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Cell wall regeneration in *Chlamydomonas*: Accumulation of mRNAs encoding cell wall hydroxyproline-rich glycoproteins

(cDNAs)

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ABSTRACT **The unicellular alga *Chlamydomonas reinhardtii* is surrounded by a cell wall composed entirely of hydroxyproline-rich glycoproteins (HRGPs). When the walls of vegetative cells are removed with the enzyme gamete autolysin (g-lysin), they regenerate a matrix within 3–4 hr. *In vitro* translation of mRNAs isolated from g-lysin-treated cells showed significant increases and decreases in abundance of several mRNAs encoding proline-rich polypeptides. Because the population of up-regulated mRNAs is likely to include species encoding cell wall components, expression of genes for two outer wall HRGPs (GP1 and GP2) was analyzed during wall regeneration by using cDNAs isolated from a *C. reinhardtii* λgt11 library. Transcripts encoding GP1 and GP2 were elevated severalfold within the first hour of regeneration, suggesting that up-regulation of HRGP mRNAs is a primary response to cell wall removal by g-lysin. Cell wall regeneration in *Chlamydomonas* provides an accessible system to study HRGP gene expression during matrix development.**

Chlamydomonas reinhardtii, a unicellular alga, has a multilayered cell wall constructed from hydroxyproline-rich glycoproteins (HRGPs), lacking the abundant carbohydrate polymers of higher plants (1, 2). Each wall layer contains a distinct set of components (3–5), which form two major domains. The inner (framework) domain maintains the overall shape of the matrix and resists extraction by chaotropic agents, perchlorate, and sarkosyl/urea solutions (6–8). By contrast, the outer domain is easily solubilized and is capable of *in vitro* assembly (5–7, 9–11). These properties have been exploited for the purification of the major outer wall HRGPs (GP1, GP2, and GP3), their biochemical and morphological characterization (5), and *in vitro* assembly of individual sublayers from purified components (11, 12).

While studies of *in vitro* assembly are contributing much to our understanding of HRGP interactions, information on HRGP gene expression during cell wall development is lacking. In this study, abundance of mRNAs encoding proline-rich polypeptides is examined during cell wall regeneration induced by the wall-degrading enzyme gamete autolysin (g-lysin). During the sexual cycle of *C. reinhardtii*, g-lysin acts to degrade gamete cell walls as a prelude to cell fusion and zygosis. Vegetative cells, which also serve as substrate for g-lysin, respond to wall removal by assembling another matrix within 3–4 hr (13, 14). Here we show that cell wall regeneration is accompanied by significant changes in levels of translatable mRNAs for proline-rich polypeptides; several transcripts increase as an early response to wall removal, while others decline. Using GP1 and GP2 cDNAs isolated from a vegetative *Chlamydomonas* λgt11 library, we demonstrate that levels of mRNAs encoding both outer wall HRGPs increase severalfold during the first hour of regeneration. These observations suggest that changes in gene expression are likely to play an important role in *Chlamydomonas* cell wall regeneration, which is a useful experimental system to study HRGP gene expression.

MATERIALS AND METHODS

Strains and Culture Conditions. Wild-type strains CC-620 (mt^+) and CC-621 (mt^-) and the flagella-less mutant *bald-2* used in these studies are available from the *Chlamydomonas* Genetics Center (Department of Botany, Duke University, Durham, NC). Gametes were harvested in nitrogen-free high-salt medium (15) after 7 days of growth on Tris acetate/phosphate (TAP) plates (16). Vegetative cells were grown in constant light in liquid TAP medium for 36–48 hr.

Preparation and Use of g-Lysin. Gametes (10^{10}) of each mating type (mt^+ and mt^-) were suspended in nitrogen-free medium (NFHSM) and mixed at a concentration of 10^7 cells per ml. After 15 min, cells were pelleted at 2000 × *g* and discarded. The g-lysin-containing supernatant was clarified by centrifugation at 40,000 × *g* for 20 min and used without further purification.

Vegetative mt^- cells (4 liters) were grown in liquid TAP to a concentration of ≈8.5×10^5 cells per ml and harvested in an IEC chemical centrifuge. Cells were briefly pelleted (1 min at 3000 rpm; Sorvall GSA) and resuspended in g-lysin at a concentration of 10^7 cells per ml. After a 20-min digestion, cells were collected by a brief centrifugation as described above, washed, and resuspended in TAP at 10^7 cells per ml. Greater than 90% of the cells were stripped of their walls, as judged by a rounded morphology and by sensitivity to detergent lysis (17).

