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Topography of *Chlamydomonas:* fine structure and polypeptide components of the gametic flagellar membrane surface and the cell wall

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Abstract. Surface polypeptide components of the flagellar membrane of *Chlamydomonas reinhardi* Dang. gametes are identified by their accessibility to in-vivo vectoral labeling by glucose oxidase-coupled lactoperoxidase-dependent ^{125}I iodination. Vectoral labeling is accomplished without observable adverse effects on cell viability or gametic function. Flagella isolated from labeled wild-type cells carry about 3% of the total incorporated label, which is found by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be distributed among 16 identifiable polypeptide bands. The most prominent surface-labeled species migrates in the M_r (relative molecular weight) 350 k region of the gel; each of the remaining iodinated polypeptides, which range in M_r from 25 k to 500 k, carries only a small proportion of incorporated label. To determine which polypeptides are unique to the flagellum and which are contaminants from the cell wall, wild-type profiles were compared with those of mutant strains and of mechanically isolated cell walls. Identification of contaminants was also facilitated by two-dimensional peptide mapping. We conclude that only 11 of the labeled bands are contributed by flagellar polypeptides; the remaining five bands are shown to be contaminants from the cell wall, and additional cell-wall polypeptides are found to co-migrate with flagellar species. A polypeptide designated as a possible membrane tubulin in preliminary studies is shown here to be different from tubulin in its peptide map. The 11 polypeptides assigned as specific flagellar surface components are candidate participants in such biological events as sexual adhesion, flagellar surface motility, and sensory signalling.

Key words: *Chlamydomonas* – Flagellum – Gamete – Membrane, flagellar – Mutant (*Chlamydomonas*).

Introduction

The flagellar surface of the unicellular eukaryote *Chlamydomonas reinhardi* Dang. engages in a number of discrete activities: it mediates the ionic fluxes thought to control forward and backward swimming [30] and phototactic behavior [48]; it sticks to a variety of surfaces [34]; it translocates attached surface particles up and down its length [7, 8, 21, 28]; it agglutinates exogenous lectins [38, 59]; it develops the capacity to recognize and adhere to flagella of opposite mating type during gametogenesis [3, 4, 19, 20]; and it presumably modulates the transmission of mating signals between the flagellar tips and the cell bodies [40, 54].

A major research goal in this and several other laboratories is to understand which flagellar surface components of *C. reinhardi* are responsible for which flagellar activities. As a first step, we have defined the polypeptides that are authentic residents of the flagellar surface. To distinguish surface molecules from internal ones, flagella were labeled in vivo using glucose oxidase-coupled lactoperoxidase-dependent iodination; they were then isolated from cell bodies by standard procedures and analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. To identify any radiolabeled cell-wall polypeptides that might contaminate flagellar preparations, we analyzed isolated cell-wall

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Abbreviations: Con A = concanavalin A; PAS = periodate-Schiff; M_r = relative molecular weight; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis

preparations, various mutant strains, and conducted mock contamination experiments. We also studied the response of the cell-wall polypeptides to autolysin, a lytic factor released during the mating reaction [12], to determine whether cell-wall contaminants could be removed by autolysin.

We conclude that the flagellar surface of *C. reinhardi* carries 11 species of polypeptides that can be resolved by one-dimensional SDS-PAGE. Two of these, the major membrane glycopolypeptide and the mastigoneme protein, have been identified in previous studies [5, 52, 60]; the remaining species are relatively minor components. We demonstrate that flagella are consistently contaminated with cell-wall polypeptides, even when isolated from alleged "cell-wall-less" strains, and we identify these contaminants by one-dimensional SDS-PAGE and by two-dimensional peptide mapping. The resultant catalogue of *Chlamydomonas* surface components should serve as a useful guide for studies of both flagellar function and cell-wall biosynthesis.

