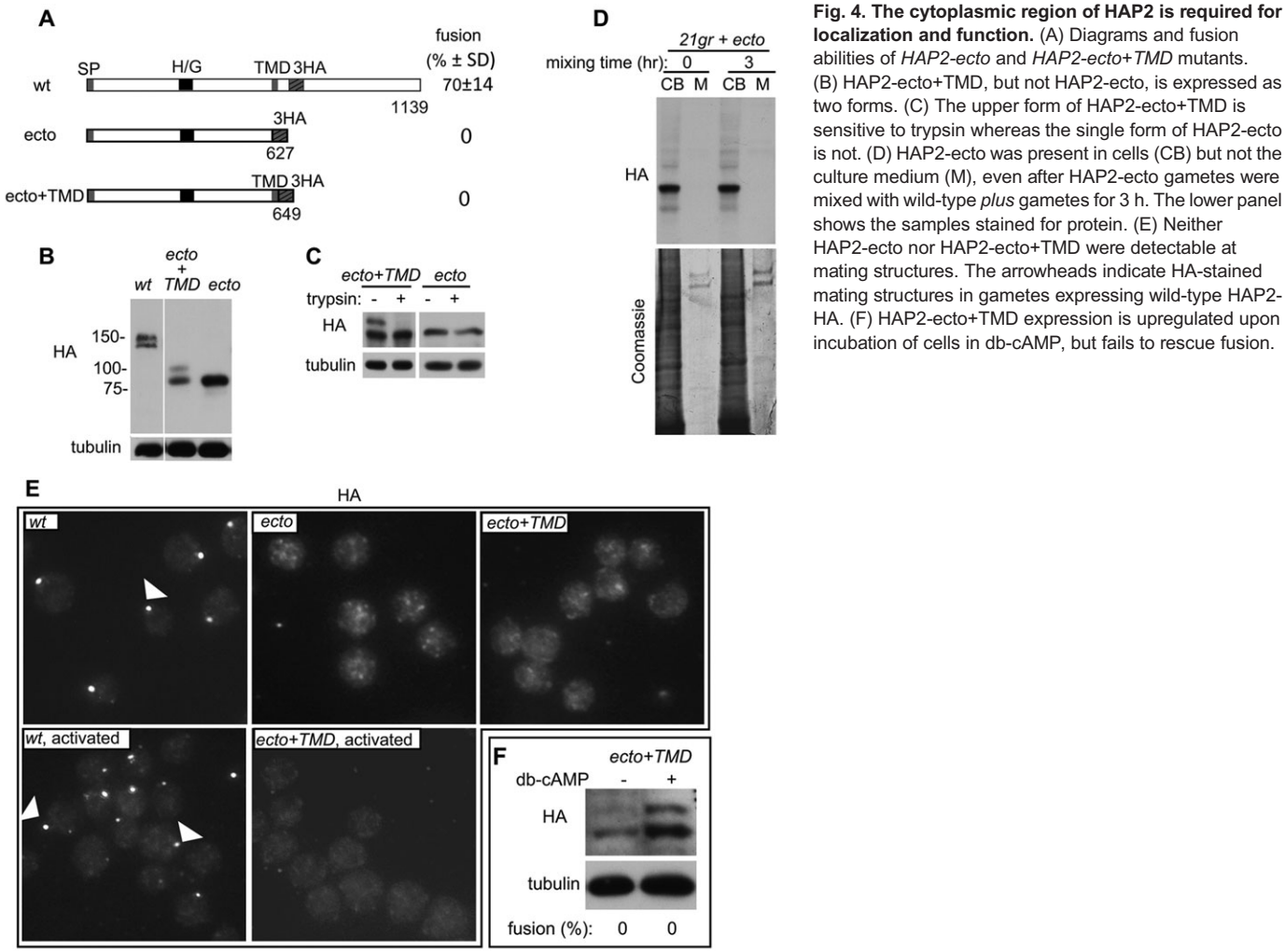

Fig. 4. The cytoplasmic region of HAP2 is required for localization and function. (A) Diagrams and fusion abilities of *HAP2-ecto* and *HAP2-ecto+TMD* mutants. (B) HAP2-ecto+TMD, but not HAP2-ecto, is expressed as two forms. (C) The upper form of HAP2-ecto+TMD is sensitive to trypsin whereas the single form of HAP2-ecto is not. (D) HAP2-ecto was present in cells (CB) but not the culture medium (M), even after HAP2-ecto gametes were mixed with wild-type *plus* gametes for 3 h. The lower panel shows the samples stained for protein. (E) Neither HAP2-ecto nor HAP2-ecto+TMD were detectable at mating structures. The arrowheads indicate HA-stained mating structures in gametes expressing wild-type HAP2-HA. (F) HAP2-ecto+TMD expression is upregulated upon incubation of cells in db-cAMP, but fails to rescue fusion.

In cells expressing HAP2-ecto+TMD, the protein was trypsin-sensitive on live cells, indicating that it was on the cell surface (Fig. 4C). On the other hand, HAP2-ecto+TMD failed to localize to the mating structure and failed to rescue gamete fusion (Fig. 4A-C,E). To test whether the absence of the protein at the mating structure was a consequence of the low expression of the upper form of HAP2-ecto+TMD, we incubated the gametes with di-butyryl cAMP (db-cAMP), a treatment that leads to upregulation of HAP2 transcription and translation (Liu et al., 2010; Ning et al., 2013). Such activated cells still lacked HAP2-ecto+TMD at the mating structure, and the activated *HAP2-ecto+TMD* cells were incapable of fusion with *plus* gametes (Fig. 4E,F). These results indicated that the HAP2 ectodomain and TMD were sufficient for trafficking the protein to the cell surface, but the cytoplasmic region was required for targeting the protein to the mating structure and rescuing fusion.

