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Cell-wall synthesis in *Chlamydomonas reinhardtii*: an immunological study on the wild type and wall-less mutants *cw2* and *cw15*

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Abstract. Cells of *Chlamydomonas reinhardtii* Dang. wild type and the cell-wall mutants *cw2* and *cw15* were grown synchronously. The two mutants secreted copious amounts of cell-wall-like glycoproteins into the culture medium in contrast to the wild type which released only minor quantities. Both the secreted proteins as well as those present in the lumen of the endoplasmic reticulum (ER) and Golgi apparatus (GA) were tested for cross-reactivity against a number of monoclonal antibodies (MACs) prepared against the 2BII glycoprotein cell-wall complex of the wild type (E. Smith et al., 1984, Planta **161**, 330–338). Of the four monoclonals applied, one, (MAC 6), did not react in either dot blot or Western blots with any of the luminal and medium proteins. By dot blotting, MAC 2 recognized polypeptides only in the wild-type medium. Neither MAC 2 nor MAC 6 were capable of recognizing polypeptides separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. However, MAC 2 recognized one of the 2BII polypeptides (135 kDa) as well as a large number of other polypeptides in wild-type and mutant media. The 135-kDa polypeptide was also detected in the luminal extracts of ER and GA membranes from the wild type and *cw2* mutant. It was also present in the GA fraction of the *cw15* mutant. If, as previously claimed, these monoclonal antibodies are indeed directed against the carbohydrate portion of the 2BII complex, our results would indicate that protein O-glycosylation is not restricted to the GA but may start in the ER. They also confirm inferences made by others that the cell-wall mutants *cw2* and *cw15* possess the capacity to synthesize and secrete the major glycoproteins of the cell wall, but, due to the lack of the W2 wall layer, are unable to assemble these components into a coherent, crystalline wall.

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Abbreviations: ER = endoplasmic reticulum; GA = Golgi apparatus; HRGPs = hydroxyproline-rich glycoproteins; kDa = kilodalton; MAC = monoclonal antibody; PAS = periodic acid-Schiff's reagent; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Key words: Cell wall (glycoprotein) – *Chlamydomonas* – Endoplasmic reticulum – Glycoprotein – Golgi apparatus – Mutant (*Chlamydomonas*, wall-less) – Protein glycosylation

Introduction

The green algal group, the Volvocales, occupies an unusual position in the plant kingdom in that their cell walls do not contain a microfibrillar polysaccharide. Instead they possess aggregates of hydroxyproline-rich glycoproteins (HRGPs), whose crystalline structure varies amongst the various species (Roberts et al. 1982, 1985; Goodenough and Heuser 1988a, b). The most frequently investigated member of this group, *Chlamydomonas reinhardtii*, has a wall consisting of several layers of interlocking fibrillar and granular elements (Goodenough and Heuser 1985). Two of these layers (W4 and W6) are readily removed by chaotropic agents, e.g. $NaClO_4$ or LiCl, and reassemble into a crystalline lattice in vitro upon dialysis (Catt et al. 1978; Goodenough et al. 1986). The major crystal-forming component, termed "2BII" or "Volvin" by Roberts et al. (1985) has been shown by Goodenough et al. (1986) to be composed of four different glycoproteins, three of which are hydroxyproline-rich.

A number of monoclonal antibodies (MACs) have been prepared against the 2BII complex (Smith et al. 1984), two of which (MAC 3 and MAC 6) were later used in an immunocytochemical study on cell-wall synthesis in *Chlamydomonas* (Grief and Shaw 1987). Because these two antibodies apparently recognize carbohydrate epitopes of 2BII glycoproteins, Grief and Shaw (1987) interpreted the almost exclusive immunolabelling of the Golgi apparatus (GA) as being evidence for a restriction of O-glycosylating reactions to this membrane compartment. This appears to be a logical conclusion in that the hydroxylation of peptidyl proline is known to occur in the endoplasmic reticulum (ER), both

in *Chlamydomonas* and higher plants (Sauer and Robinson 1985; Andreae et al. 1988a), and that this event preceeds temporally the arabinosylation of HRGPs. However, evidence has recently been presented which is at variance with this: hyp-arabinosyl transferase activity has been localized in the ER of both maize roots (Andreae et al. 1988b) and *Chlamydomonas* (Zhang et al. 1989). Since we are now able to isolate ER and GA fractions of high purity from *Chlamydomonas* we decided that the luminal contents of these fractions should be examined by immunoblotting. In addition we have carried out parallel investigations on the two wall-less mutants of *Chlamydomonas*: *CW2* and *CW15*. Our results indicate that glycoproteins occur in both the ER and the GA of this organism, and that the wall-less mutants, despite their inability to assemble a wall, secrete copious amounts of wall-like HRGPs.