Materials and methods

Strains and culture conditions. The wild-type mating-type *plus* (mt^+) strain (wt^+) used in this study was *CC-620*, a high-mating-efficiency clone of strain *137c*. Mutants employed were *CC-404* (mt^+ *cw-15*), *CC-478* (*bald-2*) and *CC-278* (mt^-, *cw-15-2-4*). All are available from the *Chlamydomonas* Genetics Center, Duke University, Durham, N.C., USA.

Gametes were harvested after 7 d of growth [36] on 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-acetate-phosphate (TAP)-agar plates [23], suspended in nitrogen-free, high-salt minimal medium (NFHSM [36]) at a cell density of $<10^8$ cells ml^{-1}, and agitated for at least 1 h on a gyrotary shaker (150 rpm) to free flagellar surfaces of any adherent material. Cells were counted using a hemacytometer, and ability to mate was assessed by mixing cells with wt^- tester gametes.

General analytic procedures. Flagella were prepared by the pH-shock method of Witman et al. [60] except that 10 mM Pipes (1,4-piperazinediethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo., USA), pH 7.4, replaced the Tris buffer. Mastigonemes were isolated by the CsCl-gradient procedure of Witman et al. [60]. Protein was determined by the method of Lowry et al. [35] with bovine serum albumin as standard. Radioactive iodine (^{125}I) was estimated using a Gamma 4000 counter with a DP5000 attachment (Beckman Instruments, Irvine, Cal., USA).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially by the method of Laemmli [32] using 4–15% linear acrylamide gradients with 3% acrylamide stacker in the microscale system devised by Matsudaira and Burgess [37]. Flagellar and cellular samples were dissolved in at least an equal volume of 3% SDS lysis buffer containing 6 M urea, 40 mM dithiothreitol, 10% (w/v) glycerol, 0.01% pyronin Y and 6.25 mM Tris-HCl, pH 6.8, and were placed in a boiling water bath for 2 min. Up to 20 µl ($\leqq 10$ µg protein) of sample was added to each gel slot. The gels were run at 150 V (constant voltage) until the bromphenol blue (Roboz Surgical Instrument Co., Washington, D.C., USA) included in the gradient gel migrated from the end of the gel (approx. 1.2 h). Protein standards employed were myosin (200 k), bovine serum albumin (68 k), ovalbumin (45 k), aldolase (40 k), α-chymotrypsin (25 k) and lysozyme (14.3 k). In some cases the M_r 350 k major membrane glycoprotein of flagella served as an internal standard. All M_r estimates are subject to the caveat that glycopolypeptides can migrate anomalously in SDS gels [49]. Following electrophoresis, gels were stained with Coomassie blue (Sigma) or periodic acid-Schiff (PAS) (Smith Chemical Co., Columbus, O., USA) [16], photographed, dried, and subjected to autoradiography using pre-flashed XR-10 or XAR-5 no screen X-ray film (Eastman-Kodak, Rochester, N.Y., USA) and Cronex XTRA Life intensifying screens (Dupont de Nemours, Wilmington, Del., USA). Autoradiograms were developed according to manufacturer's specifications.

Table 1. In-vivo conditions for radioiodination of *Chlamydomonas* gametes. wt^+ gametes were iodinated as described in Methods with the indicated omissions. After iodination and extensive washing, $3 \cdot 10^9$ cold carrier cells were added to the $1 \cdot 10^9$ iodinated cells; samples of cells were then removed for estimation of incorporated label, determination of cell numbers and flagellar preparation

Sample No.	Reaction	cpm per 10^9 cells	cpm per mg flagellar protein
I	Complete	$3.11 \cdot 10^7$	$8.52 \cdot 10^5$
II	−Lactoperoxidase	$3.75 \cdot 10^4$	$1.85 \cdot 10^3$
III	−β-D-glucose	$1.57 \cdot 10^7$	$3.69 \cdot 10^5$
IV	−Glucose oxidase	$1.24 \cdot 10^7$	$2.87 \cdot 10^5$

