Sulfur Economy and Cell Wall Biosynthesis during Sulfur Limitation of *Chlamydomonas reinhardtii*

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We have identified two novel periplasmic/cell wall polypeptides that specifically accumulate during sulfur limitation of *Chlamydomonas reinhardtii*. These polypeptides, present at high levels in the extracellular polypeptide fraction from a sulfur-deprived, cell wall-minus *C. reinhardtii* strain, have apparent molecular masses of 76 and 88 kD and are designated Ecp76 and Ecp88. N-terminal sequences of these polypeptides facilitated the isolation of full-length Ecp76 and Ecp88 cDNAs. Ecp76 and Ecp88 polypeptides are deduced to be 583 and 595 amino acids, respectively. Their amino acid sequences are similar to each other, with features characteristic of cell wall-localized hydroxyproline-rich glycoproteins; the N terminus of each polypeptide contains a predicted signal sequence, whereas the C terminus is rich in proline, alanine, and serine. Ecp76 and Ecp88 have either no (Ecp88) or one (Ecp76) sulfur-containing amino acid and transcripts encoding these polypeptides are not detected in cultures maintained on complete medium, but accumulate when cells are deprived of sulfur. This accumulation is temporally delayed relative to the accumulation of sulfur stress-induced arylsulfatase and ATP sulfurylase transcripts. The addition of sulfate back to sulfur-starved cultures caused a rapid decline in Ecp76 and Ecp88 mRNAs (half lives < 10 min). Furthermore, the *C. reinhardtii* sac1 mutant, which lacks a regulatory protein critical for acclimation to sulfur limitation, does not accumulate Ecp76 or Ecp88 transcripts. These results suggest that the Ecp76 and Ecp88 genes are under Sac1 control, and that restructing of the *C. reinhardtii* cell wall during sulfur limitation may be important for redistribution of internal and efficient utilization of environmental sulfur-containing molecules.

Sulfur is an essential macronutrient that is taken up by plant, algal, and microbial cells, primarily as the inorganic sulfate anion. Limitations for sulfur in the environment can inhibit plant growth and productivity (Grossman and Takahashi, 2001). However, plants are able to acclimate to sulfur-limited growth conditions by synthesizing enzymes that function in the efficient acquisition and utilization of both external and internal sulfur sources. Sulfur limitation may promote expression of genes encoding sulfatases and high-affinity sulfate transporters in both plants and algae (de Hostos et al., 1989; Yildiz et al., 1994; Smith et al., 1997; Takahashi et al., 2000). In *Chlamydomonas reinhardtii* and *Volvox carteri*, an extracellular arylsulfatase (encoded by the *Ars1* and *Ars2* genes in *C. reinhardtii*) can facilitate utilization of exogenous, esterified sulfate (de Hostos et al., 1988; Hallman and Sumper, 1994; Davies and Grossman, 1998). The induction of genes encoding ATP sulfurylase (*Ats1*), adenosine 5′-phosphosulfate reductase (adenosine 5′-phosphosulfate sulfotransferase), and Ser acetyltransferase during sulfur-limited growth may facilitate reduction of sulfate and the synthesis of Cys and Met (Gutierrez-Marcos et al., 1996; Setya et al., 1996; Yildiz et al., 1996; Takahashi et al., 1997). Sulfur limitation may also result in functional substitutions of proteins rich in sulfur with those having few sulfur-containing amino acids (Naito et al., 1994; Petrucco et al., 1996) and provoke protein degradation and recapture of the sulfur contained in Cys and Met (Collier and Grossman, 1992; Ferreira and Teixeira, 1992).

The acclimation of *C. reinhardtii* to sulfur limitation has been well characterized recently and has facilitated the isolation of mutants defective for the synthesis of the extracellular arylsulfatase and elevated sulfate uptake upon imposition of limitation conditions (Davies et al., 1994, 1996, 1999). One of the mutants was defective in a gene designated *Sac1*, which encodes a polypeptide that resembles an integral membrane, nutrient transporter (Davies and Grossman, 1998). It is interesting that the *Sac1* polypeptide appears to be critical for the induction of the arylsulfatase genes and the establishment of elevated rates of sulfate uptake during sulfur deprivation. Furthermore, unlike wild-type *C. reinhardtii* cells, this mutant was unable to down-regulate pho-

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tosynthetic activity and dies more rapidly than wild-type cells following exposure to sulfur deprivation. Survival of the mutant during sulfur deprivation was substantially increased if the cells were placed in the dark or treated with \(N'-(3,4\text{-dichlorophenyl})-N,N\text{-dimethylurea}\) (an inhibitor of photosystem II activity) prior to imposition of limitation conditions. These results suggest that the rapid death of the \(sac1\) strain during sulfur limitation is either a consequence of elevated levels of photosynthetically generated reactive oxygen species or altered cellular redox conditions (Davies et al., 1996).