Material and methods

Algae and culturing conditions: Chlamydomonas reinhardtii Dang. wild type (strain 137 c5) and the cell-wall mutants *cw2* and *cw15* were obtained from the Algal Culture Collection (Universität Göttingen, FRG) and the Chlamydomonas Genetics Center (Duke University, Durham, N.C., USA) respectively. All three algae were grown in the "TAP"-medium of Surzycki (1971) and synchronized according to the procedure described in Schlösser (1966). Aerated (3% CO_2) cultures were maintained in a light-thermostat at 30° C and subjected to light (2 klx): dark cycles of 12 h:12 h. The cell numbers were usually kept at $1.56 \cdot 10^6 \cdot ml^{-1}$ by daily dilution of the cultures.

Recovery of polymers released into the culture media. Algal culture (2 L) was centrifuged at $3000 \cdot g$ for 15 min to remove the algae. The supernatant was then reduced in volume to 100 ml with an ultra-filtration device (Heavy Duty Stirred Cell 4015; Amicon, Witten, FRG) using a cellulose triacetate membrane filter (SM 14539; Sartorius, Göttingen, FRG) with a cut-off at 10000 MW. The concentrated culture medium was then centrifuged at $100000 \cdot g$ for 1 h. The supernatant was carefully decanted and dialysed overnight against distilled H_2O, before freeze-drying.

Subcellular fractionation; extraction of luminal contents. Protoplasts of the wild-type cells were prepared through gamete autolysin treatment as given in Schlösser et al. (1976). Wild-type protoplasts and the mutants were exposed to digitonin and homogenized exactly as described by Andreae et al. (1988a). Homogenates were precentrifuged for 15 min at $6000 \cdot g$. The supernatant was either centrifuged at $100000\,g$ for 1 h to obtain a total membrane pellet or was added as an overlay (7 ml) to a sucrose step gradient (8 ml 15%, 8 ml 32%, 8 ml 38%, 6 ml 50%, w:w sucrose) which was centrifuged to isopycnic conditions ($100000 \cdot g$; 2 h). The compositions of the homogenizing and gradient media were exactly the same as those given in Andreae et al. (1988a); all operations were performed at 0–4° C.

Membrane fractions designated as ER or GA (see *Results*) were diluted at least threefold with low-Mg^{2+} gradient medium and centrifuged at $10000 \cdot g$ for 1 h in order to remove ribosomes. Luminal proteins were extracted from the membrane by resuspending in 4 ml of 0.1 M Na_2CO_3 at pH 11, sonicating (15 s, 200 W, micro-tip; Braun-Melsungen, FRG) and leaving to stand in an ice bath for 30 min (Fujiki et al. 1982). Luminal proteins were separated from the membranes by centrifuging at $100000 \cdot g$ for 1 h. The supernatant was then dialysed overnight against H_2O and freeze-dried.

Measurement of enzyme activities. Aliquots of resuspended membrane fractions were tested for the following enzymes as described in Andreae et al. (1988a) and Zhang et al. (1989): NADH: cytochrome *c*-reductase (EC 1.6.99.3; CCR); inosine 5-disphosphatase (EC 3.6.1.6; IDPase); prolyl-glycyl peptide, 2-oxo-glutarate: oxygen oxidoreductase, 4-hydroxylating (EC 1.14.11.2; prolyl hydroxylase); UDP-arabinose: hydroxyproline arabinosyl transferase; UDP-galactose: hydroxyproline galactosyl transferase.

Electrophoresis and immunoblotting. Polypeptides were separated under reducing conditions (50 mM dithiothreitol, DTT) by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in slab minigels ($85 \cdot 105 \cdot 1\ mm^3$; Biometra, Göttingen, FRG) as described by Depta and Robinson (1986). Both 10% and gradient (4–15%) gels were employed. Gels were stained with either Serva Blue 250 G (Neuhoff et al. 1985), silver stained (Heukeshoven and Dernick 1985) or stained for carbohydrate with periodic acid-Schiff's (PAS) reagent (Fairbanks et al. 1971).

For Western blotting; polypeptides were transferred electrophoretically (20 V, 75 mA, 12 h; wet chamber) onto nitrocellulose filters (SM 128; Sartorius, Göttingen, FRG) as described by Twobin et al. (1979). Blocking with bovine serum albumin and incubations with primary (optimal concentration $20\ \mu g \cdot ml^{-1}$) and secondary (peroxidase-coupled goat anti-rat IgGs, Sigma A-9037, dilution 1:500) antibodies were performed as already given (Andreae et al. 1988a). Bound antibody was identified with 4-chloro-1-naphthol (Vandesande 1983). For dot blotting, 3-µl sample aliquots (approx. protein concentration $0.1\ mg \cdot ml^{-1}$) were applied with a micropipette to nitrocelluose squares and left to dry at room temperature for 1–2 h. Cross-reaction with a particular antibody was determined according to the procedures described by Hawkes et al. (1982) and Smith et al. (1984).