Antibody Production. Outer wall HRGPs were extracted from intact vegetative *bald-2* cells using 1 M sodium perchlorate (5). GP1 and GP2 were purified from *in vitro*-assembled wall crystals by preparative fast protein liquid chromatography (FPLC) (5). Prior to injection into New Zealand White rabbits (18), proteins were further purified by preparative SDS/PAGE (5% curtain gel). Booster immunizations used FPLC-purified GP1 and GP2, deglycosylated with pyridine/hydrofluoric acid (19). Prior to library screening (see below), anti-GP1 and anti-GP2 IgG fractions were preadsorbed against intact *Escherichia coli* strain 1090r$^-$, and a sonicate of 1090r$^-$ coupled to Reacti-Gel 6X (Pierce).

Western Blotting. Cell wall samples were electrophoresed on 5–15% gradient SDS/polyacrylamide minigels and electroblotted onto Immobilon PVDF paper (Millipore). Duplicate lanes were excised, briefly washed with phosphate-buffered saline (PBS)/0.5% bovine serum albumin, and incubated for 1 hr with blocking buffer (PBS/5% nonfat dry milk/0.1% Tween 20). One lane was incubated with polyclonal IgG (1:500 dilution), and the other was incubated with affinity-purified

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Abbreviations: HRGP, hydroxyproline-rich glycoprotein; g-lysin, gamete autolysin.
*Deceased March 27, 1990.

antibody (1:10 dilution) for 1 hr at 25°C. Filters were washed three times for 10 min each with blocking buffer, incubated for 1 hr with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2000), and developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega).

Isolation of GP1 and GP2 cDNAs. A total of 500,000 recombinants of a vegetative *C. reinhardtii* λgt11 library (generously provided by Stephen Mayfield) were screened with each antiserum (20). Primary positives were rescreened as "microlifts" (25-mm nitrocellulose disks), using antibodies affinity-purified against Western blots of deglycosylated GP1 and GP2 (21). GP1 (λMG1.6a) and GP2 (λMG2.5d) cDNAs [0.54 and 1.3 kilobases (kb), respectively] identified by this procedure were used for immunoselection, Northern blot analysis, and DNA sequencing (see below). Clone λMG1.6a was used as a hybridization probe for isolation of cDNA λKA1.1 from a gamete *C. reinhardtii* λgt11 library (mt^+), prepared from poly(A)$^+$ RNA by the method of Gubler and Hoffman (22) using murine reverse transcriptase (BRL).

RNA Isolation. Cells (1.5×10^8) were pelleted and resuspended in RNA lysis buffer (23). Lysates were mixed in a Vortex and frozen in liquid N_2, and RNA was isolated by the method of Kirk and Kirk (23).

***In Vitro* Translation/Immunoprecipitation.** Total RNA (10 μg) was translated *in vitro* with rabbit reticulocyte lysate (Promega) in the presence of 25 μCi of [^{35}S]methionine (1 Ci = 37 GBq) or 100 μCi of L-[2,3,4,5-^{3}H]proline for 90 min at 30°C in a vol of 25 μl. A 2-μl aliquot of each reaction mixture was diluted 1:4 with 2× SDS lysis buffer and boiled for 2 min, and 5 μl was analyzed by SDS/PAGE (10). For immunoprecipitations, translation products were mixed with 160 μl of ^2H_2O and boiled for 2 min. Supernatants were clarified, made 1% SDS, and mixed with 4 vol of immunoprecipitation buffer (IPB), containing 50 mM Tris·HCl (pH 7.4), 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100. After preclearing (50 μl of a 1:1 suspension of protein A-Sepharose coated with preimmune IgG), samples were incubated with 30 μl of affinity-purified antibody/protein A-Sepharose overnight at 4°C. Beads were washed three times for 30 min each with IPB and TBS (25 mM Tris·HCl, pH 7.4/0.15 M NaCl) and solubilized with 25 μl of SDS lysis buffer, and 5-μl aliquots were analyzed by SDS/PAGE.