Previous studies have shown remarkable differences in the patterns of extracellular polypeptides that accumulate in sulfur-starved and sulfur-replete, cell wall-minus \(C.\ reinhardtii\) cells (de Hostos et al., 1988; Davies et al., 1996). The extracellular polypeptides that accumulate during sulfur limitation may function in the efficient acquisition of nutrients or reflect changes in the cell surface structure that are important for redistribution and efficient utilization of sulfur-containing amino acids. The accumulation of sulfur starvation-specific polypeptides is not observed in the \(sac1\) mutant (Davies et al., 1994, 1996).

In this study, we have identified two prominent polypeptides of 76 (Ecp76) and 88 (Ecp88) kD that accumulated in medium of sulfur-starved, \(C.\ reinhardtii\) cells that are defective for cell wall biosynthesis (the cell wall-minus strains were used to facilitate the isolation and characterization of extracellular polypeptides). We determined the N-terminal amino acid sequences of Ecp76 and Ecp88 and used this information to generate specific oligonucleotide primers that allowed for the isolation of full-length cDNA clones. Characterizations of these cDNAs have demonstrated that the Ecp76 and Ecp88 genes (accession nos. AF359251 and AF359252, respectively) are only expressed during sulfur deprivation (not during nutrient-replete growth or phosphorus deprivation), and that the Ecp76 and Ecp88 polypeptides probably function as cell wall components that are tailored for cell maintenance during sulfur-limited growth.

### RESULTS

**Extracellular Polypeptides Associated with Sulfur Deprivation**

To investigate the effects of nutritional starvation on extracellular and/or cell surface polypeptides of \(C.\ reinhardtii\), we subjected cells to −sulfur or −phosphate conditions for 24 h, concentrated the polypeptides that accumulated in the culture medium, and resolved the polypeptides by SDS-PAGE. The \(C.\ reinhardtii\) strain used in these experiments (CC425) was unable to assemble cell walls, which resulted in release of extracellular polypeptides into the medium; these polypeptides may have been associated with cell walls or located in the periplasmic space. The use of this strain facilitated the isolation and identification of extracellular polypeptides.

As presented in Figure 1, a number of polypeptides present in the medium of cells maintained under nutrient-replete conditions were absent or markedly reduced in the medium derived from cultures exposed to either −sulfur or −phosphate conditions. Furthermore, several polypeptides were observed to specifically accumulate following the imposition of either −sulfur (Davies et al., 1996) or −phosphate (Quisel et al., 1996) conditions. Polypeptides that accumulated during −sulfur growth were designated Ecp70, Ecp76, Ecp84, Ecp88, and Ecp120 (Fig. 1). The designations reflect the extracellular location of these polypeptides (extracellular polypeptides) and their apparent molecular masses (70, 76, 84, 88, and 120 kD). We were able to obtain N-terminal sequences, shown in Table I, for Ecp70, Ecp76, and Ecp88. Although we were unable to obtain an N-terminal sequence for Ecp120, Ecp84 was a mixture of Ecp88 (its mobility during SDS-PAGE may have been different because of differences in post-

![Figure 1. Extracellular polypeptides from nutrient-replete (C), sulfur-starved (−S), and phosphate-starved (−P) cells. Extracellular polypeptides were isolated from the culture medium of CC425 cells, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue. The polypeptide designations are Ecp, for extracellular polypeptide, followed by the molecular mass in kilodaltons.](image-url)
Acclimation of *Chlamydomonas reinhardtii* to Sulfur Deprivation

Table 1. N-terminal sequences of extracellular polypeptides present during −sulfur growth