In-vivo glucose oxidase-coupled lactoperoxidase-dependent iodination. Gametes (10^9) were pelleted by centrifugation (3,000 *g*, 5 min) and resuspended in 2.5 ml NFHSM; to this was added $3.7 \cdot 10^7$ Bq (1 mCi) ^{125}I ($3.7 \cdot 10^9$ Bq ml^{-1}) carrier-free $Na^{125}I$ (Amersham, Arlington Heights, Ill., USA), 50 µg lactoperoxidase (81 units mg^{-1} protein, Sigma), 50 µmol β-D-glucose (Sigma), and 12.5 units glucose oxidase (Sigma). Following thorough mixing, iodination was allowed to proceed for 15 min at room temperature with gentle agitation every 5 min. The reaction was terminated by the addition of at least 20 ml ice-cold NFHSM, pelleting of the cells by centrifugation, and a further five washes in fresh cold NFHSM medium. A three- to fivefold excess of cold gametic carrier cells was then added to facilitate subsequent manipulations.

Three microscopic criteria were used to establish that this procedure is not deleterious to *Chlamydomonas* gametes: (1) the iodinated cells appear fully viable and motile, (2) they translocate polystyrene beads along their flagellar surfaces [7] in a normal fashion, (3) they agglutinate and fuse with gametes of opposite mating type with unimpaired efficiency (>90%).

Table 1 demonstrates that the iodination procedure is dependent upon the presence of lactoperoxidase in the coupled system: if lactoperoxidase is omitted, neither the cells nor their flagella are appreciably labeled (approx. 0.2% of control levels). If either β-D-glucose or glucose oxidase is omitted, on the other hand, labeling occurs at 30–50% of control levels, presumably indicating that *Chlamydomonas* gametes generate endogenous H_2O_2.

Table 1 also shows that flagella are labeled to approx. 10^6 cpm mg^{-1} protein. Using a mean figure of $1.85 \cdot 10^9$ flagella

mg^{-1} protein [53], we calculate that about 3% of the incorporated label is associated with the flagella, the rest presumably labeling cell walls and perhaps plasma membranes.

Unfortunately, we find we can label vegetative cells to only 5–10% of these gametic levels. Addition of further H_2O_2, or inclusion of ascorbate plus copper in the reaction mixture to inhibit catalase activity [44], improves this labeling only twofold. A possible explanation is that vegetative cells might secrete soluble and preferentially labeled compounds that are not produced by gametes. For whatever reason, labeling of vegetative cells was too low to permit analysis of their surface components.

Two-dimensional peptide mapping. The method employed is a modification of that of Bordier and Crettol-Järvinen [9]. Five µg of each radiolabeled sample was separated by SDS-PAGE on 4–15% gel slabs as above. The lane was sliced out and washed for 15 min in 10 ml 20% glycerol, 0.125 M Tris-HCl, pH 6.8. Solutions of V8 protease (200 µg ml^{-1}, 400 units mg^{-1}; Miles Laboratories, Elkhart, Ind., USA), α-chymotrypsin (200 µg ml^{-1}, 49.2 units mg^{-1}; Worthington Biochemical Corp., Freehold, N.J., USA) or pronase (0.5 µg ml^{-1}, B grade, 45,000 PUK g^{-1}; Calbiochem-Behring, La Jolla, Cal., USA) were prepared by addition of 0.4 ml of a fivefold excess of protease to 1.6 ml of a 2% solution of low-melting-temperature agarose (Sigma) in 0.125 M Tris-HCl, pH 6.8, at 40° C (1 mM ethylenediaminetetraacetic acid (EDTA) was included in the case of V8 protease). Each solution was drawn up into 100-µl microcapillary tubes and allowed to set at 0–4° C. The protease-containing agarose gel was laid upon the first-dimension lane already in place over a second-dimension SDS-PAGE 4–15% acrylamide slab (with 4% acrylamide stacker), and the assembly secured with additional molten agarose containing stacking gel buffer. Protein standards and approx. 2.5 µg of the sample under test were added to separate wells at the ends of the gel. Electrophoresis was carried out at 150 V until both the sample and the protease were concentrated within the stacking gel; the current was then stopped for 30 min to allow proteolytic digestion; after this, electrophoresis was continued as usual. Following Coomassie staining of protein bands (to ensure that the protease was equally distributed and had equal access to all regions of the first-dimensional gel), the gel was dried and subjected to autoradiography.