Protein determination. Protein was determined according to the method of Petersen (1977) or Bradford (1976).

Electron microscopy. Fractions for fixation were taken from homogenates which were *not* previously stabilized with glutaraldehyde, and were processed according to the procedure of Heupke and Robinson (1985). Thin sections were post-stained with uranyl acetate and lead citrate and examined in a Philips EM 400 operating at 80 kV.

Chemicals and antibodies. Chemicals used in this investigation were usually of the highest purity grade commercially available and were purchased from either Sigma (Munich, FRG), Serva (Heidelberg, FRG) or Merck (Darmstadt, FRG). The monoclonal antibodies prepared against the 2BII glycoprotein complex of *C. reinhardtii* were kindly supplied by Dr. Peter Shaw, John Innes Institute, Norwich, UK; their production is described in Smith et al. (1984). Perchlorate-extracted *C. reinhardtii* cells walls were provided by Dr. Winfried C. Lang (Universität Kaiserslautern, FRG).

Results

Analysis of polymers released into the growth medium. Proteins are secreted into the growth medium by the wild type and the two mutants of *Chlamydomonas reinhardtii*, but the amounts and compositions are different. The mutants release more than twice the amount of protein per cell over a 24-h period than the wild type (Table 1). The relatively high levels of measurable hydroxyproline in these protein mixtures indicates the presence of HRGPs. This is substantiated by SDS-PAGE (Fig. 1). As previously demonstrated (e.g. by Catt et al. 1976; Imam et al. 1985) perchlorate extracts of the wild-type

Table 1. Comparison of the amounts of protein and peptidyl hydroxyproline released into the medium during one growth cycle (24 h) by synchronous cultures (cell density = $1.56 \cdot 10^6$ ml^{-1}) of the wild type and the mutants *cw2* and *cw15* of *Chlamydomonas*. Only polymers larger than M_r 10 kDa were taken into consideration

	Wild type	*cw2*	*cw15*
Protein (mg·l^{-1})	1.01	2.1	2.3
Hydroxyproline (mmol·l^{-1})	4.6	10	11
Hydroxyproline (mmol·(mg protein)$^{-1}$)	4.6	4.8	4.8

wall electrophorese into five polypeptides: three major bands at 245, 150 and 136 kilodaltons (kDa) and two, very much weaker, bands at 210 and 200 kDa. All react positively with PAS, indicating their glycoprotein nature. Glycosylated polypeptides co-electrophoresing with these are recognizable in gels of both *cw2* and *cw15* media proteins. Surprisingly, they are hardly recognizable in gels of the wild type. We ascribe their relative lack in the wild-type media to their effective incorporation into the cell wall.

Below 100 kDa there are a number of polypeptides common to all three media (80, 58, 50, 35, 28, 23, 14 kDa), the wild-type medium being especially enriched in these smaller polypeptides. This is in accordance with the observation of Voigt (1985a), that the major radioactively labelled polypeptides released into the medium by *C. reinhardtii* wild type after incubating with [^{3}H]proline and [^{35}S]methionine have molecular masses of 35, 44, and 49 kDa.

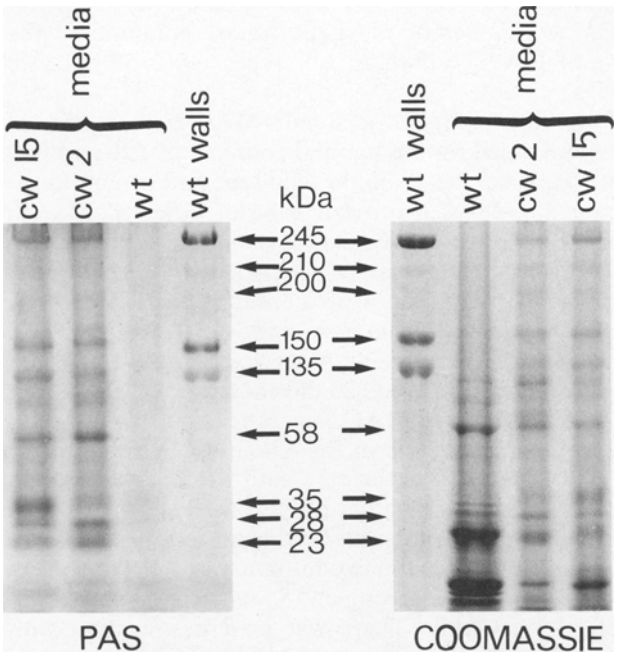

Fig. 1. Analysis by SDS-PAGE (gradient gel, 4–15% acrylamide) of proteins secreted into the culture medium by the wild-type (*wt*) and *cw2* and *cw15* mutants of *Chlamydomonas reinhardtii*. The major polypeptides of perchlorate-extracted wild-type walls (mainly 2BII glycoproteins) were also run for comparison

