Plant Physiol. (1995) 108: 703–712

Mutations Affecting *O*-Glycosylation in *Chlamydomonas reinhardtii* Cause Delayed Cell Wall Degradation and Sex-Limited Sterility[1]

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We describe a mutation, *gag*-1, that affects in a temperature-dependent manner a specific type of *O*-glycosylation in the green alga *Chlamydomonas reinhardtii*. In the mutant, all the major glycoproteins, in particular cell wall proteins, show a decreased apparent molecular weight in polyacrylamide gels, and their antigenicity is affected. The mutant forms multicellular aggregates (palmelloid colonies) at the restrictive temperature due to the delayed release of the daughter cells from the mother cell wall after mitosis. In addition, the mutation causes sterility by preventing sexual agglutination. In contrast to the other phenotypes, the sterility phenotype is temperature independent, and it is expressed only by cells of the plus mating type. We show that *imp*-8, a previously described nonagglutinating sex-limited mutation, causes the same glycosylation defect and is allelic to *gag*-1. Thus, expression of mt^+ agglutinability appears to require the specific type of *O*-glycosylation that is defective in these mutants. More generally, these observations show that a sex-limited phenotype can be caused by a mutation in a gene that is not itself sex limited in its expression.

O-Glycosylation is a widespread posttranslational modification of secreted proteins and the extracellular domains of membrane proteins, both in plants and in animals. A wide variety of carbohydrates, ranging from single monosides to complex, branched oligosaccharides, can be linked by an *O*-glycosidic linkage to various hydroxylated amino acid residues (Hyp, Ser, or Thr) in the protein. Due to its chemical diversity, *O*-glycosylation can be expected to play a multiplicity of roles in different proteins (Poulsen, 1989), but our knowledge of these functions remains sketchy. *O*-Linked sugars make a major contribution to the structural properties of proteins of the extracellular matrix, in particular the extensins of the plant cell wall (Kieliszewski and Lamport, 1994). They can also participate in molecular recognition processes at the cell surface, for example in selectin (Picker et al., 1991). In addition, they contribute to the stability and resistance to proteolysis of membrane-bound (Kozarsky et al., 1988; Reddy et al., 1989; Carlsson et al., 1993) and secreted (Kim et al., 1994) proteins.

In principle, genetic analysis could prove helpful in the study of both the role of *O*-linked sugars in proteins and the biochemical process of *O*-glycosylation itself, via the cloning of enzymatic and regulatory components. However, with the notable exception of yeast mannan biosynthesis (Ballou, 1990; Häusler et al., 1992), mutations affecting *O*-glycosylation have not been systematically searched for, perhaps due to the difficulty of predicting the mutant phenotypes. Thus, only a few mutations affecting *O*-glycosylation have been identified in other organisms (Kozarsky et al., 1988; Greer et al., 1989). The present study describes a mutation of the unicellular green alga *Chlamydonomas reinhardtii* that causes a specific defect in *O*-glycosylation. As a result, mutant cells are affected in at least two cellular processes in which glycoproteins are involved, i.e. cell wall release and sexual recognition.

The cell wall of *C. reinhardtii* is composed primarily of rod-shaped Hyp-rich glycoproteins homologous to plant extensins (Catt et al., 1976; Goodenough et al., 1986; Woessner and Goodenough, 1989). This finding has emphasized the ancestry of the glycoprotein components of the plant cell wall and has made *Chlamydomonas* a valuable model for the study of the structure and function of the extensins. *Chlamydomonas* Hyp-rich glycoproteins are extensively *O*-glycosylated: sugars constitute approximately 65% of the chaotrope-soluble fraction of the wall, mainly as Ara, Gal, and Man, with some Xyl and Glc (Catt et al., 1976; O'Neill and Roberts, 1981). Methylation analysis indicates the presence of short-branched oligosaccharides in *O*-glycosidic linkage with Hyp and probably Ser residues. The absence of amino sugars suggests that *N*-glycosidic linkage does not occur (O'Neill and Roberts, 1981). In the wall, Hyp-rich glycoproteins are arranged in concentric layers anchored on a network of radiating fibers (Roberts et al., 1972; Goodenough and Heuser, 1985). The outer layer, W6, has a crystalline structure. Layers W4 to W6 can be solubilized in chaotropic agents and reassemble readily upon dialysis (Catt et al., 1976; Adair et al., 1987). In contrast, the inner W2 layer is amorphous and insoluble in chaotropes. Isodityrosine cross-links may participate in wall insolubilization (Waffenschmidt et al., 1993).

In continuously growing cultures of *C. reinhardtii*, each cell usually undergoes two or three successive rounds of cell division, leading to the formation of four to eight cells encased in the mother cell wall. Each cell then forms a new

[1] This work was supported by the Centre National pour la Recherche Scientifique (URA 1187 and UMR 9922).

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Abbreviations: PAS, periodic acid Schiff; WT, wild type.

wall, after which hatching occurs (i.e. the disassembly of the mother cell wall), leading to release of the daughter cells from the zoosporangium. Hatching requires the action of an endogenous protease known as vegetative autolysin (Jaenicke et al., 1987), presumably acting on W2 components, as has been shown for the gametic autolysin responsible for the shedding of the gamete wall during mating (Imam et al., 1985; Matsuda et al., 1985; Jaenicke et al., 1987). We show here that the *gag*-1 mutation prevents normal release of the daughter cells after cell division, causing the formation of large aggregates of cells (palmelloid colonies).