Each polypeptide in flagellar and wall preparations exhibits unique and reproducible behavior in these two-dimensional systems, arguing that we are identifying bona-fide surface polypeptides and not collections of aggregates and proteolytic products such as, for example, those that plague students of gap-junction proteins [24, 25]. For example, the 17 recognizable wall polypeptides display the following range of responses: (a) complete digestion with loss of the polypeptide and its fragments from the gel (e.g. W4, Fig. 13C); (b) complete digestion yielding a discrete series of labeled fragments (e.g. W1, Fig. 13C); (c) partial digestion yielding the original polypeptide in reduced amount and a diagnostic series of fragments (e.g. W12, Fig. 13A); and (d) partial digestion with no observable fragment (e.g. W6, Fig. 13C). A similar series of responses is given by the flagellar components.

Location of conconavalin A (Con A)-binding proteins on SDS gels. In a modification of the method of Adair [1], 5-µg samples of flagellar proteins were separated on SDS-PAGE microslabs; the gels were fixed and SDS was removed in 25% isopropanol, 10% acetic acid. After overnight washing in deionized water, the gel was treated for 1 h with 0.25% glutaraldehyde (Polysciences, Warrenton, Pa., USA), washed extensively with deionized water, and equilibrated in potassium phosphate-buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin. The gel surface was drained and 0.6-cm strips of Miracloth (Chicopee Mills, Milltown, N.J., USA) laid over each lane. Aliquots of 100 µl of ^{125}I-Con A in PBS (± 1.0 M α-methylglucopyranose) were added to appropriate strips. After 4 h incubation at room temperature the strips were removed, the gel surface gently washed with a stream of deionized water, and the gel electro-destained, the Con A-treated surface adjacent to DE-81 paper (Whatman, Clifton, N.J., USA). Current was applied using a 12-V battery charger for 2 h such that unbound species were transferred. The gel was then dried and subjected to autoradiography.

Iodinated Con A was prepared by incubating 2 mg Con A (Sigma) in the presence of $7.4 \cdot 10^6$ Bq Na^{125} I, 30 mM β-D glucose, 50 µl preswollen Enzymobeads (Bio-Rad, Richmond, Cal., USA) in PBS in a total volume of 312 µl for 15 min at room temperature. Enzymobeads and free ^{125}I were removed by spinning aliquots of approx. 100 µl of the incubation mixture for 3 min through approx. 1 ml packed Biogel P6DG (Bio-Rad) pre-equilibrated in PBS and pre-spun in punctured microfuge tubes [56]. This procedure was repeated twice and the recovered label was shown to be associated with Con A by trichloroacetic-acid (TCA) precipitation and SDS-PAGE autoradiography.

Preparation of crude autolysin. Equal numbers of wt^+ and wt^- gametes were mixed at a final density of $4 \cdot 10^7$ cells ml^{-1}. The cells were allowed to mate for 1 h and were then pelleted by centrifugation at 3,000 *g* for 5 min. The supernatant was pelleted again at 18,000 *g* for 10 min and passed through a 3.5- to 5-µm glass filter. The filtrate was spun at 100,000 *g* for 1 h and the supernatant dialyzed against distilled water overnight and lyophilized. The lyophilized material was resuspended in a small volume of NFHSM to yield a crude active autolysin preparation about 20-fold more concentrated than in the mating medium.