Motifs in the cytoplasmic region target HAP2 to the fusogenic membrane patch

To identify cytoplasmic regions that influenced HAP2 localization and function, we generated several additional mutant forms of the protein. The fusion ability of cells expressing HAP2 lacking the C-terminal 31 amino acids (HAP2-1108; Fig. 5) was indistinguishable from that of cells expressing wild-type HAP2 (1139 residues). Cells with HAP2 lacking the C-terminal 85 residues (HAP2-1054, Fig. 5A-C) expressed less of the upper form of the protein (and also possessed a much smaller fragment of uncertain derivation), but trafficked the upper form to the cell surface and supported low levels of fusion (Fig. 5C). Although we did not detect HAP2-1054 at the mating structure in resting gametes, it became detectable there when gametes were induced to express higher amounts of the protein by incubation in db-cAMP (Fig. 5D,E), and the db-cAMP-activated cells were capable of wild-type levels of fusion. Thus, the C-terminal 85 residues influenced localization, but were not essential for localization or fusion competency.

Analysis of *hap2* gametes expressing a HAP2 that was C-terminally truncated to residue 760 (HAP2-760) showed that the protein was expressed at the cell surface (Fig. 5A-C). However, we did not detect the protein at the mating structures of resting or activated gametes, and activated gametes showed less than 2% fusion (Fig. 5A,D,E). Addition of the C-terminal 998-1139 fragment to HAP2-760 resulted in some surface expression of the upper form of HAP2-Δ761-997 (Fig. 5A-C), but no localization to the mating structure was detected in resting or activated cells (Fig. 5D-E) and activated *HAP2-Δ761-997* gametes showed only very low levels of fusion (6%) (Fig. 5A).