We have tested the proteins in the growth media for cross-reactivity against four monoclonal antibodies prepared against the 2BII complex. In dot blots (Fig. 2a), one monoclonal (MAC 6) reacted so weakly (even against the cell-wall standard) that it could not be used for Western blotting. However, MAC 2 cross-reacted with both the cell-wall standard and the wild-type-medium proteins, but showed no reaction with the *cw2* or *cw15* mutant-medium proteins in dot blots. Unfortunately, as already noted by Smith et al. (1984), MAC 2 will not work on Western blots, a fact confirmed in this investigation. On the other hand, MAC 3 and MAC 20, functioned in both dot-blotting and Western-blotting experiments.

In dot blots they cross-react much less strongly with proteins in the *cw2* media than in the *cw15* or wild-type media. In Western blots, MAC 3 cross-reacts very strongly with the 135- and 150-kDa polypeptides of the 2BII complex in the cell-wall standard and with two similar bands in the *cw2* and *cw15* gel lanes, but with only one band (135 kDa) in the wild-type lane (Fig. 2b).

There is also a faint cross-reaction of MAC 3 with a group of polypeptides around 220 kDa present in the medium of the wild type. To our surprise MAC 3 also reacted with numerous other polypeptides in all three media, in particular with the 23-, 35-, and in the case of *cw*15, 58-kDa polypeptides. Whilst curiously not recognizing the cell-wall standard, MAC 20 does cross-react with a number of polypeptides in each of the media. It does not appear to recognize clearly any polypeptides above 120 kDa.

Subcellular fractionation and enzyme activities. Endomembranes can be isolated from *C. reinhardtii* without thylakoid contamination by gently rupturing detergent-pretreated protoplasts (Andreae et al. 1988a) followed by sucrose-density-gradient centrifugation. In order to obtain larger quantities of endomembrane fractions for further analysis more easily, we modified this procedure by substituting a step for a linear gradient. Material collecting at the 38/50% interface had the highest activity of the ER marker cytochrome *c*-reductase (CCR) and the lowest activity of the GA marker inosine 5-diphosphatase (IDPase) and contained a correspondingly large number of ribosome-studded membranes, as shown in the electron microscope (Fig. 3a). By contrast, material at the 15/32% interface had the highest IDPase activity, had little measurable CCR activity, and was mainly made up of GA membranes (Fig. 3b). Rough ER was not detectable in this fraction by electron microscopy; cisternal elements of the GA were not recognizable in the 38/50% interface fraction. Material which collected at the 32/38% interface showed considerable cross-contamination by ER and GA membranes. This fraction was discarded. The ER and GA fractions were obtained in an identical manner from the two mutants, *cw*2, *cw*15; the distribution of marker enzymes was the same as for the wild type (results not shown).

Endomembranes from the wild type and the mutants were tested for key enzymes which participate in the