Glycoproteins are also involved in cell recognition at mating. Sexual reproduction in *C. reinhardtii* involves differentiation of haploid vegetative cells into mating-competent gametes, which are then capable of agglutinating and fusing with gametes of the opposite sex (Harris, 1989). The resulting zygote generally enters dormancy rapidly, after which it can undergo meiosis to yield four haploid clones. Reproduction is heterothallic and isogamous, since two stable, genetically distinct mating types are present (called plus and minus) with identical morphologies. A single genetic locus with two alleles (mt^+ and mt^-) defines the mating type (Smith and Regnery, 1950) (i.e. is responsible for the selectivity of the sexual recognition and fusion processes). The mechanism for this selectivity rests in part on the presence at the surface of the gametic flagellae of mating type-specific agglutinins (reviewed by Adair, 1985). Agglutinins are high M_r glycoproteins with strong similarities to cell wall glycoproteins in their shape, amino acid composition, and glycosylation pattern (Cooper et al., 1983). In vitro studies have shown that only mt^- gametes will bind to purified mt^+ agglutinins, and only mt^+ gametes will bind to mt^- agglutinins (Adair et al., 1982). The molecular basis for this interaction is unknown; mt^+ and mt^- agglutinins may bind to each other or alternatively to specific receptors at the flagellar membrane surface. In the present study, we show that the *gag*-1 mutation abolishes agglutinability of mt^+, not mt^-, gametes, probably by preventing proper production of the mt^+ agglutinin molecule.

MATERIALS AND METHODS

Chlamydomonas reinhardtii strain 137c was grown on Tris/acetate/phosphate medium (Harris, 1989) under 500-lux continuous illumination, in some cases followed by nitrogen starvation in nitrogen-free medium (Vallon et al., 1993). Mutant strains obtained from the *Chlamydomonas* Culture Collection at Duke University (Durham, NC) were CC463 (*imp*-2 mt^+), CC464 (*imp*-2 mt^-), CC474 (*imp*-8 mt^+), CC475 (*imp*-8a mt^+), and CC476 (*imp*-8a mt^-). Fluorescence microscopy, sexual crosses, and genetic analysis were performed using standard protocols (Harris, 1989). For zygotic colony analysis, the meiotic products were not separated after germination, and the cells were tested together without further cloning.

SDS-polyacrylamide gel analysis was performed in the system of Laemmli (1970), using 8% acrylamide in the resolving gel. PAS staining was performed according to Konat et al. (1984). For immunoblotting, gels were transferred to nitrocellulose (BA85, Schleicher & Schuell) using a semi-dry electrotransfer apparatus (Bio-Rad) according to the manufacturer's instructions. Immunostaining was performed with specific antibody and chemiluminescence (ECL, Amersham).

For thin-section EM, cells were harvested by centrifugation, resuspended in 60 mM cacodylate buffer, pH 7.4, and an equal volume of the same buffer containing 4% glutaraldehyde and 4% tannic acid (AR, Malinckrodt, St. Louis, MO) was added dropwise over 15 min at room temperature. Fixation was continued for another 2 h, after which the cells were rinsed in buffer, progressively dehydrated in ethanol, and embedded in Epon-Araldite. Sections were stained with saturated aqueous uranyl acetate (30 min) and Reynold's lead citrate (12 s).

RESULTS

The *gag*-1 Mutation Affects the Electrophoretic Mobility of the *C. reinhardtii* Glycoproteins

Protein M, the L-amino acid oxidase of *C. reinhardtii*, is located in the periplasm and is induced during nitrogen starvation (Bulté and Wollman, 1992; Vallon et al., 1993). In the course of the study of this glycoprotein, we observed that one of our control strains, otherwise WT for photosynthesis or amino acid oxidase production, carried an unsuspected mutation affecting protein glycosylation. We named this mutation *gag*-1, for reasons explained below. When cells carrying this mutation were grown at high temperature (32°C) and their total proteins were analyzed by SDS-PAGE and PAS staining, all the PAS-positive bands appeared to be shifted down by 3 to 4 kD with respect to WT (Fig. 1, arrows). Additional Coomassie blue staining of the gel indicated that only PAS-positive bands were affected by the mutation. When mutant cells were grown at the permissive temperature (18°C), the electrophoretic pattern was indistinguishable from that of WT. At 25°C (not shown), an intermediate position of glycoproteins was observed, with either smeared bands at an intermediate position or the occurrence of both the high- and low-mobility forms.

Among the major glycoproteins affected by this mutation, we found Mα and Mβ, the two subunits of the amino acid oxidase, plus three major bands that are present in a cell-wall-enriched preparation (not shown). On the basis of their electrophoretic mobility, the top band can be identified as GP2 and the lower two bands as GP3 (Goodenough et al., 1986). Both have been shown to belong to the W4 and W6 layers of the cell wall. The shift in electrophoretic mobility of GP2 in the mutants is better observed when gels are allowed to run for a longer time (not shown). GP1 and GP1.5, the high M_r components of the wall, are not sufficiently resolved in this gel system to assess whether they are also affected by the mutation.

Not all glycosylation was abolished at 32°C in these proteins, as was apparent from the fact that the bands were still PAS-positive. This suggested that a specific subset of sugar residues was missing in the mutants. Mα and Mβ from WT together may carry both *N*- and *O*-linked sugars,