Although HAP2-1054, HAP2-Δ761-997 and HAP2-760 were not detectable at the mating structures in resting gametes, we detected prominent intracellular staining (Fig. 5E). The smaller fragments of HAP2-1054 and HAP2-760 were not sensitive to trypsin, confirming that they were intracellular (Fig. 5C); optical sectioning also confirmed that the stain was internal in *HAP2-760*

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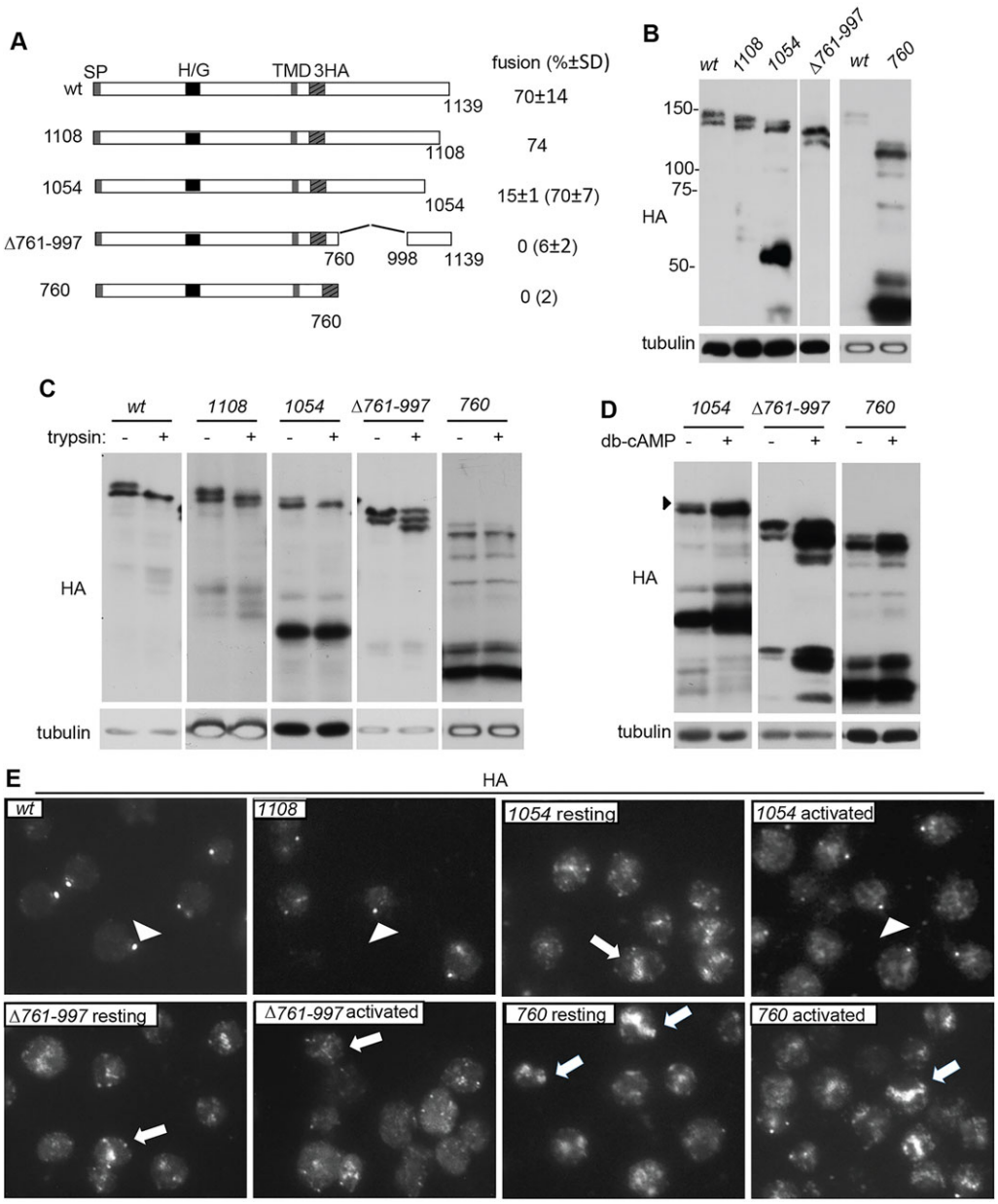