Preparation of mechanically isolated cell walls. Following the procedure of Minami [41], as modified by Dr. D. Stuart (Washington University), 10^9 live *bald-2* gametes that had been ^{125}I-surface labeled in vivo were pelleted by centrifugation and resuspended in 5 ml of 10 mM Tris-HCl, pH 7.8, in the presence of 5 g of glass beads (16–200, average diameter 0.2 mm). The mixture was agitated in a vortex mixer for 90 s to elicit removal of cell walls from the cells. The beads were allowed to settle, and the supernatant was decanted. The beads were then washed two more times and the three supernatants pooled and spun at 1,500 *g* for 1.5 min to pellet the cells. The supernatant was made 0.5% (w/v) in Nonidet P-40 (BDH Chemicals, Poole, U.K.) and the walls pelleted by centrifugation at 1,500 *g* for 6 min. This crude wall fraction was resuspended in 20 ml of 22% sucrose containing 10 mM Tris-HCl, pH 7.8, and 5-ml aliquots were laid over Tris-buffered 25% sucrose pads and spun in an HB-4 swinging bucket rotor (DuPont Co., Sorvall Biomedical Div., Wilmington, Del., USA) at 367 *g* for 60 s to pellet residual cell bodies. The top 6 ml of the gradients were collected and made 0.4% in Nonidet P-40. The walls were pelleted at 18,000 *g* for 5 min, washed several times in 0.4% Nonidet P-40, 10 mM Tris-HCl, pH 7.8, and finally collected in 5% sucrose, 10 mM Pipes, pH 7.4.

Electron microscopy. Cells were washed in water, pelleted, applied to the surface of a piece of fixed lung [27] and quick-frozen, deep-etched, and rotary-replicated as described by Heuser [26]. Replicas were photographed using a 100CX electron microscope (JEOL, Tokyo, Japan) operating at 100 kV; the negatives were photographically reversed before printing.