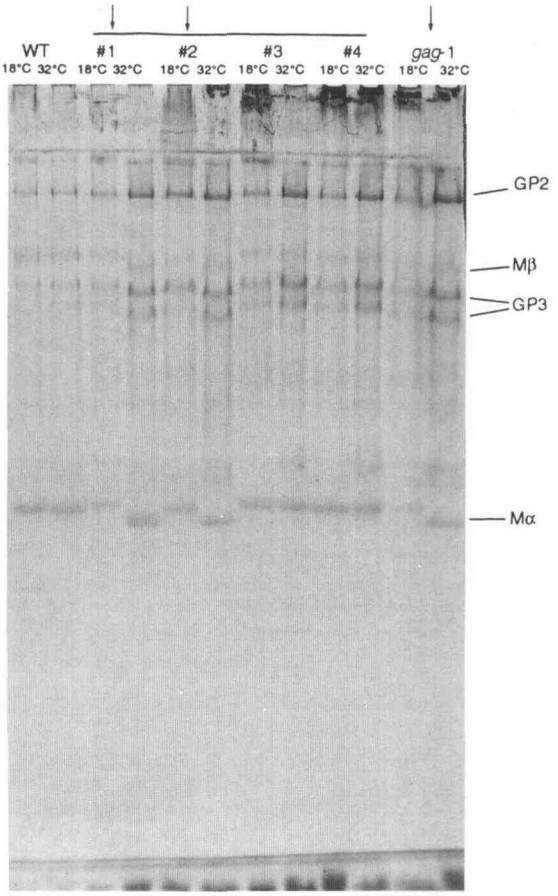

Figure 1. Altered electrophoretic mobility of glycoproteins in *gag*-1 mutants. SDS-polyacrylamide gel, stained with PAS, of total proteins from cells grown at 18 or 32°C and starved in nitrogen-free medium to allow accumulation of amino acid oxidase. Shown are the WT strain (left) and a *gag*-1 mutant (right), together with their progeny (four clones derived from a single tetrad). Arrows indicate *gag*-1 strains.

as judged from the sugar analysis of protein M (O. Vallon and F.-A. Wollman, and B. Fournet and Y. Leroy, University of Lille, France, unpublished data). But the other proteins affected, those of the cell wall, seem to carry exclusively *O*-linked sugars (Catt et al., 1976; O'Neill and Roberts, 1981). Therefore, the mutation is believed to affect a specific step in the addition of *O*-linked sugars. We cannot exclude, however, that the mutation primarily affects another type of modification necessary for proper *O*-glycosylation.

The *gag*-1 Mutation Affects Antigenicity of the Glycoproteins

Antibodies have been raised to the purified subunits of the amino acid oxidase, Mα and Mβ (L. Bulté, Thèse de l'Univerité Paris 6). The two antibodies have similar reactivity patterns (Fig. 2 shows data with the Mα antibody, but similar results were obtained with the Mβ antibody); they both recognize very strongly Mα and Mβ, as well as the major cell wall glycoproteins GP2 and GP3, plus other weaker bands that are not readily detected by PAS staining but may also be glycoproteins of lower abundance. This suggests that the antibodies recognize a major carbohydrate epitope present on the glycoproteins of *C. reinhardtii*. Indeed, preincubation of the blots with periodic acid to destroy the carbohydrate moiety of the proteins (Woodward et al., 1985) strongly decreased reactivity with the Mα and Mβ antibodies (not shown).

Immunoblotting experiments with the Mα antibody were performed on the *gag*-1 mutants (Fig. 2). In all of the mutant strains that we analyzed, immunoreactivity was normal when cells had been grown at 18°C but was markedly lower than in WT when they had been grown at 32°C. In the mutant strains, residual reactivity at 32°C was at the position of the WT glycoproteins, not of the higher mobility (mutant) forms, and was probably due to small amounts of normal glycoproteins that were still being produced at the restrictive temperature. We conclude that the *gag*-1 mutants are affected in the addition of the same carbohydrate moiety that is responsible for the bulk of the immunoreaction with the Mα and Mβ antibodies.

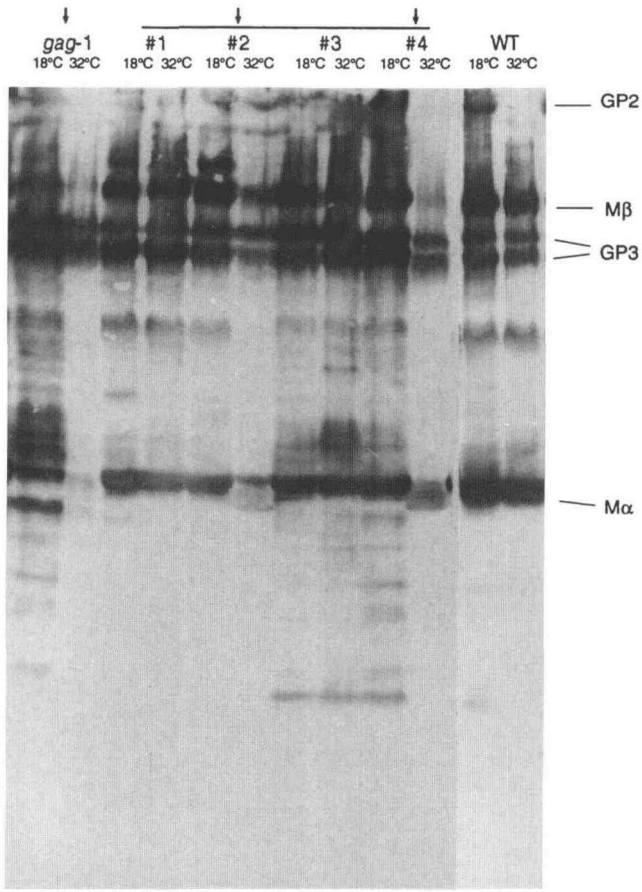

Figure 2. Altered antigenicity of glycoproteins in *gag*-1 mutants. Immunoblot of total proteins from cells grown at 18 or 32°C and nitrogen starved, reacted with the antibody to Mα. Shown are a *gag*-1 mutant (left) and the WT strain (right), and four clones derived from a single tetrad (different from that of Fig. 1). Arrows indicate *gag*-1 strains.