Fig. 5. The cytoplasmic domain contributes to both HAP2 targeting to the mating structure and to its function in fusion. (A) Diagrams and fusion abilities of HAP2 cytoplasmic domain mutants. Results in parentheses show fusion of gametes that had been activated with db-cAMP before mixing with wild-type *plus* gametes. (B) All of the mutant HAP2 proteins were expressed as two forms. Tubulin is shown as a loading control in the lower panels. (C) Immunoblots showing effects of trypsin treatment on the upper form of HAP2 in the mutants. The basis for the only partial trypsin sensitivity of the upper band in the *HAP2-Δ761-997* gametes is unknown, as is the origin of the third band in that sample. (D) The amounts of HAP2-1054, HAP2-Δ761-997 and HAP2-760 protein (arrowhead) increased when gametes were activated with db-cAMP. (E) Immunolocalization of HAP2 in the cytoplasmic region of mutants. Arrowheads indicate mating structure localization. Arrows show internal staining.

cells (see Movies 1-3 in the supplementary material). Taken together, these results indicated that the HAP2 segment comprising residues 761-997, which contains low complexity regions and is enriched in repetitive sequences, was essential for localization to the mating structure.

A cysteine dyad near the TMD regulates the fusion capacity of HAP2

As shown in Fig. 2B, the cytoplasmic regions of HAP2 homologs in members of all major evolutionary groups possess multiple cysteine residues near the TMD, suggesting that this multi-cysteine motif was a feature of the ancient HAP2. Because inspection of HAP2 alignments (Fig. 2B) indicated that the HAP2 proteins of many species contain a dicysteine motif in the cytoplasmic region near the TMDs, we tested for a possible role of the *Chlamydomonas* CC661-2 dyad in fusion. Cells expressing HAP2 with just the first of the cysteines mutated, HAP2-C661S, localized the protein to the mating structure, and were somewhat reduced in fusion capacity. Protein location and fusion capacity of cells expressing the protein with the second of the cysteines mutated, HAP2-C662S, were indistinguishable from wild type. Simultaneous mutation of both cysteines to serines (HAP2-CC661-2SS), however, strongly impaired fusion, even in gametes activated by db-cAMP (Fig. 6A). When *minus* gametes expressing wild-type HAP2 are mixed with wild-type *plus* gametes, fusion occurs rapidly, and pairs of gametes adhering by their mating structures are rarely detected in fixed samples examined by light microscopy. In the mixtures of *HAP2-CC661-2SS minus* gametes with wild-type *plus* gametes, however, many pairs were observed, indicating that membrane adhesion at the mating structures was unaffected in the mutant, but that fusion was blocked (Fig. 6E). Notably, except for being impaired in fusion ability, the properties of the HAP2-CC661-2SS mutant form of the protein were indistinguishable from those of wild type HAP2. HAP2-CC661-2SS was on the cell surface as assessed by trypsin sensitivity, and it was detected at the mating structure at levels