Northern Blot Analysis. Total RNA (10 μg) from control and g-lysin-treated cells at various times during wall regeneration was separated on a 1.2% formaldehyde/agarose gel (24) and blotted onto Nitro-plus paper (MSI). Blots were hybridized (18) with ^{32}P-labeled GP1 and GP2 cDNA inserts (≈10^9 cpm/μg), prepared by the random-primer method (25). Final washes were 0.1× SSC/0.5% SDS at 65°C. RNA loadings were verified by ethidium bromide staining of duplicate lanes and by hybridization with a cDNA insert (MG1.10a) that encodes a constitutively expressed mRNA (W.S.A., unpublished data).

DNA Sequencing. Phage cDNA inserts were excised with *Eco*RI and subcloned into pGEM-4 (Promega). Sequences were determined by the dideoxynucleotide chain-termination method (26) using Sequenase (United States Biochemical). Both strands were sequenced, using SP6, T7, and synthetic oligonucleotide primers. Open reading frames were identified by using the program DNA INSPECTOR II (Textco) and the published codon usage of *C. reinhardtii* nuclear genes (27, 28).

RESULTS

g-Lysin Regulation of mRNAs Encoding Proline-Rich Polypeptides. *Chlamydomonas* cells stripped of their cell walls with g-lysin rapidly regenerate another matrix over 3–4 hr (13, 14). To examine the expression of genes encoding proline-rich polypeptides during cell wall regeneration, total RNA was isolated at various times after wall removal and was translated *in vitro* with [^{35}S]methionine or [^{3}H]proline. Fig. 1 shows that changes in relative abundance of several transcripts encoding proline-rich products are apparent within 15 min of recovery from g-lysin treatment. By 45–60 min several proline-rich products have increased severalfold, in a relatively coordinate fashion. Especially prominent are a doublet of ≈160 kDa and a broad band of ≈100 kDa, the latter being almost undetectable in translations in which [^{35}S]methionine was used. Similar results have been reported in abstract form by Su *et al.* (29). Interestingly, g-lysin treatment results in decreased levels of certain other proline-rich products (Fig. 1, asterisks). Down-regulation by g-lysin has not been previously reported.

Isolation of GP1 and GP2 cDNAs. While it is reasonable to assume that mRNAs encoding cell wall HRGPs are among the transcripts up-regulated by g-lysin, *Chlamydomonas* is known to possess at least one additional class of HRGPs—sexual agglutinins (30, 31). Therefore, to determine whether mRNAs for known cell wall HRGPs accumulate during wall regeneration, rabbit polyclonal antibodies to two outer wall HRGPs (GP1 and GP2) were used to isolate GP1 and GP2 cDNAs from a vegetative *C. reinhardtii* λgt11 cDNA library. Initial screens identified several candidate clones for each;

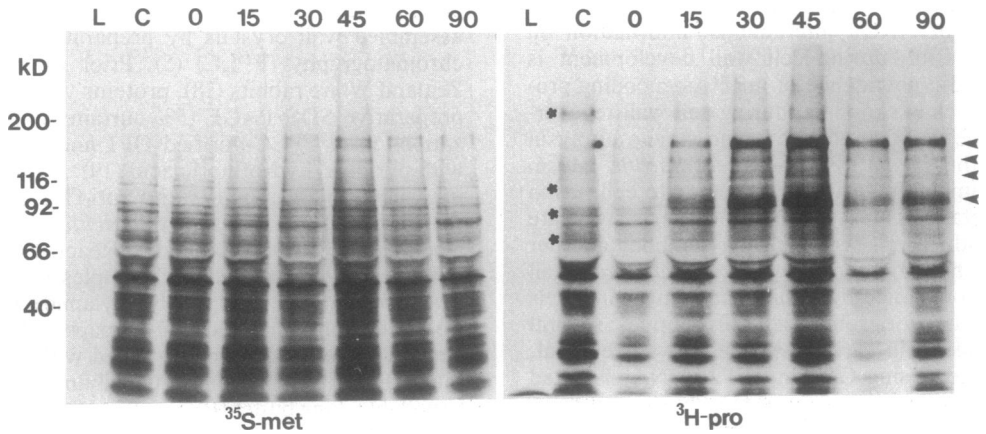

FIG. 1. Total RNA (10 μg) isolated from untreated controls and cells at the indicated times (min) during cell wall regeneration were translated *in vitro* with [^{35}S]methionine (*Left*) or [^{3}H]proline (*Right*). Translation products were separated by SDS/PAGE on a 5–15% gel and were detected by autoradiography. Lanes: L, lysate control (no added RNA); C, untreated controls. Arrowheads indicate bands that increase during regeneration; asterisks indicate bands that decrease. Cells were transferred to regeneration medium at time 0 (i.e., 20 min after addition of g-lysin).