The *gag*-1 Mutation Causes Palmelloid Colony Formation

Interestingly, the *gag*-1 strains also showed an "aggregated" phenotype in liquid cultures. When grown at 32°C, *gag*-1 strains displayed extensive clumping of the cells, which formed macroscopic aggregates either in suspension or adhering to the glass vessel. This corresponds to a classical, yet poorly understood, phenomenon appearing in some *Chlamydomonas* strains, usually referred to as palmelloid colony formation (Harris, 1989). This phenomenon was not observed in liquid cultures of *gag*-1 strains at 18°C, nor in Gag^+ strains at 18°C or 32°C. Electron microscopic examination of palmelloid-containing cultures (Fig. 3) showed that cells generally occurred in large groups, surrounded by the remnants of several (up to five) successive cell walls. Control experiments with WT cells or *gag*-1 cells grown at 18°C showed mostly single cells encased in a single wall and some groups of four or eight cells (not shown).

In palmelloid structures, cohesion of the group was primarily due to the incomplete degradation of the cell walls of previous generations, so that, for example, the 16 cells formed by two rounds of 4-fold division could remain encased in a common wall. In addition, even when the ancestral wall was ruptured, the walls of sister cells were often found to adhere over long stretches to each other and also to that of the previous generation. This allowed the formation of very large groups, larger than could be encased in a single wall. Palmelloid colony formation thus appears to result both from delayed degradation of the mother cell wall and from increased adhesion between walls.

A similar phenomenon was also observed on agar plates. At the restrictive temperature (at 32°C or more markedly at 37°C), cells formed irregular clumps instead of the usual "mashed pea soup" appearance. Although the temperature

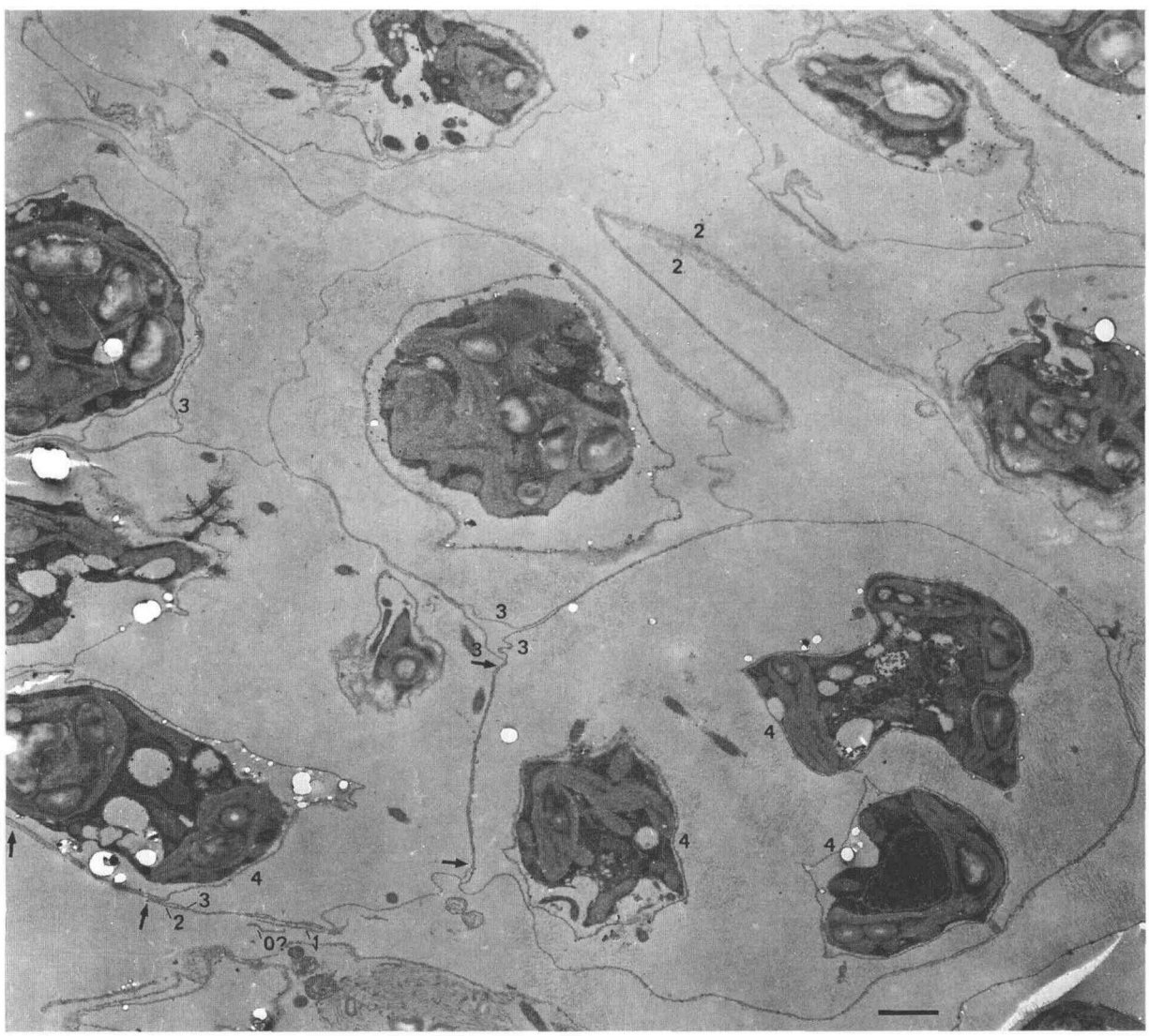

Figure 3. Ultrastructure of a palmelloid colony. Thin section of a palmelloid colony from a *gag*-1 strain grown at 32°C in liquid TAP medium. Figures next to each wall indicate the generation in which it was produced in chronological order. Arrows mark zones of adhesion between cell walls. The cell appears to have shrunk away from the cell wall, an artifact of the procedure that we have been unable to avoid. Bar = 1 μm.

dependence of the phenotype was more variable in plate than in liquid cultures (see below), culturing at 32°C on plates proved to be a useful screen in the genetic analysis of the *gag*-1 mutation.