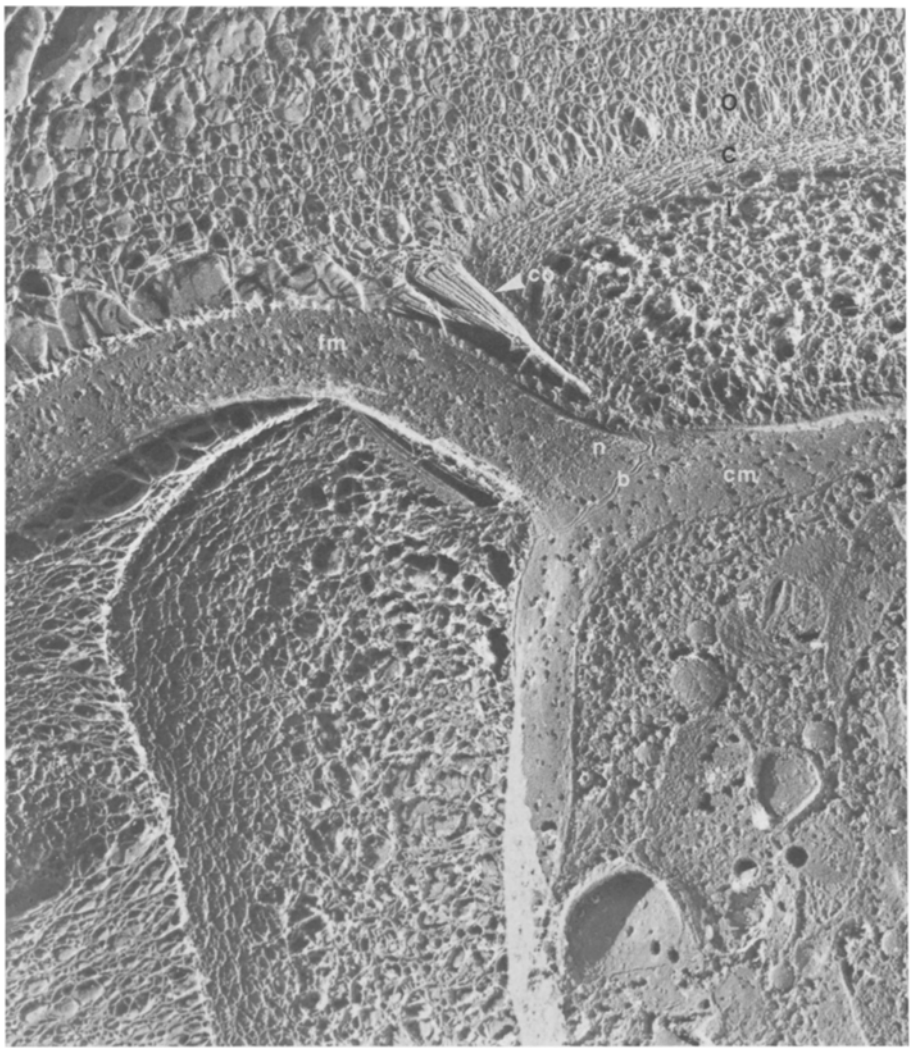

Fig. 1. Cell surface of wild-type *C. reinhardi* gamete. The cell membrane (*cm*) and flagellar membrane (*fm*) have been exposed by freeze-fracture and then subjected to deep-etching; this creates large pits at the sites of most intramembranous particles. Etch-resistant, however, are the particles comprising the necklace (*n*) and bracelet (*b*) arrays. Etching reveals details of the cell wall. Using the nomenclature of Roberts [46], the wall is seen to be comprised of a loose inner layer (*I*), a dense central layer (*C*), and a loose outer layer (*O*). Both the outer and the inner surfaces of the ridged flagellar collar (*co*) are also exposed by etching. Micrograph provided by J.E. Heuser. × 50,000

Results

Morphology of the Chlamydomonas surface

Figures 1–3 show the fine structure of wild-type *plus* (wt^+) gametes as visualized by deep etching of quick-frozen specimens [26]. The cell in Fig. 3 was pre-treated with a preparation of autolysin, the wall-removing substance that is secreted by gametes during the mating reaction [12]. Figure 4 shows a deep-etched replica of a "cell-wall-less" *cw-15* gamete [13]. The following points pertinent to our labeling studies are illustrated by these micrographs:

1) The three major layers of the *Chlamydomonas* cell wall [46] are designated in Fig. 1 as inner (I), central (C) and outer (O). The tangential fracture in Fig. 2 provides additional resolution of each of these layers. The inner layer consists of a loose network of fibrous material. The central layer is a triplet structure [46], with two dense fibrous meshworks separated by a medial zone of more loosely organized fibers. The outer layer is composed of long fibers that anchor in the outer zone of the triplet layer and stream out into the medium. Since these loose outer fibers will be directly exposed to surface-labeling reagents, they are the most likely radiolabeled contaminants of flagellar preparations.

2) The collar (Co) through which the flagellum (fm) emerges is seen to be a rigid, grooved structure [45, 46] that makes close contact with the flagellar base. By electron microscopy, then, the wall-collar complex forms a barrier that undoubtedly hinders direct access of large macromolecules (e.g. lactoperoxidase) to the plasma-membrane surface.

3) Autolysin treatment removes the bulk of the cell wall, including the collar (Fig. 3). A sparse ar-