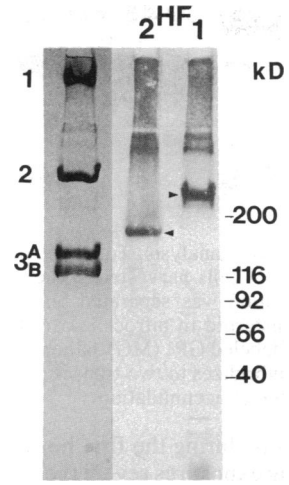

FIG. 2. Deglycosylated GP1 and GP2. FPLC-purified GP1 and GP2 were deglycosylated with pyridine HF, fractionated by SDS/PAGE, and electroblotted onto PVDF paper. Arrowheads denote GP1 and GP2 bands used for antibody purification. Left lane, outer cell wall crystals, showing native (glycosylated) forms of the three major outer wall HRGPs (GP1, GP2, and GP3 A and B). Middle lane, HF-GP2. Right lane, HF-GP1.

however, preliminary analysis of each set indicated the presence of more than one sequence. To identify bona fide GP1 and GP2 clones, initial "positives" were rescreened with antibodies affinity purified from Western blots of deglycosylated GP1 and GP2 (21). Bands used as affinity reagents are shown in Fig. 2 (arrows). Microlift screening of primary clones unambiguously identified two GP2 (λMG2.5d, λMG2.4b) clones and one GP1 (λMG1.6a) clone as true positives. Clones λMG1.6a [493 base pairs (bp)] and λGP2.5d (833 bp) were analyzed further as described below.

Verification of Clone Identities. To verify the identities of λMG1.6a and λMG2.5d cDNAs, the procedure outlined above was reversed; i.e., fusion protein expressed by each clone was adsorbed to nitrocellulose and was used to affinity purify the corresponding antibody. Fig. 3 is a Western blot comparing IgG before and after affinity purification. Lanes A and C show that, prior to selection, each antiserum reacts with multiple cell wall components, a consequence of immunodominant carbohydrate epitopes shared by the primary immunogens (31). By contrast, antibodies affinity purified against λMG1.6a and λMG2.5d fusion proteins display strong and specific reactivity toward GP1 and GP2, respectively (lanes B and D).

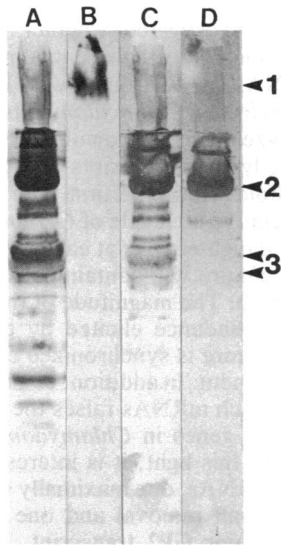

FIG. 3. Western blot of outer wall HRGPs. GP-β-galactosidase fusion proteins expressed by cDNA clones λMG1.6a (GP1) and λMG2.5d (GP2) were adsorbed to nitrocellulose and used as affinity reagents for isolation of corresponding antibodies. Starting antisera and affinity-purified antibodies were used to probe Western blots of cell wall crystals (see Fig. 2). Lanes: A, unselected anti-GP1; B, anti-GP1 selected by λMG1.6a; C, unselected anti-GP2; D, anti-GP2 selected by λMG2.5d.

DNA Sequencing. λMG1.6a and λMG2.5d inserts were subcloned into the plasmid vector pGEM-4 and sequenced in their entirety. Fig. 4*A* shows the complete sequence of MG1.6a. Three features are noteworthy. First, the indicated open reading frame conforms to the strongly biased codon usage of *C. reinhardtii* nuclear genes (27, 28) and predicts a peptide containing 26% proline, including a central region (underlined) that is 50% proline. Second, within an extended Pro-$(Xaa)_n$-Pro (n = 1 or 2) region of this domain are two $Xaa(Pro)_3$ sequences. $Xaa(Pro)_2$, $Xaa(Pro)_3$, Pro-$(Xaa)_{1-2}$-Pro motifs, recently identified in cDNAs encoding *Chlamydomonas* zygote

A

5'
TTAGCTCCAGAACCCGAAGAACAACGGGCGCGGTACAACTACCGCGTGCGGCGTTAGAAGGTCAA
Leu Ala Pro Glu Pro Glu Glu Gln Arg Ala Arg Val Gln Leu Arg Val Arg Pro Leu Glu Gly Gln