Genetic Analysis of the *gag*-1 Mutation

The genetic nature of the lesion was analyzed. In 12 complete tetrads from a cross involving a *gag*-1 and a Gag^+ strain, the aggregated-palmelloid phenotype and the shift in electrophoretic mobility of the glycoproteins at 32°C were always found to segregate together in two of the four meiotic products (one such tetrad is shown in Fig. 1). In addition, 37 haploid clones, either from incomplete tetrads or from germination of pooled zygotes, were analyzed throughout the course of this study, and a strict correlation between the two phenotypes at 32°C was always observed. The third phenotype described above, i.e. reduced reactivity of the glycoproteins with the antibodies to Mα and Mβ, was analyzed in a subset of 4 tetrads. It was found to co-segregate with the other two phenotypes (one of those tetrads is shown in Fig. 2). Taken together, these results strongly suggest that the three phenotypes are caused by a single nuclear mutation or by tightly linked mutations.

The *gag*-1 Mutation Causes Sterility in mt^+ Strains

An additional phenotype conferred by the *gag*-1 mutation pertains to mating behavior. In a cross between a *gag*-1 mt^- and a Gag^+ mt^+ strain, the Gag^+ products mated either as plus or minus, whereas the *gag*-1 products either mated as minus or were sterile. With regard to mating behavior, glycoproteins, and aggregation, three categories of tetrads could be distinguished; these are described in Table I. Type A tetrads could readily be identified as parental ditypes. Types B and C could be recognized as nonparental ditypes and tetratypes, respectively, provided two simple assumptions were made. First, the mutation causing the aggregated phenotype also causes sterility in genetically mt^+ cells only, and second, it is unlinked to mating type. Among the 10 tetrads analyzed, the number of parental ditypes, nonparental ditypes, and tetratypes in this cross was 1:1:8.

Table I. *Types of tetrads obtained in a cross between a gag-1 mt^- and a Gag^+ mt^+ strain*

Tetrad Type	Phenotype		
	Aggregation	Position of glycoproteins	Mating as
Type A	Yes	Low	Minus
	Yes	Low	Minus
	No	High	Plus
	No	High	Plus
Type B	Yes	Low	Sterile
	Yes	Low	Sterile
	No	High	Minus
	No	High	Minus
Type C	Yes	Low	Minus
	Yes	Low	Sterile
	No	High	Minus
	No	High	Plus

The mating defect in *gag*-1 mt^+ strains involves the agglutination step. These strains did not agglutinate with either mt^+ or mt^- tester gametes (whether the testers were genetically *gag*-1 or Gag^+). This effect was observed with plate- or liquid-grown cells, irrespective of the temperature at which the cells had been grown and differentiated (18, 25, or 32°C) and of the temperature at which mating was performed (18 or 25°C). Note that when cells had been grown at 32°C, matings were performed after the clumps had been disrupted with a Pasteur pipette, taking only motile cells for the test, so that lack of agglutination could not be ascribed to the palmelloid phenotype itself. In contrast, *gag*-1 mt^- cells grown at any temperature mated apparently with the same efficiency as their Gag^+ mt^- or Gag^+ mt^+ siblings (mating of gametes differentiated at 32°C was less efficient than at 25 or 18°C, but this was true for WT cells also). We were unable to identify in these *gag*-1 mt^- strains any abnormality in their mating behavior, efficiency of zygote formation, germination of zygotes, or survival of progeny.

In view of this third phenotype, we decided to call the mutation *gag*-1, for "glycoproteins, aggregation, and sexual agglutination."

The *imp*-8 Mutation Causes the Same Phenotypes as *gag*-1

Goodenough et al. (1976) have characterized a series of mutants impaired in sexual agglutination (a subclass of the so-called *imp* mutants). Recombination and complementation studies indicate that these mutations are found at two distinct loci, called *sag*-1 and *sag*-2, neither of which are linked to *mt* (Goodenough et al., 1978; Adair et al., 1983). Most remarkably, the mutant phenotype is expressed only in mt^+ cells; genetically, mt^+ cells are sterile unless the sexual response is artificially stimulated (Pasquale and Goodenough, 1987), but mt^- carriers are perfectly fertile and form normal progeny. In view of the similarity between the sterility phenotypes of the *gag*-1 and *sag* mutants, we wondered whether any of the latter could be allelic to *gag*-1. We have analyzed the glycoproteins of several of these mutants, carrying either *imp*-2, representing the complementation group *sag*-1, or *imp*-8, the single mutation found at the *sag*-2 locus.

As judged by SDS-PAGE and PAS staining (Fig. 4), the glycoproteins of the two *imp*-2 strains we tested (mt^+ and mt^-) appeared normal at 18°C as well as at 32°C. These strains were not aggregated on plates or in liquid culture at 32°C. In contrast, the three strains with mutations at the *sag*-2 locus that we obtained from the Culture Collection at Duke University (the original *imp*-8 mt^+ and two derivatives obtained by crossing with WT, *imp*-8a mt^+ and *imp*-8a mt^-) showed the same shift in glycoprotein mobility (Fig. 4) and altered reactivity to the Mα and Mβ antibodies (not shown) as *gag*-1 mutants, with the same temperature dependence. In addition, they were found to form palmelloid colonies in liquid cultures at 32°C but not at 18°C. Thus, the three phenotypes associated with the *gag*-1 mutation are also associated with the *imp*-8 mutation. This is further evidence that they result from a single genetic lesion and