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecp76</td>
<td>Lys-Leu-Leu-Pro-Leu-Val-Pro-Ala-Asp-Phe-Ala-Thr-Leu-Tyr-Asp-Pro-Pro-Thr-Tyr-[Gln/Glu]-Glu-Lys-(Leu/Arg)</td>
</tr>
<tr>
<td>Ecp88</td>
<td>Ala-Tyr-Leu-Ser-Pro-Asp-Ala-Phe-Asp-Thr-Ile-Leu-Pro-Ala-Thr-Asp-Glu-Glu-Lys-Ile-Ala-Gln-Thr-Thr-(Trp)</td>
</tr>
<tr>
<td>Ecp70 (Ars)</td>
<td>Ala-Asp-Thr-Lys-Lys-Pro-Asp-Val-Val-Ile-Phe-Thr-Asp-Asp-Gln-Asp-Ala-Ile-Gln-(Gln)-Ser-[Thr/Ser]-His-Pro-(His)</td>
</tr>
</tbody>
</table>

cDNAs for Ecp76 and Ecp88

Fragments of cDNA clones encoding Ecp76 and Ecp88 were generated by PCR. DNA templates used for amplification were from recombinant λZAPII libraries harboring *C. reinhardtii* cDNAs that were synthesized from RNA isolated either from cells grown under nutrient replete, −phosphate, or −sulfur conditions (Davies et al., 1996). The forward primer used for the amplification was either the T3 or SK primer whereas the reverse primers were degenerate oligonucleotides that were designed using information derived from N-terminal amino acid sequences of Ecp76 and Ecp88. Nested PCR was performed (described in “Materials and Methods”) to help establish whether the fragments amplified during the initial PCR represented segments of the genes of interest. As presented in Figure 2, dominant, specific amplified fragments, marked with asterisks, were observed following primary PCR when template DNA was from the −sulfur cDNA library. Amplification of an Ecp76-specific fragment also resulted in the generation of numerous nonspecific fragments that migrated along the entire length of the agarose gel, no matter which template DNA was used (from nutrient replete-, −phosphate-, or −sulfur-grown cells). The specific fragments were purified and sequenced, and it was determined that the species marked with the asterisks in Figure 2 were derived from the Ecp76 and Ecp88 genes. These amplified fragments were hybridized to plaques from the −sulfur λZAPII cDNA library to isolate clones containing full-length Ecp76 and Ecp88 cDNAs. The isolated clones were used for Southern-blot hybridizations, which demonstrated that Ecp76 and Ecp88 hybridized to each other to some extent, but have no other homologs on the genome that are highly similar (hybridizations were performed at high stringency, data not shown).

Based on the deduced polypeptide sequences, Ecp76 and Ecp88 are comprised of 583 and 595 amino acids, respectively. Their deduced molecular masses are 62.3 and 63.9 kD, respectively, which is considerably less than the apparent molecular masses calculated from SDS-PAGE. These polypeptides show significant similarity to each other (Fig. 3), and also have features that are characteristic of polypeptides localized to the cell wall. Each of these polypeptides has an N-terminal leader sequence of 30 amino acids (boxed in Fig. 3) that may serve as a signal sequence involved in extracellular secretion. The alignment of Ecp76 and Ecp88 reveals conserved regions scattered along the entire length of the polypeptides, suggesting that these polypeptides serve a similar function. Although searching databases did not uncover polypeptides with strong sequence similarities to Ecp76 or Ecp88 over their entire lengths, it did reveal significant matches with regions of Hyp-rich glycoproteins (HRGs) of plants and algae (Wannenschmidt et al., 1993; Woessner et al., 1994; Rodriguez et al., 1999). The discrepancy between the apparent molecular masses determined by SDS-PAGE and the masses deduced from the cDNA sequence suggest that Ecp76 and Ecp88 are modified; previous work established that most of the *C. reinhardtii* extracellular polypeptides are glycosylated (Goodenough and Heuser, 1985; de Hostos et al., 1988; Adair and Snell, 1990; Ferris et al., 2001; A.R. Grossman, unpublished data). There is a strong bias in both Ecp76 and Ecp88...
polypeptide sequences against sulfur-containing amino acids. Ecp88 contains no sulfur amino acids, whereas Ecp76 contains a single Met (and no Cys residues; Table II). Furthermore, both polypeptides are highly enriched for Leu, Ala, Gly, and Pro; a high proportion of these amino acids are present in several of the HRGPs (Adair and Appel, 1989; Woessner and Goodenough, 1989; Adair and Apt, 1990; Woessner et al., 1994).