CCCGGTCCACCACCGCAGCTACGGTCACCCCATCGGCCGTTACCCCCACCACGGTATCCAGTACTTCGG
Pro Gly Pro Pro Pro Gln Leu Arg Ser Pro His Arg Pro Leu Pro Pro Pro Arg Tyr Pro Val Leu Arg

TCCCGTGGACTAGACCGACGCCCTAGTGTAACCGCCGCTACCCGTGCGCCTACTCCCACCGTGGTCACA
Ser Arg Gly Leu Asp Arg Arg Pro Val STOP

ACAGTGCTGGTCGAGCTACTGAGCTCATCTAGTCGCGCCCGAAGAGCCAGCGCTTTCGCGTGCGCCTA
CCACTGCAATCGCTGCCTCGGGTGCCACGCCGGTGGGCTCTTGTGTCGGACCGGGGTCGCTCCCAAGGA
CGACCCGAACCCGTAGGTCGGGAAGTCCAACGGGAACGTGAACCACGGCCGCTACAGCCCGAGCGGC
TGGTACGCGCTCCGCCTCGGCCCGGACCAGCTCCACGGCAAGTCCAGCAGCTTCCGCTACCGTACCGTA
CGTAGTCGGGTTTGGTCGAACTGC3'

ORF 2

Pro Pro Leu Pro Val Arg Leu Leu Pro Pro Trp Ser Gln His Val Leu Val Glu Leu Arg Ala His Leu Val
Ala Pro Glu Glu Pro Ala Leu Ser Leu Arg Leu Pro Leu Gln Ser Leu Pro Arg Val Pro Arg Arg Trp Pro
Leu Val Ser Asp Arg Gly Arg Ser Gln Gly Arg Pro Glu Pro Val Gly Arg Glu Val Gln Arg Glu Arg Glu
Pro Arg Pro Leu Gln Pro Glu Arg Leu Val Arg Ala Pro Pro Arg Pro Gly Pro Ala Pro Arg Gln Gln Gln
Leu Pro Leu Pro

B

5'
CGTGGTCTGAACGCTTACTGCGCTATTCAGAACCGCACCACCAACGCTTGGGCTTGCAACCTGGGC
Arg Gly Leu Asn Ala Tyr Cys Ala Ile Gln Asn Arg Thr Thr Asn Ala Trp Ala Cys Asn Leu Gly

GTGAACACTCCCACTGCTTGCGAGACCACCGATCTCTGGTCCACGGACGCCACTGAGTGCTCTCTG
Val Asn Thr Pro Thr Ala Cys Glu Thr Thr Asp Leu Trp Ser Thr Asp Ala Thr Glu Cys Ser Leu

CCTCAGGTCCCCGGCTTCCCCGTGCCCGCGAACACCTTCCCGTAGACGGACACTTGCCAGTACCAGGGT
Pro Gln Val Pro Gly Phe Pro Val Pro Ala Asn Thr Phe Pro Tyr Thr Asp Thr Cys Gln Tyr Gln Val

GCTCGCCTGCCCCTGGTCTAGAAGGGCACTACCACCGTTGGCGGCTTCCTGGTCCTGTTCAAGGAC
Ala Arg Leu Pro Leu Val Tyr Lys Gly Thr Thr Thr Val Gly Gly Phe Leu Val Leu Phe Lys Asp

TACGCCGACAACATCTACTACATCTCTGTCGCTGAACCGCGACTGACGGTGCCGCCAACCAGCCGGAT
Trp Ala Asp Asn Ile Trp Trp Thr Leu Ser Leu Asn Ala Thr Glu Arg Ala Ala Asn Gln Pro Asp

GGCCAGTGGCTGTACGTGGAGCCCACCCTGCCCGGTCAGACCGGCCCCACGCGCGCCGTCTACCTGTGG
Gly Gln Trp Leu Tyr Val Glu Pro Thr Leu Pro Gly Gln Thr Gly Pro Ser Gly Ala Val Tyr Leu Trp

GACTCTCCCCCGGACAGCTCCAAGCCTCTGTCGGTCCAGGCCTCCGTCCAGGACTACATGATTGAG
Asp Ser Pro Pro Asp Ser Ser Lys Pro Leu Ser Val Gln Gly Ser Val Gln Asp Tyr Met Ile Glu