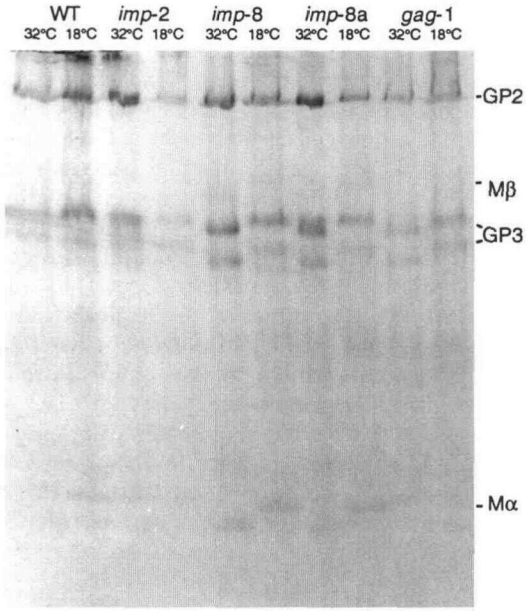

Figure 4. Glycosylation patterns in *imp* mutants. SDS-polyacrylamide gel, stained with PAS, of total proteins from cells grown at 18 or 32°C and nitrogen starved. WT, *imp*-2 (CC464), *imp*-8 (CC474), *imp*-8a (CC475), and *gag*-1 strains are presented.

raises the possibility that the two mutations lie in the same gene.

At this point, we noted that aggregation on plates was observed at 32°C in the *imp*-8a mt^- strain but not in the *imp*-8 mt^+ and *imp*-8a mt^+ strains. In subsequent crosses involving the *imp*-8a mt^- strain, aggregation in liquid cultures co-segregated normally with the glycoprotein defect and sex-limited sterility, but aggregation on plates was observed in only a fraction of the *imp* progeny (irrespective of *mt*). Conversely, crosses involving *gag*-1 and Gag^+ strains frequently yielded *gag*-1 clones that showed aggregation on plates already at 18°C, while maintaining the usual temperature dependence for the glycoprotein defect and palmelloid formation in liquid cultures. In general, the intensity and temperature dependence of the aggregated phenotype appears to be much more variable in plate than in liquid cultures. These observations suggest that the genetic context can modulate the expression of the aggregated phenotype in plate cultures.

The *imp*-8 Mutation Is Allelic to *gag*-1

To assess whether the *gag*-1 and *imp*-8 mutations were allelic, complementation experiments were carried out. In a first step, derivatives of our *gag*-1 mutant and of the *imp*-8a mt^- strain were constructed that also carried the *arg*-2 or *arg*-7 mutations. They were used to isolate vegetative diploids (Ebersold, 1967), taking advantage of the fact that *arg*-2 and *arg*-7 are able to complement each other yet show very little recombination. Three sexual crosses were performed: (1) Gag^+ *arg*-2 mt^+ × *gag*-1 *arg*-7 mt^-; (2) *imp*-8a *arg*-2 mt^- × Imp^+ *arg*-7 mt^+; and (3) *gag*-1 *arg*-2 mt^- × *imp*-8a *arg*-7 mt^+.

Crosses 1 and 2 were designed to determine whether the phenotypes associated with the *gag*-1 and *imp*-8 mutations were recessive or dominant. Cross 3 was meant to test whether the two mutations could complement each other in diploids. In the latter cross, due to sterility of the mt^+ parent, we had to resort to the dibutyryl-cAMP-isobutylmethylxanthine treatment described by Pasquale and Goodenough (1987) to enhance mating of infertile strains.

Mating mixtures were placed on Tris/acetate/phosphate plates, and large colonies appearing after 4 d in bright light were picked onto TAP plates and analyzed for mating type. All 12 per experiment behaved as mt^-, as expected from diploids (Ebersold, 1967), a strong indication that the selection scheme for diploids had been successful. A fraction of these colonies was examined by fluorescence microscopy after 4′,6-diamidino-2-phenylindole staining and showed a high number of nucleoids, confirming that they were true diploids (Tsubo and Matsuda, 1984). They were analyzed by SDS-PAGE and PAS staining. As shown in Figure 5, diploids resulting from cross 3, between the *gag*-1 and the *imp*-8a strain, all displayed the mutant electrophoretic pattern of glycoproteins, whereas *gag*-1/Gag^+ and *imp*-8a/Imp^+ diploids resulting from crosses 1 and 2 were all phenotypically WT. In addition, the 12 clones from cross 3 were found to be aggregated on plates at 32°C (but not at 18°C), whereas the 24 clones from crosses 1 and 2 were not aggregated at either temperature. This showed (a) that the two mutations were recessive for the glycosylation and aggregation phenotypes, and (b) that they did not complement each other and therefore probably affected the same gene. Our experiments, using diploids obtained by sexual crosses, did not allow us to assess the mt^+-limited sterility phenotype. However, Adair et al. (1983) have shown, using PEG-induced fusion between mt^+ strains to produce a diploid mt^+ background, that the sterility phenotype associated with *imp*-8 is recessive, i.e. that heterozygous diploids agglutinate normally, and that homozygous mutants express the nonagglutinating phenotype.

Additional evidence for the identity of the *gag*-1 and *sag*-2 genes was obtained in recombination tests. We analyzed clones obtained by dissecting tetrads from a dibutyryl-cAMP-isobutylmethylxanthine-stimulated cross beween a *gag*-1 and an *imp*-8 strain (only 10 clones from incomplete tetrads). All of these clones showed aggregation at 32°C and altered glycoproteins, and all of them either mated as minus or were sterile. Since neither *gag*-1 nor *imp*-8 is linked to *mt*, this indicated that no recombination had occurred between the *gag*-1 and *imp*-8 mutations. To circumvent the paucity of zygotes in these crosses and to reduce lethality, zygotes were left to germinate and the progeny was analyzed together without further cloning. None of these zygotic colonies (42 colonies total) showed self-crossing when allowed to form gametes in nitrogen-free medium overnight, and no agglutination was observed when they were challenged with mt^- tester gametes. This showed that none of the progeny had restored a functional plus mating type, again indicating the absence of recombination between the two mutations.