**Regulation of Ecp76 and Ecp88**

Accumulation of transcripts for Ecp76 and Ecp88 under different conditions was determined by RNA gel-blot hybridizations. We initially examined induction of mRNAs encoding Ecp76, Ecp88, Ars2, and Ats1 in a sak1-complemented mutant strain, as shown in Figure 4 (SacI/H11001, SacI/H11002). This strain is equivalent to wild-type cells with respect to sulfur stress responses (although it is derived from CC425 and lacks a cell wall) and is the control for evaluating the effect of the sak1 mutation (see “Introduction”) on expression of the Ecp76 and Ecp88 genes. The results presented in Figure 4 suggest that the accumulation of Ecp76 and Ecp88 polypeptides is a consequence of mRNA accumulation during sulfur-limited growth. Kinetic analyses of transcript accumulation demonstrated that mRNAs encoding Ars2 (Davies et al., 1999) and Ats1 (Yildiz et al., 1996) accumulated at an earlier time following the imposition of sulfur conditions than that of Ecp76 and Ecp88. These results suggest that the breakdown of organic sulfur by arylsulfatase and subsequent uptake and reduction of sulfate is necessary during the early stages of sulfur deprivation (beginning during the first 2 h), with the synthesis of the putative alternative cell wall polypeptides occurring somewhat later.

As shown in Figure 5, the kinetics of accumulation of mRNAs encoding Ecp76, Ecp88, Ars2, and Ats1 in the cell wall-containing, wild-type strain of *C. reinhardtii*, CC125, are similar to those presented in Figure 4. Hence, the accumulation of transcripts encoding a number of different sulfur starvation-induced polypeptides appears to be similar in the cell wall plus and cell wall minus strains. Furthermore, after transferring sulfur-starved wild-type cells back to nutrient-replete medium, there was a rapid decline in the levels of all of the induced transcripts characterized; the transcripts decayed with half lives of less than 10 min, with an exceptionally rapid loss of transcripts encoding Ecp88 and Ars2 (Fig. 5).

The sak1 mutant (Davies et al., 1994; 1996) was previously shown to be unable to increase its capacity for sulfate uptake and was defective for the induction of Ars2 and Ats1 mRNA accumulation during sulfur starvation (Davies et al., 1994; Yildiz et al., 1996). Figure 4 shows that sulfur deprivation of the sak1 mutant (sak1, −S) does not elicit accumulation of Ecp76, Ecp88, and Ars2 mRNA and only causes very low level induction of Ats1. Hence, the Sak1 gene product is absolutely required for activation of Ecp76 and Ecp88 and for modulating cell wall composition as cells become sulfur starved.

Figure 3. Comparison of deduced amino acid sequences of Ecp76 and Ecp88. Identical amino acids are highlighted with a black background, whereas conserved amino acids are highlighted with a gray background. The putative signal sequences of the two polypeptides are boxed.
DISCUSSION

Photosynthetic organisms have evolved a number of different mechanisms that enable them to survive sulfur-limited growth for extended periods of time. These mechanisms are biochemically and temporally distinct; they represent a staged process that is tuned to the extent of sulfur deprivation experienced by cells. Over the course of sulfur starvation, expression of several genes that encode enzymes or polypeptides responsible for acquisition and assimilation of sulfur are induced. Such genes encode sulfate transporters (Yildiz et al., 1994; Smith et al., 1997; Takahashi et al., 2000), arylsulfatase (de Hostos et al., 1988), and enzymes required for the reduction and assimilation of sulfate (Gutierrez-Marcos et al., 1996; Setya et al., 1996; Yildiz et al., 1996; Takahashi et al., 1997). The accumulation of these polypeptides following sulfur deprivation would increase the cell’s capacity to access and take up the limited amount of sulfate in the environment and facilitate the synthesis of Cys and Met. When external sulfur sources are exhausted, organisms must also reallocate sulfur present in structural/functional components of the cell; this reallocation may help sustain cellular activities and facilitate acclimation processes. Degradation of proteins and metabolites occur under such circumstances (Collier and Grossman, 1992; Ferreira and Teixeira, 1992). For example, during sulfur deprivation, there is marked degradation of ribulose-1,5-bisphosphate carboxylase (Ferreira and Teixeira, 1992; Esquivel et al., 2000), the enzyme of the reductive pentose phosphate pathway that initiates the fixation of CO₂. In addition, sulfur-limited plants preferentially express proteins having few Cys and Met residues. These changes have been shown to occur for seed storage proteins and vascular plant enzymes that function in secondary metabolism (Naito et al., 1994; Petrucco et al., 1996). Plant and algal cells also have high levels of sulfur-containing lipids such as sulfoquinovosyl diacylglycerol, which may be important for photosynthetic function under certain environmental conditions. In some photosynthetic organisms, the level of this lipid is sensitive to the nutrient conditions. For example, in photosynthetic bacteria and plants the levels of sulfolipids increase and phospholipids decrease during phosphorus limitation (Benning, 1998). It is not known how sulfur limitation affects cellular lipid composition and membrane activities, but it is an area worth exploration.