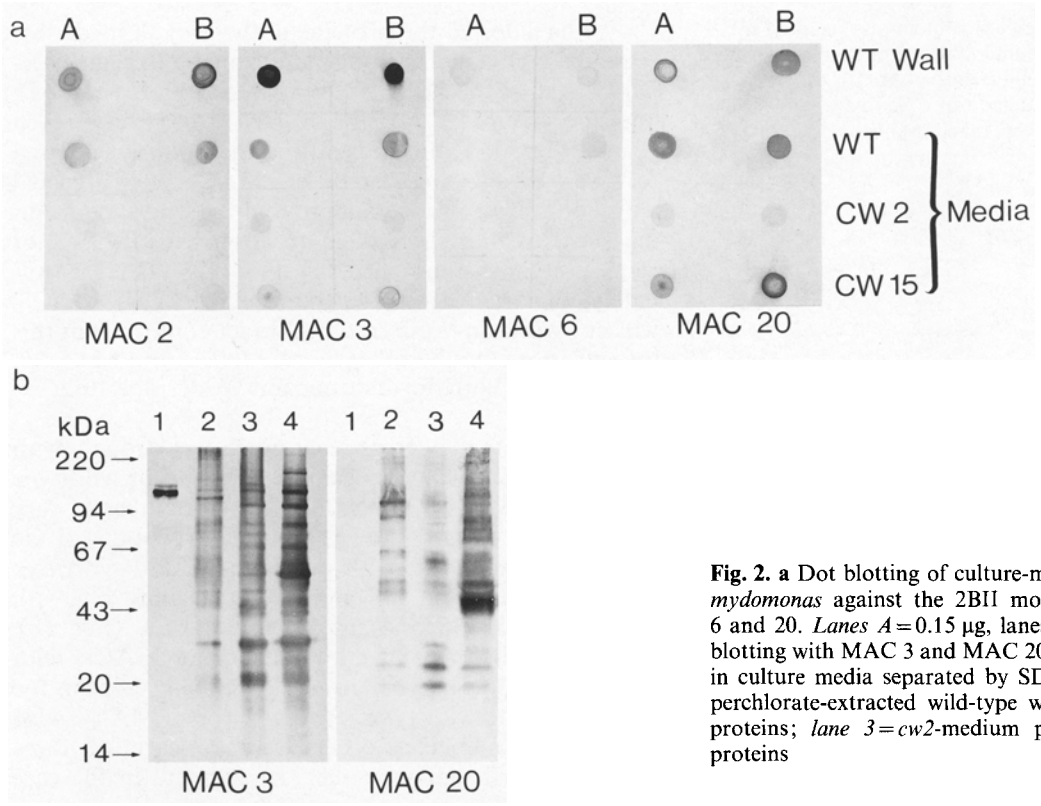

Fig. 2. a Dot blotting of culture-media secretory proteins of *Chlamydomonas* against the 2BII monoclonal antibodies MAC 2, 3, 6 and 20. *Lanes A* = 0.15 µg, lanes *B* = 0.30 µg protein. **b** Western blotting with MAC 3 and MAC 20 antibodies against polypeptides in culture media separated by SDS-PAGE (10% gels). *Lane 1* = perchlorate-extracted wild-type walls; *lane 2* = wild-type-medium proteins; *lane 3* = *cw2*-medium proteins; *lane 4* = *cw15*-medium proteins

post-translational modification of HRGPs (Table 2). In the case of prolyl hydroxylase and hydroxyproline-arabinosyl transferase the wild-type cells possessed much higher activities (expressed both as relative and specific activities) than the two mutants. With galactosyl transferase the mutants had higher relative activities than the wild type but the specific activities for this enzyme were similar for all three organisms.

Table 2. Comparison of relative (rel.) and specific (sp.) activities (act.) of prolyl hydroxylase, arabinosyl transferase and galactosyl transferase in total membrane fractions (6–100 kg) prepared from the wild type and the mutants *cw2* and *cw15* of *Chlamydomonas*. Synchronized cultures were employed with a total cell number, in each case, of $9.4 \cdot 10^8$. Cells were harvested at the fourth hour of darkness

Enzyme	Wild type	*cw2*	*cw15*
Prolyl hydroxylase			
rel. act. (nkat)	2.5	1.3	2.0
sp. act. (nkat·(mg protein)$^{-1}$)	4.6	0.93	1.7
Arabinosyl transferase			
rel. act. (nkat)	$1.8 \cdot 10^{-6}$	$6.7 \cdot 10^{-7}$	$7.2 \cdot 10^{-7}$
sp. act. (nkat·(mg protein)$^{-1}$)	$1.3 \cdot 10^{-5}$	$1.9 \cdot 10^{-6}$	$2 \cdot 10^{-6}$
Galactosyl transferase			
rel. act. (nkat)	$2 \cdot 10^{-6}$	$4.2 \cdot 10^{-6}$	$3.9 \cdot 10^{-6}$
sp. act. (nkat·(mg protein)$^{-1}$)	$1.4 \cdot 10^{-5}$	$1.2 \cdot 10^{-5}$	$1.1 \cdot 10^{-5}$

Analysis of luminal contents of ER and GA membranes. The results of SDS-PAGE of the luminal contents of ER and GA membranes from the wild type and the two mutants are given in Fig. 5. Interestingly, polypeptides larger than 100 kDa are, in all cases, poorly represented. A number of polypeptides are common to the ER and GA of all three cell types (100, 58, 54, 18, 14 kDa).

Dot blots with monoclonals MAC 2, 3, 6 and 20 were performed on the luminal contents of ER and GA membranes isolated from the wild type and two mutants. Once again, MAC 6 proved to be of little value: even against the cell-wall standard the cross-reaction was very weak (Fig. 4). Interestingly, MAC 2 cross-reacted only with the GA membranes of the mutant *cw*2. However, MAC 3 recognized proteins in both the ER and the GA of all three algae and, with the exception of the GA of the mutant *cw*2, MAC 20 did the same.