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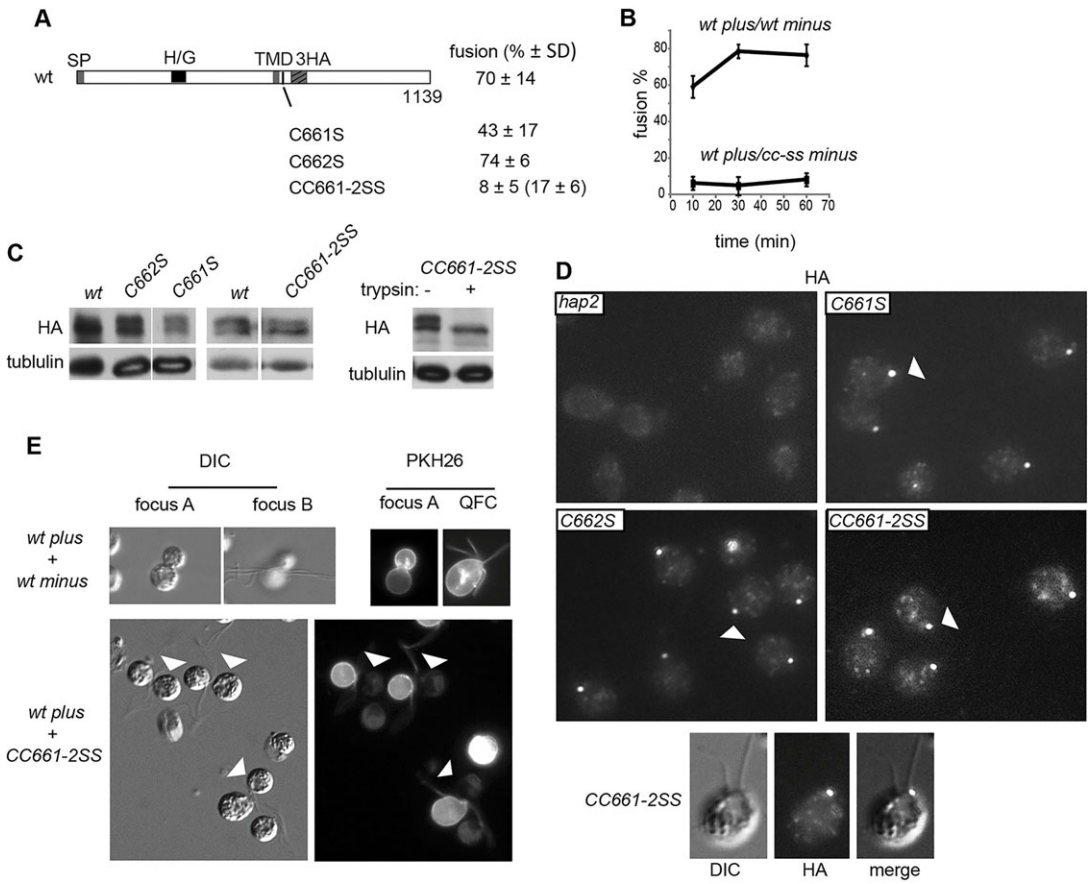

Fig. 6. The membrane proximal cysteine pair (CC661-2) regulates the fusion capacity of HAP2. (A) The location of the pair of cysteines and the fusion abilities of the three cysteine mutants. Results in parentheses show fusion of *CC661-2SS* gametes activated with db-cAMP before mixing with wild-type *plus* gametes. (B) Quantification of fusion at the indicated times after mixing with wild-type *plus* gametes. (C) Immunoblots of the HAP2 cysteine mutants. The upper form of HAP2-CC661-2SS is trypsin sensitive (right panels). (D) Immunofluorescence shows that all three HAP2 cysteine mutant proteins localize at the mating structure (arrowheads). The lower panels are higher-magnifications views documenting that HAP2-CC661-2SS is localized at the mating structure, which is at the apical end of the cell between the two flagella. This cell is resting on its side. (E) Membrane merger fails between *HAP2-CC661-2SS minus* gametes and wild-type *plus* gametes. Wild-type *plus* cells were labeled with PKH26 and then mixed with wild-type *minus* gametes (upper panels) or *HAP2-CC661-2SS minus* cells (lower panels). Whereas the PKH26 dye transferred rapidly after mixing wild-type *minus* and wild-type *minus* gametes, no dye transfer was detected when PKH26-labeled *plus* gametes were mixed with *HAP2-CC661-2SS minus* gametes (arrowheads), even though they formed pairs of cells that were tightly adherent at their mating structures. QFC, quadriflagellated cell.