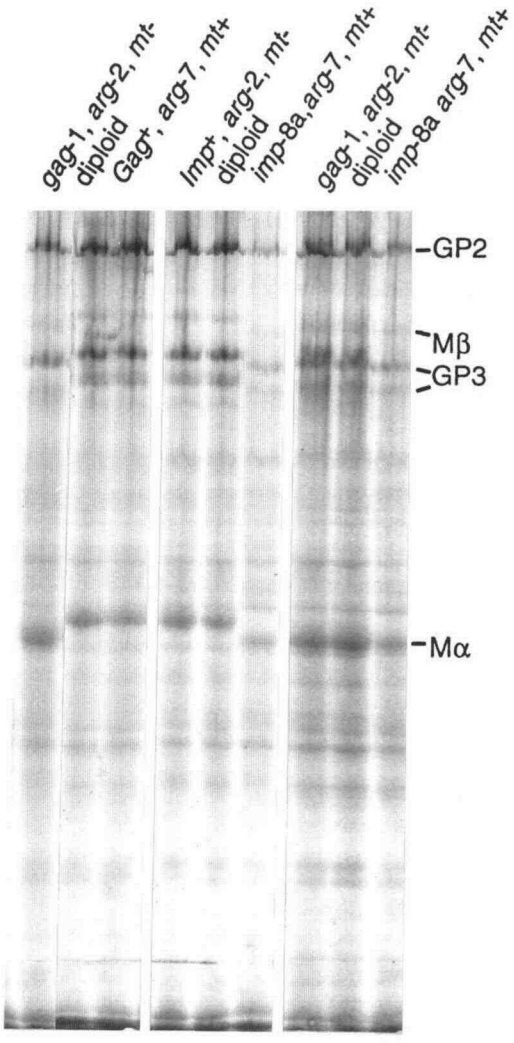

Figure 5. Complementation analysis of *gag*-1 and *imp*-8. SDS-PAGE of total proteins from cells grown at 32°C and nitrogen starved. For the three different crosses described in text, one diploid strain is shown between its two parents, whose genotypes are indicated. PAS staining was followed by dilute Coomassie blue.

DISCUSSION

In the present study, we describe a mutation affecting the major *O*-glycosylated proteins of *C. reinhardtii*. This phenotype is accompanied by a defect in the release of daughter cells and by mt^+-limited sterility. Perfect co-segregation of these three phenotypes in crosses, and the fact that they also accompany an independent mutation that we show to be allelic to *gag*-1, strongly suggest that the three phenotypes are caused by a single mutation and not by tightly linked mutations. Although the possibility remains that the *gag*-1 and *imp*-8 mutations affect two (or more) closely linked genes of unrelated functions (for example as large deletions), we favor a more straightforward model in which a single gene product is affected. This product may of course be a common regulator of several distinct pathways controlling either glycosylation, hatching, or sexual recognition, but we will primarily entertain the more simple hypothesis that a single defect in glycosylation is the direct cause of the delayed separation of daughter cells and lack of sexual agglutination.

Glycoproteins and Wall Degradation

The observed deleterious effect on hatching of a mutation affecting *O*-glycosylation is not unexpected, if only because of the prevalence of glycoproteins in the *Chlamydomonas* cell wall (Catt et al., 1976). Although no gross change could be detected in the appearance of the mutant wall in thin sections, its molecular assembly may be altered in a subtle way. Changes in the physicochemical properties of its surface may lead to the observed increased propensity of the mutant walls to adhere to other walls, via both the inner and the outer surfaces.

In addition, the absence of specific oligosaccharides in the mutant wall may be responsible for an increased resistance to degradation. Hatching of the zoosporangium can be obtained in vitro by the action of the well-characterized gamete lysin, a protease known to be involved in the shedding of the gametic wall at mating (Matsuda et al., 1985). In vivo, however, a distinct sporangial autolysin appears to be involved in the opening of the zoosporangium. This enzyme has been partially purified and seems to act primarily as a protease (Jaenicke et al., 1987). Its substrate(s) has not been precisely identified, but the wall proteins are the most likely candidates. Interestingly, the oligosaccharide side chains of the wall proteins appear to play a crucial role in wall lysis (Waffenschmidt et al., 1988). Altered side chains in the *gag*-1 glycoproteins may render them more resistant to the action of the autolysin. Alternatively, and perhaps concomitantly, aggregation may result from improper production of enzymes involved in wall degradation, if they are themselves *O*-glycosylated.

Glycoproteins and Sexual Recognition

Our unexpected finding that mutations affecting *O*-glycosylation in *C. reinhardtii* cause sex-limited sterility has some important implications in our understanding of sex determination in this alga. Heterothallism in *C. reinhardtii* rests on the existence of a single mating-type locus comprising a number of genes between which meiotic recombination is impaired (Gilham, 1969). Some of these genes have "housekeeping" functions, whereas others play an obvious role in sexuality. Two kinds of sex-determination genes can be expected to reside in the *mt* locus (Galloway and Goodenough, 1985): genes encoding mating-type specific components of the sexual apparatus, and genes encoding regulator proteins governing (positively or negatively) the expression of other sex-related genes located outside the *mt* locus itself. Molecular analysis of the mating-type locus has provided evidence for the presence of both types of genes (Ferris and Goodenough, 1994). For example, the mt^+-specific *imp*-1 gene may encode a structural protein of the mating structure or a positive regulator of such genes, whereas the mt^--specific *imp*-11 gene may be a negative regulator of several mt^+-specific genes.