GACCGCAACTCGTACAAGCGCTGGTCTTGCGCCACCTACAGGACTAGCCTGAACAACTACTGCGCC
Asp Arg Asn Ser Tyr Lys Arg Trp Ser Cys Ala Thr Tyr Lys Thr Ser Leu Asn Asn Tyr Cys Ala

CCCGGCTACGTGTTCGACGCGGCCGGCATTTGCCAGCCCAAGGCTGGTGCCACCCAGGGCCCCCTGCCC
Pro Gly Tyr Val Phe Asp Ala Ala Gly Ile Cys Gln Pro Lys Ala Gly Ala Thr Gln Gly Pro Leu Pro

TCCAAGGATCTGTCGGGCGCGGCTGGATCCTCTCTCTTCCTGGCGGCCGAGTACAGCATCGTCAAG
Ser Lys Asp Leu Ser Gly Ala Ala Gly Ser Ser Leu Phe Leu Ala Ala Gln Tyr Ser Ile Val Lys

TACTCGGTCTCCCAGATCGGCACTGCTGAGACCGGCTTCTACTGCGGTGACCCGCGCACCAGCATG
Tyr Ser Val Ser Gln Ile Gly Thr Ala Gln Thr Gly Phe Tyr Cys Gly Asp Pro Arg Thr Ser Met

ATCGCCGGCCAGACCCTGCAGACCCTGGCCCTTCAGGGCGGCGTCGTCACCCTGGCCGCCATCCCTGGC
Ile Ala Gly Gln Thr Leu Gln Thr Leu Ala Leu Gln Gly Gly Val Val Asn Leu Ala Ala Ile Pro Gly

TTCTTCCTGCCCCCAGCTGCAAGACCA3'
Phe Phe Leu Pro Pro Ala Ala Arg Pro

FIG. 4. (*A*) DNA and predicted amino acid sequences of MG1.6a. The indicated open reading frame predicts a peptide containing 26% proline, including a proline-rich (50%) central domain (underlined) with two $Xaa-(Pro)_3$ sequences. A second potential open reading frame (ORF 2) would encode a peptide containing 23% proline and containing several sequence motifs typical of *Chlamydomonas* HRGPs (underlined). A second GP1 cDNA (KA1.1) was sequenced in the bracketed region, using the indicated primer (boldface) to confirm the presence of the TAA stop codon. (*B*) DNA sequence of MG2.5d. The indicated open reading frame encodes a peptide containing 8% proline, including the indicated Leu/Pro, $Xaa-(Pro)_2$, and Pro-$(Xaa)_{1-2}$-Pro sequences (underlined).

and *Volvox carteri* inner wall HRGPs (32, 33), may be diagnostic features of these proteins. Finally, immediately following the indicated stop codon (TAA) is a second potential open reading frame. This sequence would encode a peptide with 26% proline, including an additional motif (Pro-Leu/Leu-Pro sequences) shared by several *Chlamydomonas* HRGPs, including the tetrapeptide Leu-Leu-Pro-Pro. The possible significance of this tetrapeptide is discussed below.

Fig. 4*B* shows the nucleic acid and derived protein sequences of MG2.5d. The predicted peptide contains 8% proline, with relatively proline-rich sequences interspersed with proline-poor regions, a feature not unexpected given the complex morphology of GP2, which contains both fibrous and globular domains (5). Present are two $Xaa(Pro)_2$ sequences (Ser-Pro-Pro and Leu-Pro-Pro), a 10-amino acid $Pro-(Xaa)_{1-2}$-Pro sequence and several Leu-Pro/Pro-Leu sequences (underlined). Of the 23 prolines, 7 are either preceded or followed by leucine.

Up-Regulation of GP1 and GP2 mRNAs During Cell Wall Regeneration. GP1 and GP2 messenger abundance following g-lysin treatment was analyzed by two methods. First, antibodies affinity purified against λMG1.6a and λMG2.5d fusion proteins were used to immunoprecipitate products of *in vitro* translation. Fig. 5 shows that both antibodies precipitate products significantly more abundant in translations of mRNA from regenerating cells (arrows). Note that the major translation product precipitated by anti-GP1 migrates faster than its GP2 counterpart—i.e., the opposite from what is observed with the native and HF-deglycosylated proteins (see Figs. 2 and 3). This reflects the fact that GP1 (native and deglycosylated) display an abnormal electrophoretic behavior in SDS gels (5, 11). The native protein, for example, migrates in the 4% stacking gel (Fig. 2). This unusual property, shared by both sexual agglutinins of *Chlamydomonas* (31, 34, 35), may reflect the extended, rod-like morphology and high hydroxyproline content of these HRGPs (4, 5, 11).