C. reinhardtii contains a proteinaceous cell wall that is rich in sulfated, Hyp-containing glycoproteins or HRGPs (Miller et al., 1972, 1974; Roberts, 1974; Catt et al., 1976; Roberts et al., 1980; Adair and Apt, 1990; Ferris et al., 2001); these proteins often contain Ser-Pro and/or X-(Pro)₃ repeat units, are frequently rich in Cys residues (Adair and Apt, 1990; Woessner and Goodenough, 1992; Woessner et al., 1994; Rodriguez et al., 1999; Suzuki et al., 2000), although not always (Woessner and Goodenough, 1989), and specific

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Ecp76</th>
<th>Ecp88</th>
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<tbody>
<tr>
<td>Ala</td>
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<tr>
<td>Val</td>
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</table>

Table II. Amino acid composition of mature Ecp76 and Ecp88
Amino acid residues of the signal peptide are not included. Amino acids containing sulfur are in bold.

Figure 4. Accumulation of mRNA encoding Ecp76, Ecp88, arylsulfatase (Ars2), and ATP sulfurylase (Ats1). mRNA accumulation in the Sac1-complemented strain (Sac1+/H11001) and the sac1 mutant strain (Sac1) was evaluated by RNA blot hybridizations following transfer of the cells to −sulfur medium for 1, 2, 4, and 8 h. Equal amounts of RNA from starved and unstarved cells were resolved on agarose gels, blotted to nitrocellulose membranes, and probed with DNA fragments specific for Ecp76, Ecp88, Ars2, Ats1, and 18S rRNA. The 18S rRNA probe was used to confirm equal RNA loading.
HRGP family members are present in vegetative cells, gametes, and zygotes of the various members of the volvocales (Woessner et al., 1994; Rodriguez et al., 1999; Suzuki et al., 2000;). The cell wall HRGPs form a crystal structure with a central trilaminar morphology embedded in a meshwork of fibrils (Hills et al., 1975; Goodenough and Heuser, 1985; Goodenough et al., 1986; Adair et al., 1987). Cross-linking among the components of the wall may occur through the formation of isodityrosine bridges (Waffenschmidt et al., 1993) and as a consequence of transglutaminase activity (Waffenschmidt et al., 1999).

In this study, we identified two extracellular polypeptides, Ecp76 and Ecp88, that specifically accumulate when C. reinhardtii cells are starved for sulfur. These polypeptides, which have strong similarity to each other, have a high proportion of Pro, Ala, and Ser residues. A number of C. reinhardtii cell wall polypeptides contain significant stretches of alternating Pro and Ser residues. Ecp88 has an extended stretch of prolines at its C terminus (amino acid residues 541–588) that alternate with Ser, Thr, or Ala. Furthermore, like cell wall polypeptides, Ecp76 and Ecp88 contain a number of Asn X Ser/Thr potential glycosylation sites; for Ecp76, these sites are at amino acids 250, 348, 426, 482, and 497, whereas for Ecp88 they are at amino acids 90, 242, 417, 475, and 490. It was previously demonstrated that most extracellular polypeptides of C. reinhardtii are glycosylated (Miller et al., 1972, 1974; Goodenough and Heuser, 1985; Adair and Snell, 1990); glycosylation of Ecp76 and Ecp88 was suggested based on the observation that the apparent molecular masses of the polypeptides were significantly greater than those predicted from the amino acid sequences, and that tunicamycin, an inhibitor of protein glycosylation, prevents the accumulation of Ecp76 and Ecp88 (de Hostos et al., 1988). The extracellular Ars was shown to have at least three glycosylation sites; the polysaccharide moieties could be removed by endoglycosidase F (de Hostos et al., 1988). Glycosylation may augment the resistance of extracellular polypeptides to proteolytic attack and may also play a role in maintaining the specific architecture of the extracellular matrix.