In Western blots, MAC 3 cross-reacted strongly with a 135-kDa polypeptide in the ER and GA fractions of the wild type. As was the case with the culture-medium proteins a number of other polypeptides, especially one at 43 kDa, were recognized by this antibody. The membrane fractions from the two mutants, with the exception of the ER fraction from *cw*15, reacted similarly but much more weakly. There was no cross-reaction with the ER of *cw*15. By contrast, MAC 20 recognized a polypeptide at 135 kDa in both ER and GA fractions from *cw*15, but it cross-reacted more stongly with a polypeptide at 94 kDa. It did not cross-react with endomembrane fractions from either the wild type or *cw*2 mutant.

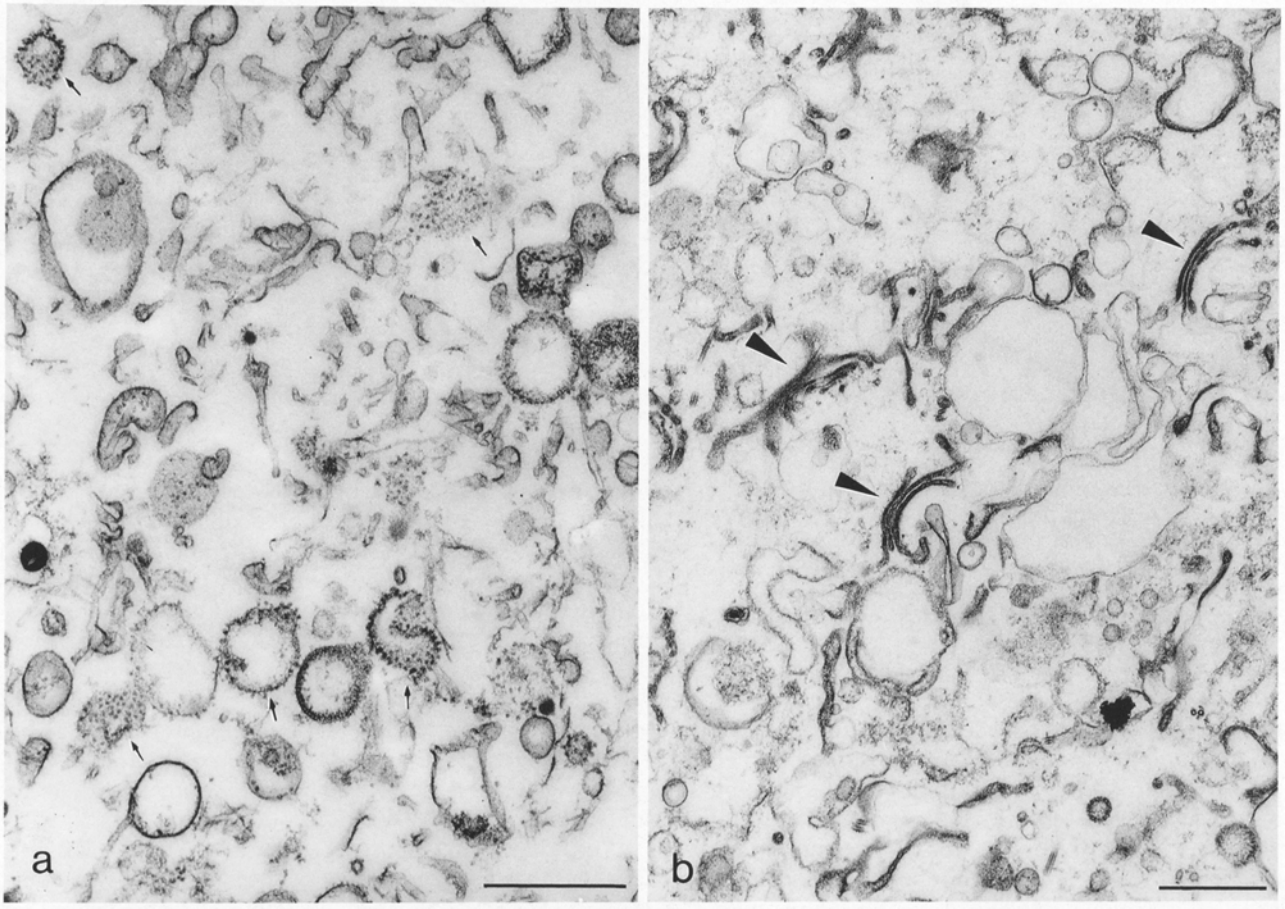

Fig. 3a, b. Thin sections of membrane material of *Chlamydomonas* collecting at the 38/50% interface, mainly rough ER (*arrows*, **a**), and 15/32% interface, mainly GA cisternae (*arrowheads*, **b**). *Bar* = 0.5 μm