Accumulation of mRNAs encoding GP1 and GP2 during cell wall regeneration was also assessed by Northern blot analysis. In Fig. 6, total RNA isolated from untreated controls and cells harvested at various times during wall regeneration (10 μg per lane) was probed with ^{32}P-labeled inserts excised from plasmids pMG1.6 and pMG2.5d. Both cDNAs hybridize strongly with transcripts whose abundance increases dramatically during the first hour of cell wall regeneration. While long exposures reveal the presence of GP1 and GP2 transcripts in untreated controls (data not shown), low levels of cell wall mRNAs in asynchronously growing cultures suggest a tight regulation during the *Chlamydomonas* life cycle, a conclusion supported by recent studies of synchronous cultures (W.S.A. and S. Waffenschmidt, unpublished data). Note that MG1.6a hybridizes with two transcripts that display differential rates of accumulation after wall removal. The larger species (≈3.5 kb) is maximally up-regulated at the end of g-lysin treatment (time 0 in Fig. 6), while the smaller mRNA (≈3.3 kb) increases in parallel with the major GP2 transcript (≈5.0 kb), reaching maximal levels by 60 min. Longer exposures also reveal weak hybridization of MG2.5d to a second transcript (≈5.2 kb) whose abundance increases with the same kinetics as the 5.0-kb species.

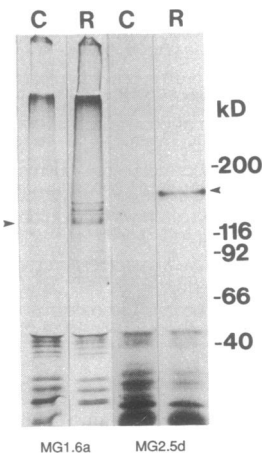

FIG. 5. *In vitro* translation/immunoprecipitation of RNA from control (lanes C) and regenerating (lanes R) cells. Total RNA (10 μg) from untreated controls and from cells regenerating new cell walls (60-min time point) was translated *in vitro*, and products were immunoprecipitated with affinity-purified anti-GP1 (*Left*) or anti-GP2 (*Right*). Arrowheads denote products specifically precipitated by antibodies purified against the indicated fusion proteins. Several lower by the indicated clones. Low molecular weight polypeptides are nonspecifically precipitated by both antisera.

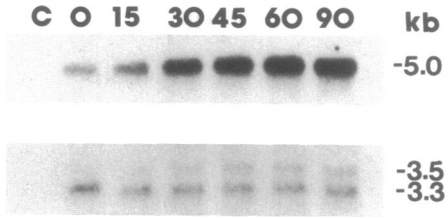

FIG. 6. Northern blot analysis. Total RNA (10 μg) from control cells (lane C) and from cells harvested at the indicated times (min) during wall regeneration was separated on a 1.2% formaldehyde agarose gel and transferred to nitrocellulose. Duplicate blots were hybridized with ^{32}P-labeled GP1 (MG1.6a) or GP2 (MG2.5d) inserts. Note that MG1.6a hybridizes to two transcripts (3.3 and 3.5 kb) that display different rates of accumulation.

DISCUSSION

This report demonstrates that cell wall regeneration in *Chlamydomonas* is accompanied by marked alterations in abundance of translatable mRNAs encoding proline-rich polypeptides; several transcripts increase severalfold as an early response to cell wall removal, while others decline. Since the *Chlamydomonas* cell wall is composed entirely of HRGPs (1–3), we investigated whether the group of up-regulated mRNAs includes species encoding known cell wall components. cDNAs for two outer wall HRGPs (GP1 and GP2) were isolated from a *Chlamydomonas* λgt11 library and were used to analyze the regulation of their mRNAs after cell wall removal by g-lysin. Transcripts encoding both HRGPs increased severalfold within the first hour of regeneration.