There are a number of reasons why C. reinhardtii may change its complement of cell wall polypeptides. Specific cell surface polypeptides may be important functional components of cell walls such as those present in differentiated gametes or zygotes (Woessner and Goodenough, 1992; Rodriguez et al., 1999). Nutrient limitation has been shown to alter C. reinhardtii cell wall morphology and reduce the ability of the zooplankters Daphnia pulex and Daphnia magna to digest the alga (Van Donk et al., 1997). The putative cell wall polypeptides Ecp76 and Ecp88 are probably tailored to survival during sulfur-limited growth. This is reflected in the finding that the mature Ecp polypeptides have a single sulfur amino acid between them. The lack of sulfur-containing amino acids in these polypeptides was first suggested by in vivo labeling experiments in which extracellular polypeptides were labeled with $^{35}$SO$_4^-$ during exposure to sulfur conditions; incorporation of label into Ecp76 and Ecp88 was not detected (de Hostos et al., 1988). Substitution of sulfur-rich cell wall components (e.g. a number of the cell wall polypeptides in zygotes and vegetative cells contain several methionines and cysteines; Adair and Appel, 1989; Woessner et al., 1994; Rodriguez et al., 1999; Suzuki et al., 2000) with polypeptides that have either no or few sulfur-containing amino acids is likely to help conserve sulfur during periods of need and possibly allow for reallocation of sulfur present in dominant cell wall polypeptides of unstarved cells.

The accumulation of Ecp76 and Ecp88 mRNA was triggered by sulfur starvation conditions. However,
both the Ecp76 and Ecp88 transcripts increased in abundance more slowly than those encoding Ars2 and Ats1; this kinetic feature was observed for both CC425, the cell wall-minus strain used for most of the studies presented in this manuscript, and CC125, a cell wall-plus, wild-type strain. The results suggest that the function(s) of the Ecp76 and Ecp88 polypeptides is not likely to be related to acquisition and assimilation of external sulfur. The time lag of induction may reflect the difference in the extent of sulfur limitation critical for triggering expression from the Ecp76 and Ecp88 genes. Furthermore, levels of Ecp76 and Ecp88 mRNAs rapidly declined when sulfate was added back to cultures of sulfur-starved cells. A similar rapid decline in transcripts encoding Ars2 and Ats1 was observed.

Sulfur deprivation caused essentially no accumulation of Ecp76 and Ecp88 transcripts in the sac1 mutant. The Sac1 protein has significant similarity to integral membrane transport proteins (Davies and Grossman, 1998). However, it appears to strongly influence both the specific responses to sulfur growth (responses important for scavenging the limiting nutrient) and the general responses, which enable organisms to coordinate cellular metabolism with growth potential (Davies et al., 1996). A yeast (Saccharomyces cerevisiae) regulatory molecule that resembles a transporter was shown to be critical for the metabolism and utilization of Glc (Liang and Gabel, 1996). The finding that Ecp76 and Ecp88 are under Sac1 control reinforces the suggestion that Sac1 is involved in controlling essentially all aspects of sulfur limitation responses in C. reinhardtii.

MATERIALS AND METHODS

Strains and Growth Conditions

Chlamydomonas reinhardtii cells were grown in Tris-acetate-phosphate (TAP) medium at 25°C at a light intensity of 100 μmol photons m⁻² s⁻¹, as previously described (Davies et al., 1994). Medium devoid of sulfur (−sulfur) was prepared by replacing sulfate in the TAP medium with chloride (Davies et al., 1994). Medium devoid of phosphate (−phosphate) was prepared by replacing the potassium phosphate in the TAP medium with potassium chloride (Quisel et al., 1996). Genotypes of the strains used for this study are as follows: CC125 (mt⁺, nit1, and nit2), CC425 (mt⁻, cw15, nit1, nit2, and arg7-8), ars5-4 (mt⁺, cw15, nit1, and sac1::ARG7), ars5-4-C11 (mt⁺, cw15, nit1, sac1::ARG7, and Sac1+; Davies et al., 1996).