equivalent to that of wild-type gametes (Fig. 6A-D). Similar localization and functional results were obtained with three independent *HAP2-CC661-2SS* transformants. Previously, we have shown that *hap2* mutant gametes adhering by their mating structures failed to exhibit membrane lipid exchange with their *plus* partners (Liu et al., 2008). In fluorescent lipid experiments with *HAP2-CC661-2SS* gametes, we also found no evidence for lipid mixing (e. g. hemifusion) between the adherent gametes (Fig. 6E). Thus, the dicysteine motif of *Chlamydomonas* HAP2 regulates the capacity of the protein to accomplish membrane fusion.

DISCUSSION

Although the membrane protein HAP2 is essential for gamete membrane fusion in disparate evolutionary groups, the molecular features of the protein that regulate its location and function are poorly understood. The present study demonstrates that the ectodomain is necessary and, along with the TMD, sufficient for delivery of the protein to the cell surface in *Chlamydomonas*, and that the cytoplasmic domain contains motifs that target the protein to the site of fusion and regulate its activity in the membrane fusion reaction.

The finding that HAP2-ecto+TMD was expressed on the cell surface demonstrated that the cytoplasmic region of the protein was dispensable for trafficking through the secretory pathway and is consistent with the earlier findings that the ectodomain of *Plasmodium* HAP2 is sufficient for fusion (Mori et al., 2010). These results also indicated that the as yet uncharacterized post-translational modification that underlies the appearance of two forms of HAP2 must occur on the ectodomain or TMD. On the other hand, the result that HAP2 containing the first 55 residues of the ectodomain along with the TMD and cytoplasmic region failed to reach the cell surface makes it likely that the ectodomain actively participates in trafficking. Possibly, HAP2 forms homomeric or heteromeric complexes via features of its ectodomain, including glycoylation sites, that are important for targeting to the cell surface (Isaji et al., 2006; Gupta et al., 2008). Similarly, mutations in the H/G domain impaired (C366A) or prevented (PbCr, ΔDKVG and L391G/Q394P) HAP2 expression at the cell surface, possibly because the mutations altered the structure of the protein and prevented normal trafficking. These findings indicate that assessing functional properties of regions of HAP2 is complex, and being able to conclude that a domain is essential in the membrane fusion

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reaction per se requires demonstrating that the mutant form of the protein is expressed at the cell surface.

It is more difficult to provide an explanation for the results that mutations of any of three conserved residues (two of them charged) within the H/G domain (D367, K368, G372) had no influence on HAP2 fusion capacity. Possibly, our bioassays do not assess the full range of the fusion capacity of the protein, or perhaps these residues make subtle contributions to structural features of the protein that are preserved when only a single conserved residue is altered.

Our combined results, that HAP2 containing only the ectodomain+TMD was expressed at the surface, but was not targeted to the fusogenic membrane patch and that adding the contiguous 111-residue cytoplasmic segment (as found in *HAP2-760*) also failed to rescue targeting to the mating structure or fusion, indicated that targeting was influenced by even more C-terminal regions of HAP2. The additional finding that *HAP2-1054* rescued localization and fusion capacity in activated gametes indicated that residues between 760 and 1054 were crucial for trafficking to the mating structure. A test of that model in the experiments with *HAP2-Δ761-997* confirmed that the 761-997 segment played a crucial role in localization. The ability of both *HAP2-760* and *HAP2-Δ761-997* to rescue any fusion (albeit at very low levels) after gamete activation (compared with the *hap2* mutant, which is completely incapable of fusion), suggests that as long as the HAP2 proteins are at the cell surface, the low amounts (~0.1% of the total) that would likely be present at the mating structure could function in fusion. By contrast, the failure to detect any fusion in cells expressing HAP2-ecto+TMD, which was present on the cell surface, emphasizes the importance of cytoplasmic motifs in the fusion reaction.