Discussion

Since treatments which altered, reduced or eliminated the oligosaccharide side chains of the 2BII cell-wall glycoprotein complex of *C. reinhardtii* prevented antibody recognition, Smith et al. (1984) concluded that most, if not all of the monoclonal antibodies raised against 2BII were directed against sugar-containing epitopes. Of the six groups of monoclonals characterized, only two (groups III: MACs 3, 5 and V: MACs 12, 15, 17) were capable of recognizing 2BII polypeptides separated by SDS-PAGE. This was taken by Smith et al. (1984) to indicate the existence of simple sequence determinants for these two groups, whereas the epitopes recognized by the other groups appeared to be formed by at least two polypeptides. Our results are in partial agreement with these observations: MACs 2, 6 and 20 (representing groups II, IV and VI respectively) in our hands also did not react in Western blots against 2BII. However MAC 3, in contrast to the results of Smith et al. (1984), reacted predominantly with the smallest 2BII polypeptide (135 kDa). Unexpectedly, MAC 20 recognized a

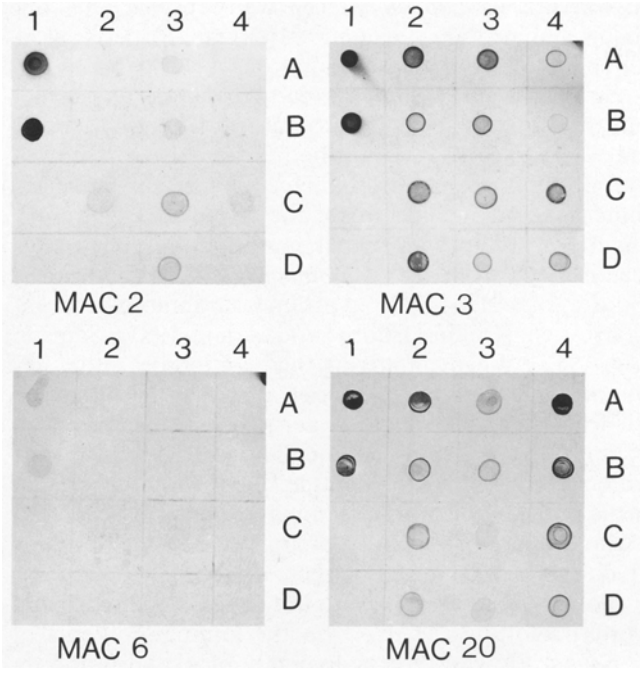

Fig. 4. Dot blotting of endomembrane luminal proteins of *Chlamydomonas* against monoclonals MAC 2, 3, 6 and 20. *Lane* = perchlorate-extracted wild-type walls; *lane 2* = wild-type endomembranes; *lane 3* = *cw2* endomembranes; *lane 4* = *cw15* endomembranes. *A, B* = endoplasmic reticulum (0.3 and 0.15 μg protein, respectively). *C, D* = Golgi apparatus (0.3 and 0.15 μg protein, respectively)

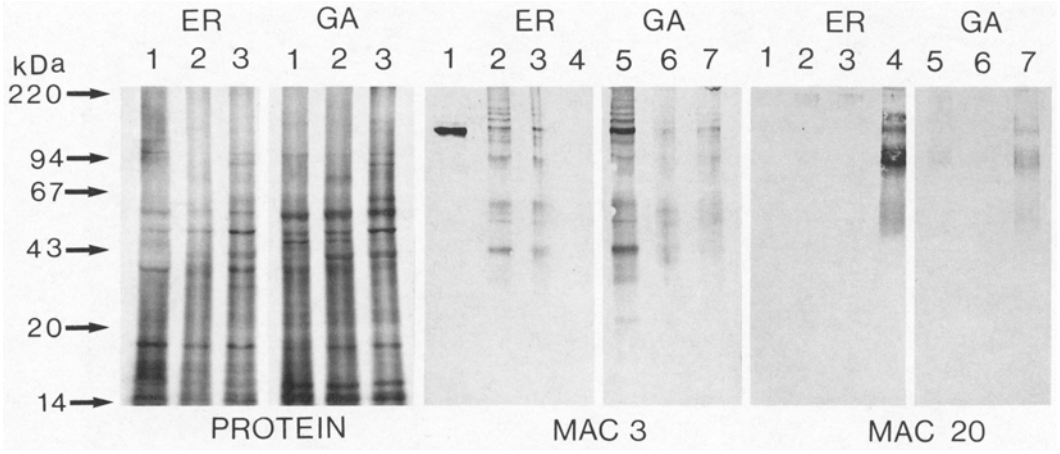

Fig. 5. Analysis by SDS-PAGE (10% gels) and Western blotting of luminal proteins of *Chlamydomonas*, with monoclonals MAC 3 and MAC 20. *Lanes 1, 2* and *3* in the protein detection are wild type, *cw2* and *cw15*, respectively. In the Western blots, *lanes 1* were perchlorate-extracted wild-type walls, *lanes 2* and *5* wild-type luminal proteins, *lanes 3* and *6 cw2* luminal proteins, *lanes 4* and *7 cw15* luminal proteins. Endoplasmic-reticulum and Golgi-apparatus fractions are marked *ER* and *GA*