Purification and Sequencing of Extracellular Protein

Extracellular polypeptides were isolated from cultures (approximately 250 mL) of CC425 that were in the logarithmic phase of growth, as described previously (Davies et al., 1994). In brief, algal cells were grown either on complete medium or transferred to −sulfur (Davies et al., 1994) or −phosphate (Quisel et al., 1996; Wykoff et al., 1998) medium. Sulfur deprivation conditions were maintained for 24 h and phosphorus deprivation for 48 h before cultures were made 100 mM NaCl, 1 mM benzamidine-HCl, 1 mM e-amino caproic acid (protease inhibitors) and cells immediately separated from the medium by centrifugation at 4°C for 5 min at 4,000g. The medium was recentrifuged to remove remaining debris, the supernatant saturated to 80% (w/v) with ammonium sulfate, which precipitates essentially all extracellular proteins, and the precipitate was resuspended by brief sonication in 1 to 2 mL of 0.1 M Na₂CO₃-0.1 M dithiothreitol. The protein suspension was then made 2% (w/v) SDS/5% (w/v) Suc/0.1% (w/v) bromphenol blue, boiled for 1 min, and polypeptides in the sample resolved by SDS-PAGE on a 7.5% to 15% (w/v) polyacrylamide gel (de Hostos et al., 1988). Polypeptides were eluted from the gel and the amino terminal sequences determined by the Protein Micro Analytical Laboratory (California Institute of Technology, Pasadena).

Cloning of the cDNAs

Phage DNA was prepared from the control, −sulfur (Davies et al., 1996), and −phosphate (Wykoff et al., 1999) cDNA libraries of C. reinhardtii. The cDNA fragments generated for library preparation were cloned directionally between EcoRI and XhoI sites of the AZAP II vector (Stratagene, La Jolla, CA). One hundred nanograms of recombinant DNA was used as a template for PCR amplification of 5' ends of cDNAs representing the Ecp76 and Ecp88 genes. The Expand Long Template PCR System (Roche Diagnostics, Indianapolis) was used for the amplification reaction together with the T3 and Ecp76-R (5'-AGCTTCTCTCTG/C/G[A/T][A/G/C/A][G/T][G/C/A][G/G]-3') primers for Ecp76 and the T3 and Ecp88-R3 (5'-GT/G/C/GT/C/T[G/C/A][G/C/G/A][ATC/T/C/T-C'-3']) primers for Ecp88. Primary PCR was performed by the following two-phase program: five cycles of low stringency amplification (95°C for 30 s, 40°C for 1 min, and 68°C for 1 min) followed by 30 cycles of high stringency amplification (95°C for 30 s, 50°C for 1 min, and 68°C for 1 min). For Ecp76, 1 μL of the primary PCR product was re-amplified with the SK and Ecp76-R primers under high stringency conditions for 30 cycles to obtain an Ecp76-specific hybridization probe. For Ecp88, 1 μL of the primary PCR product was used for nested PCR, which was performed using high stringency conditions for 30 cycles. SK and Ecp88-R4 (5'-TAGAT[G/C/G][T/C/G/A][A/G/C/A][G/G/A/T/[C/T-C'-3']) primers were used to prime the nested amplification. Amplified fragments were purified from agarose gels, cloned into pGEM-T easy (Promega, Madison, WI), and sequenced. Full-length cDNA clones were obtained from the −sulfur cDNA library (Davies et al., 1996) by hybridization screening using Ecp76- and Ecp88-specific amplification products following standard protocols (Sambrook et al., 1989).

RNA Blot Hybridization

Cells (either strain CC425 or CC125) were grown in TAP medium to mid-logarithmic phase and subjected to −sul-
fur conditions as described previously (Davies et al., 1994). The procedures for extracting, transferring, and probing the RNA were performed according to Davies et al. (1992). cDNA inserts specific for Ecp76, Ecp88, Ars2 (Davies et al., 1999), Ats1 (Yildiz et al., 1996), and P-92 (18S rRNA, obtained from the Chlamydomonas Genetics Center) were labeled with ³²P-DCTP by the random priming method. Nitrocellulose membranes were washed under high stringency conditions (Sambrook et al., 1989) and exposed to X-Omat x-ray film (Kodak, Hollywood, CA).

Data Deposition

The sequences reported in this paper have been deposited in the GenBank/EMBL/DDBJ database (accession nos. AF359251 and AF359252).

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