Finally, our discovery that mutation of the pair of cysteines in the ancient, multi-cysteine motif near the TMD had no effect on HAP2 localization to the mating structure, but strongly crippled fusion further indicated that the cytoplasmic region of *Chlamydomonas* HAP2 participates in regulating the fusion reaction. In future experiments, it will be interesting to determine whether the multi-cysteine motif in HAP2 proteins of other organisms also are important for fusion. The mechanism through which the dicysteine motif exerts its control is unknown, but one possibility is that it becomes palmitoylated, which thereby allows it to interact with the lipid bilayer. Several integral membrane proteins become palmitoylated on membrane-proximal cysteine residues, including integrins, tetraspanins and viral fusion proteins (Yang et al., 2004; Delandre et al., 2009; Veit, 2012; Shmulevitz et al., 2003) (see supplementary material Table S1). On the other hand, the motif might be unrelated to palmitoylation, and serve a HAP2-specific function that was lost during evolution in some species. Experiments to determine whether *Chlamydomonas* HAP2 is palmitoylated on CC661-2 should help in our understanding of the function of the dicysteine motif.

In light of our results and knowing that *Arabidopsis* HAP2 trafficking is temporally and spatially complex, it will be interesting to determine whether the previously described HAP2 mutants that failed to support seed formation in *Arabidopsis* indeed are capable of being targeted to the cell surface, or if they failed to function because their trafficking was disrupted. If the histidine-rich motif of the cytoplasmic domain of *Arabidopsis* HAP2 functions directly during fusion as proposed (Wong et al., 2010; Wong and Johnson, 2010), possibly, the multi-cysteine motifs originally fulfilled this function, but were replaced by the histidine-rich motif during evolution of higher plants.

MATERIALS AND METHODS

Cells and cell culture

Chlamydomonas reinhardtii wild-type strains *21gr* (mating type *plus*; mt+; CC-1690), *6145c* (mating type *minus*; mt−; CC-1691) and *B215* (mt−; nitrate reductase-deficient strain, Greg Pazour, University of Massachusetts, USA) were used for these experiments. HAP2 mutant *40D4* (available from the *Chlamydomonas* Culture Collection) was the *hap2* mutant strain used as recipient for the transgenes described below and was generated by insertional mutagenesis of strain *B215* using the nitrate reductase gene (see methods and Fig. S1 in the supplementary material). Cells were treated with trypsin to assess surface expression of HAP2 as described previously (Liu et al., 2008). Gamete fusion was assessed by determining the number of cells that had formed zygotes after being mixed with wild-type *plus* gametes for 30 min and was expressed as percent fusion using the following equation: (2×number of zygotes)/[(2×number of zygotes)+(number of unfused gametes)]×100. At least three sets of 100 randomly selected cells were counted in at least two independent experiments, except for *HAP2-1108*, for which quantification from only a single experiment is shown.

Taxonomic categories of HAP2 proteins

HAP2 homologs were found by PSI-BLAST (Altschul et al., 1997) searches. Representative HAP2 homologs were selected to sample major phylogenetic groups of eukaryotes. The transmembrane regions of them were predicted by Phobius (Käll et al., 2004). Sequence alignment were produced by MAFFT (Katoh et al., 2002) followed by manual adjustment. The dendrogram among the selected species (Fig. 2B) was made according to consensus knowledge of phylogenetic relationships among eukaryotes (Burki, 2014).