During the vegetative life cycle of *C. reinhardtii*, cell walls are assembled by daughter cells at each mitotic cycle. Thus, an asynchronous culture will contain cells in various stages of cell wall formation. The magnitude of the increase in GP1 and GP2 mRNA abundance elicited by g-lysin, therefore, suggests that the culture is synchronized for cell wall development by this treatment. In addition, coincident appearance of multiple proline-rich mRNAs raises the possibility that at least some cell wall genes in *Chlamydomonas* are coordinately regulated. In this light, it is interesting that MG1.6a hybridizes to two mRNAs, one maximally up-regulated as an early response to wall removal and one that increases in parallel with the major GP2 transcript. If coordinate up-regulation of the latter mRNAs is at the level of transcription, cell wall regeneration would resemble the well-studied process of flagellar regeneration in this alga. Following deflagellation, cells grow new flagella in ≈1 hr, a process requiring new protein synthesis (36). Among the many proteins whose synthesis increases dramatically are the flagellar tubulins (37, 38), whose four unlinked genes (39–41) are coordinately up-regulated at least partly at the level of transcription (42).

Plant HRGPs, while unrelated to HRGPs of animal cells (the collagens), nonetheless perform similar structural, developmental, and recognition functions. Compared with animal HRGPs, however, relatively little is known about plant HRGP interactions during matrix assembly. The *Chlamydomonas* outer wall is ideally suited to such studies; it has a highly organized lattice structure and a relatively simple composition (three HRGPs), and it can be assembled *in vitro* (5, 6, 11) from purified molecules (5, 11). In addition, *Chlamydomonas* is an excellent genetic system from which several cell wall-deficient mutants have been isolated. Isolation of GP1 and GP2 cDNA clones now adds an additional tool for analysis of HRGP structure–function. For example, identification of Xaa(Pro)$_{2-3}$ sequences in MG1.6a and a recently characterized *Chlamydomonas* zygote wall cDNA (32) suggests that this sequence motif is a general feature of the more fibrous HRGPs of this alga. In analogy with the Ser-(Pro)$_4$ repeats of higher plant extensins (43), Xaa-(Pro)$_3$ sequences may participate in polyproline II helix formation.

The presence of a second potential open reading frame in the MG1.6a sequence is intriguing; several explanations can be entertained. (*i*) It is coincidental, a consequence of the high G+C content (63%) of *C. reinhardtii* nuclear DNA. This is unlikely for two reasons. First, the open reading frame conforms to the biased codon usage of this alga, encoding a peptide (26% proline) with several sequence motifs diagnostic of *Chlamydomonas* HRGPs, including a predicted Leu-Leu-Pro-Pro tetrapeptide. Interest in this peptide derives from partial protein sequences of three *Chlamydomonas* HRGPs—GP2 CnBr peptide C-66, plus agglutinin, and minus agglutinin (W.S.A. and S. Siegel, unpublished data). All three have a Leu-Leu-Hyp-Hyp tetrapeptide, preceded by hydroxyproline in both agglutinins and by proline in C-66. Second, similar sequences are not found at the most 3′ end of the mRNA, as determined by partial sequencing of a second GP1 cDNA (λKA1.1) isolated from a gamete λgt11 library (unpublished data). (*ii*) The stop codon is a cloning artifact. This possibility has been ruled out by sequencing the corresponding region of the KA1.1 insert (Fig. 4*A*, brackets). (*iii*) Ribosomes read through the stop codon because of extensive secondary structure in this region. This is conceivable, given the high G+C content; however, there is, as yet, no precedence for this in *Chlamydomonas*. Also, TAA is the preferred stop codon. (*iv*) GP1 is a truncated version of an ancestral gene. This possibility has not been ruled out. (*v*) More than one GP1-related mRNA is derived from a single gene by differential splicing. This interesting possibility remains to be evaluated; it would be consistent with the presence of two transcripts hybridizing to MG1.6a, but the number of *Chlamydomonas* GP1 genes remains to be determined.

The predicted peptides encoded by MG1.6a and MG2.5d are clearly proline-rich (26% and 8%, respectively) and contain motifs characteristic of *Chlamydomonas* HRGPs. The proline content of the small sample of the GP2 coding sequence represented by MG2.5d is, however, below reported values (5) for the native protein (14.7% hydroxyproline, 7.7% proline), suggesting that the region represented by this cDNA lies outside the small fibrous (proline-rich) domain visualized by electron microscopy (4, 5). More detailed structural analysis of GP1 and GP2 requires more complete sequences. Finally, while similar sequence motifs are clearly shared by the three *Chlamydomonas* cell wall HRGPs analyzed to date, extensive sequence homologies have not been found. It is possible, therefore, that *Chlamydomonas* possesses multiple HRGP gene families.

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