large number of polypeptides presented in the wild-type and mutant-cell culture media as well as some polypeptides present in the lumen of mutant *cw*15 endomembranes. In this context one notes that group IV monoclonals are the only antibodies raised by Smith et al. (1984) capable of recognizing the wall of *Lobomonas pyriformis*, a related volvocalean alga. Interestingly, SDS-PAGE of *Lobomonas* cell walls reveals only one major polypeptide (around 210 kDa; Roberts et al. 1981).

Smith et al. (1984) have already remarked on the fact that the epitopes recognized by group III (e.g. MAC 3) and VI (e.g. MAC 20) antibodies are also present in glycoproteins present on the flagellar surface. These are numerous and include some, the so-called agglutinins, which are involved in sexual reproduction. They have been isolated from both *C. reinhardtii* and *C. eugametos* and are, like 2BII, hydroxyproline-, arabinose- and galactose-rich (Collin-Osdoby and Adair 1985; Samson et al. 1987). When employing these antibodies either immunocytochemically (e.g. Grief and Shaw 1987) or on cell fractions as we have done, it cannot be therefore assumed that a cross-reaction is indicative of the sole presence of a 2BII polypeptide. Our Western blots of media and endomembrane luminal proteins indicate that a whole range of (glyco)-proteins possess determinants which can be recognized by these antibodies.

Positive reactions by both dot (MACs 3 and 20) and Western blotting (MAC 3) on the luminal contents of *C. reinhardtii* wild-type endomembranes qualify the results of Grief and Shaw (1987). Whilst GA luminal proteins tended to react more strongly there was, nevertheless, a very clear reaction with luminal proteins extracted from rough-ER fractions. In both fractions, MAC 3 identified a 135-kDa polypeptide corresponding to that present in the 2BII complex. It also recognized three smaller polypeptides at around 44, 57 and 65 kDa, which may correspond to those polypeptides which Voigt (1985b) was able to detect in LiCl-extracts of intact cells of *C. reinhardtii*. This indicates that glycosylated proteins are indeed present in the ER, and puts this observation in agreement with the recent in-vitro localization of hyp-arabinosyl transferase in this fraction (Zhang et al. 1989). It is also in accordance with data on O-glycosylation of collagen in animal cells (reviewed in Kivirikko and Myllylä 1982) whereby the activities of galactosyl and glycosyl transferases were found to be highest in the rough ER (Harwood et al. 1975).

In 1972, Hyams and Davies induced 79 cell-wall mutants of *C. reinhardtii*. Some of these, including the mutant *cw*15, were described by them as being devoid of a cell wall. Others, including *cw*2, produced a wall which appeared to have no connections with the plasma membrane. Later studies (Lang and Chrispeels 1976; Adair et al. 1987) have, however, shown that the mutant *cw*2 also cannot produce an organized cell wall. In thin section (Zhang and Robinson 1986) mutants *cw*2 and *cw*15 are naked protoplasts, but in deep-etch, rotary-shadowed preparations, small fibers emanating from the plasma membrane can be discerned (Monk et al. 1983; Adair et al. 1987). These have been interpreted as being proteins normally attributed to the W 1 and W 7 layers of the wild-type wall (Adair et al. 1987; Monk 1988). The ability to form a wall lies, according to Adair et al. (1987) in the presence of polypeptides constituting the so-called W 2 layer. These polypeptides are apparently not synthesized by *cw*2 and *cw*15 mutants, although these cells are equipped with enzymes for HRGP synthesis, and 2BII glycoproteins (amongst others) are detectable both in the lumen of their endomembranes and are secreted into the culture medium. The W 2 layer is also implicated in autolysin action (Goodenough and Heuser 1985), whereby the target for this enzyme appears to be a 220-kDa polypeptide (Monk et al. 1983; Matsuda et al. 1985; Imam and Snell 1988). Bands above 200 kDa were not seen in Western blots of any of the endomembrane fractions which we tested. However, a group of faint bands, around 220 kDa, recognized by both MAC 3 and MAC 20, were detected in the wild type but not in the mutant media. Whether this repre-

sents the W 2 200-kDa polypeptide is unclear, since we do not yet know if it can be recognized by monoclonal antibodies which have been prepared against 2BII glycoproteins.

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