The hypothesis of master regulator genes involved in determination of the mating type rests in part on the existence of mutations causing sex-limited sterility but unlinked to the *mt* locus. The mt^--limited *gam*-1 mutation (Forest and Togasaki, 1975) represents an example of such a gene, as do the mt^+-limited mutations in the *sag*-1 and *sag*-2 genes. Indeed, the sex-limited expression of the sterility phenotype of *sag* mutants led Goodenough et al. (1978) to propose that the *sag*-1 and *sag*-2 genes are transcriptionally or posttranscriptionally controlled by a sex-specific regulator gene of the mt^+ locus. Our finding, however, that the glycosylation defect of *gag*-1 (*sag*-2) mutants is expressed irrespective of the mating type implies that the gene is transcribed in both mating types, i.e. is not under control of the mating-type locus. Formally, the *sag*-2 mutations can be viewed as epistatic to the mt^+ locus, in the sense that they prevent full expression of the mt^+ phenotype.

This finding may lead us to consider more carefully the hypothesis of positive regulator genes governing sex determination in *Chlamydomonas*. In particular, the possibility should be considered that the other known genes with sex-limited sterile alleles, such as *sag*-1 and *gam*-1, or the *Chlamydomonas monoica mtl*-2 gene (VanWinkle-Swift and Hahn, 1986), are also expressed in both mating types, but are required for fertility in only one of them. This question will be answered only when the genes are cloned, or if mating-type-independent phenotypes are found to be associated with these mutations.

How can a defect in glycosylation result in sterility? A simple hypothesis is that a component of the mt^+ mating apparatus requires this specific type of *O*-glycosylation, either for proper assembly or for full activity. The sexual agglutinin, belonging to the family of Hyp-rich glycoproteins (Cooper et al., 1983), is an obvious candidate for such a role. Experiments with the purified mt^+ agglutinin have shown that *O*-linked carbohydrates are critical for its function (Collin-Osdoby et al., 1984), and analysis of the *imp*-8 mutant shows that it lacks agglutinin activity and the corresponding polypeptide (Adair et al., 1983). In contrast, the mutant has a normal complement of a series of other gamete-specific fibrillar molecules of the flagellar surface, known as short canes, loops, and crescents (Goodenough et al., 1985). The primary, if not sole, reason for the lack of agglutinability of the *imp*-8 and *gag*-1 mutants, therefore, appears to be a defect in agglutinin production.

Note that the specific effect of these mutations on the plus mating type closely resembles the specific effect of tunicamycin, an inhibitor of *N*-glycosylation, on agglutinability of mt^+, not mt^-, gametes (Matsuda et al., 1981; see, however, Ray and Gibor, 1982). The two effects may be related, since drugs affecting *N*-glycosylation can also indirectly alter *O*-glycosylation patterns (Naim, 1994).

Nature of the *gag*-1 Mutation

What is the exact nature of the *gag*-1 mutation? At present, and in the absence of a comparison of the structure of *O*-linked sugars in the WT and mutant strains, we can only state that the mutation affects an enzymatic system involved in the addition of one or several types of sugar residues, without preventing other glycosyltransferase reactions. The product of the *gag*-1 gene may be a glycosylation enzyme or a specific factor required for such an activity. As a result of the mutation, glycoproteins are smaller in M_r and fail to express reactivity to antibodies raised to normal glycoproteins.

The temperature dependence of the general glycosylation defect can at first be interpreted as an indication that the mutation itself is temperature sensitive, i.e. that it results in the production of a protein with reduced thermal stability. Alternatively, the mutant gene product may be stable but inactive at high temperatures. One problem with this category of hypotheses comes from the observation that the sterility phenotype is already fully expressed at 18°C, whereas the bulk of the glycoproteins appears normal. Nevertheless, this could be explained if the mutant gene product was not fully functional at 18°C. At this temperature, a small fraction of the glycoproteins could escape proper glycosylation, although too little to be detected by PAS staining. In the case of the agglutination-related protein, such improperly glycosylated molecules could impair the function of the normal proteins by associating with them in a nonfunctional configuration. Indeed, the agglutinin has been hypothesized to function as a dimer (Adair et al., 1983). A related hypothesis is that the mutation would alter not only the stability of the enzyme at 32°C but also its specificity at 18°C. At the latter temperature, cell wall proteins and amino acid oxidase subunits would be properly glycosylated, but the agglutination-related molecule would not.

Although these models remain reasonable working hypotheses, the available genetic evidence seems to argue against them. The two independent mutants that have been obtained in this gene show the same combination of temperature-dependent glycoprotein defect and constitutive sterility. Since the mutation does not seem to alter viability, even at the restrictive temperature, loss-of-function mutations should not be counter selected and would be expected to arise much more frequently than would temperature-sensitive mutations in this precise range of temperatures. In addition, even if null mutations could not be recovered for some unknown reason, it would remain surprising that two independent conditional mutations affecting enzyme stability at 32°C would also specifically affect agglutinin glycosylation at 18°C.

Therefore, we are led to formulate other types of models, all based on the assumption that the two mutations observed in the *gag*-1 (*sag*-2) gene are null mutations. First, the WT gene product could be a cofactor or modulator of the considered glycosylation enzyme, necessary for maintaining the enzyme (or its substrates) in the proper configuration. This factor would be necessary at high temperatures but dispensable for glycosylation of most of the substrates at 18°C. The agglutinin molecule would show a more stringent requirement for this factor, so that it could not be processed by the enzyme in its absence, even at 18°C.

An alternative hypothesis is that two parallel pathways, or two isoenzymes, can concur to this specific type of

O-glycosylation. One of the pathways would be constitutively defective in the *gag*-1 mutants. The other enzyme would take over function at low temperatures, explaining the normal glycosylation pattern observed at 18°C. However, it would be unable to fully glycosylate the proteins when the temperature was raised, because it would become unstable or inactive. Only the first pathway, abolished in the mutant, would be capable of producing the functional mt^+-specific protein required for sexual agglutination.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of F. Lacarrière for tetrad analysis and M. Recouvreur for EM. We thank J. Girard-Bascou for guidance in experimental genetic techniques and for fruitful discussion.

Received November 29, 1994; accepted February 15, 1995.
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