Plasmid construction and transformation into *Chlamydomonas*

A modified wild-type HAP2 cDNA (GI:288563867) from strain *6145c* was used to generate the mutant strains for these experiments. Wild-type HAP2 protein is listed under accession number ABO29824.2 in GenBank. A *Bgl*II site was inserted into the first exon of the *HAP2* gene in the HAP2-HA plasmid (pYJ58) (Liu et al., 2008) using standard PCR weaving methods. Genomic *HAP2* DNA between *Bgl*II and *Nru*I in the HAP2-HA plasmid was replaced with a *HAP2* c-DNA oligonucleotide also containing the first intron of the *HAP2* gene generated by standard PCR methods (see methods in the supplementary material for the plasmid sequence). The resulting plasmid pHAP2-HAm was cut with *Spe*I and the *aphVIII* paromomycin resistance gene (Sizova et al., 2001) generated by PCR was inserted using the In-fusion Dry-down PCR Cloning Kit from Clontech (California). The *aphVIII* gene was amplified with primers P82 and P83 using plasmid pSI103 (Sizova et al., 2001) as a template (P82, 5′-CACAGGTT-GCACTAGTGATCCCCCGGGCTGCAGGAATT-3′; P83, 5′-TGGCGC-CTTAACTAGTCAGCCCGCCCATGGAGAAAGAG). All of the *HAP2* mutant transgenes were generated from the pHAP2-HAm plasmid using standard PCR weaving methods and were confirmed by DNA sequencing. The *HAP2* transgene plasmids containing the *aphVIII* paromomycin resistance gene were transformed into *hap2* mutant *40D4* using electroporation (Shimogawara et al., 1998) and transformants that grew on culture plates containing paromomycin (Sigma) were screened for rescue of fusion (Liu et al., 2008) or by PCR for the presence of the transgenes; positive transformants were confirmed with immunoblotting.

Assaying for appearance of HAP2-ecto protein in the culture medium

The *HAP2-ecto* gametes (2×10^8 cells/ml) were mixed with an equivalent number of wild-type *plus* gametes (*21gr*) for 3 hours. The mixed gametes underwent flagellar adhesion and gamete activation, and failed to fuse. The culture was centrifuged (3000 ***g*** for 5 min) and the gametes and the supernatants (medium) were collected separately for immunoblotting. Cells were suspended at 1×10^7 cells/100 µl in HMDEK buffer (Liu et al., 2010) containing a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail, Roche Applied Science) and an equal volume of HMDEK buffer with protease inhibitor was added to the supernatants. Equivalent regions of the samples were mixed with an equal volume of 2× SDS-PAGE running buffer and proteins were analyzed by SDS-PAGE and immunoblotting as described previously (Misamore et al., 2003).

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Immunoblotting and fluorescent microscopy

Gametes (1×10^7 cells) suspended in 25 µl HMDEK buffer containing a protease inhibitor cocktail were mixed with an equal volume of 2× SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting. Anti-HA monoclonal antibody (Roche Applied Science) and monoclonal anti-acetylated tubulin antibody (Sigma) were used as primary antibodies for immunoblots. Differential interference contrast (DIC) microscopy and immunofluorescent staining of *HAP2-HA* transgenic cells were as previously described (Belzile et al., 2013). The gametes were applied to glass slides as described, fixed in ice-cold methanol for 20 min, and stained with anti-HA monoclonal antibody. For immunofluorescent staining of activated gametes, the activated gametes were suspended in 80 mM sucrose in 20 mM HEPES (pH7.0) before being applied to glass slides and were prepared for immunofluorescence as described previously (Belzile et al., 2013). All images were obtained with a 63× objective lens. Membrane lipid mixing was detected in experiments using lipid dye PKH26 (Sigma). Wild-type *plus* gametes were incubated with PKH26 (Liu et al., 2008) and then mixed with the indicated *minus* gametes. Samples were observed under fluorescence and DIC microscopy. Images were prepared using Image J (NIH) and Adobe Photoshop (Adobe Systems). Methods for generating supplementary image stacks are described in the supplementary material.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.L. designed the experiments in collaboration with W.J.S. J.P. and N.G. provided guidance in evolutionary analysis of HAP2 and constructed the HAP2 dendrogram. Y.L. performed the experiments, and Y.L. and W.J.S. analyzed the results and prepared the manuscript.

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Supplementary material

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