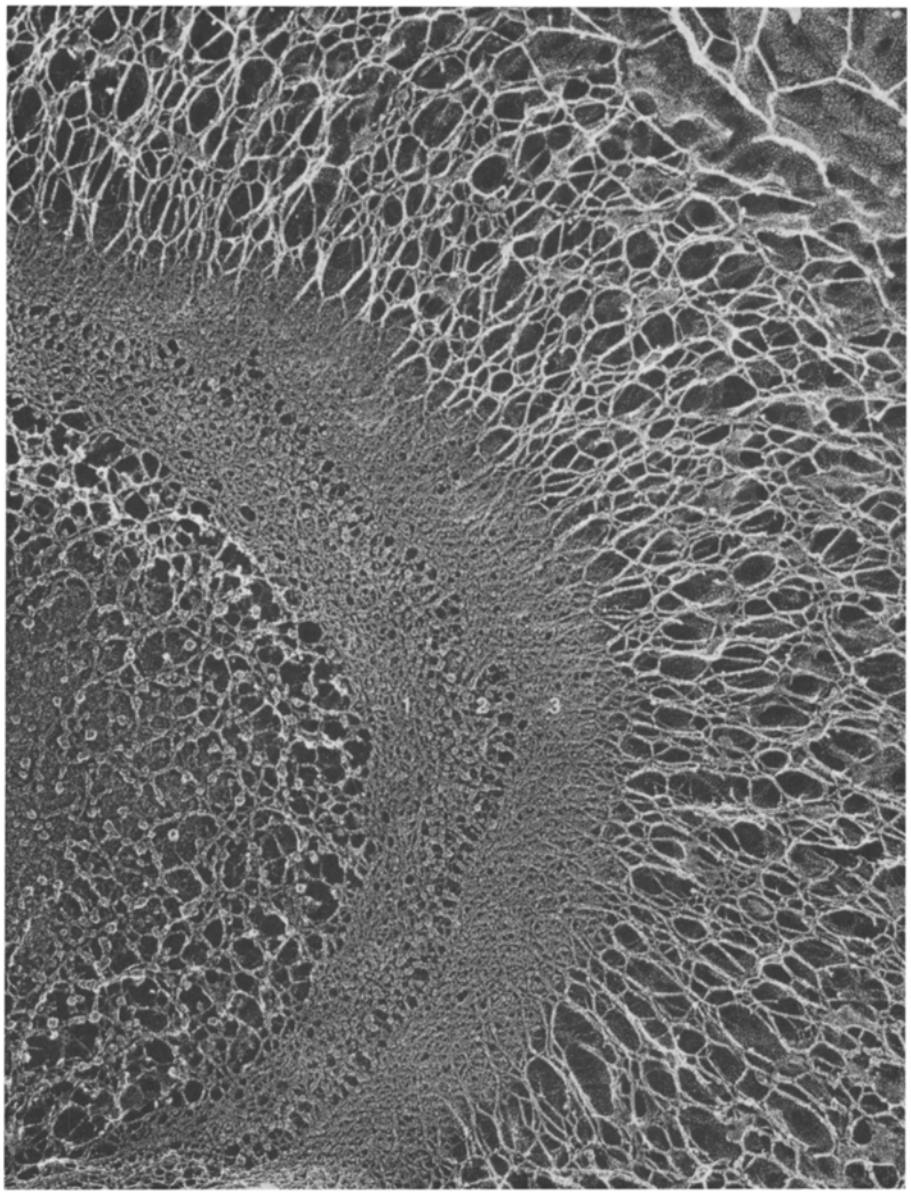

Fig. 2. Cell wall of wild-type *C. reinhardi* gamete. The wall has been fractured tangentially. The dense central layer (compare Fig. 1) is resolved into three zones, labeled 1–3. The inner layer (left) appears as a trabeculum overlying the cell surface membrane; the outer layer (right) appears as a sunburst of long anastomosing fibers. Micrograph provided by J.E. Heuser. × 71,000

ray of fibers remains, however, on the surface of the cell membrane (Fig. 3f); these most closely resemble the inner-layer meshwork and the long fibers of the outer layer.

4) The "wall-less" *cw-15* strain clearly lacks the central triplet layer and the collar (Fig. 4). A substantial layer of fibers continues to surround the cell body, however; those resemble the components of the outer layer in the wild type (Figs. 1, 2). Therefore, both *cw-15* and autolysin-treated wt^+ cells continue to carry cell-wall fibers that are accessible to iodination and are likely contaminants of flagellar preparations.

5) Flagella are seen in two views. In Figs. 1 and 4, the internal fracture face of a flagellar membrane (fm) is present, while in Figs. 3 and 5 the true outer surface of the flagellar membrane is exposed by deep-etching. This surface is seen to carry a dense array of projections that are absent from the contiguous etched surface of the cell membrane (Fig. 3). The projections presumably correspond to the "sheath" or "fuzzy coat" seen in conventional images of the *Chlamydomonas* flagellar membrane [5, 45, 52, 60]. Since the predominant component of isolated flagellar membranes is the PAS-positive major membrane glycoprotein [5, 52, 60, and this paper], we propose that most of the projections are platinum replicas of individual or aggregated

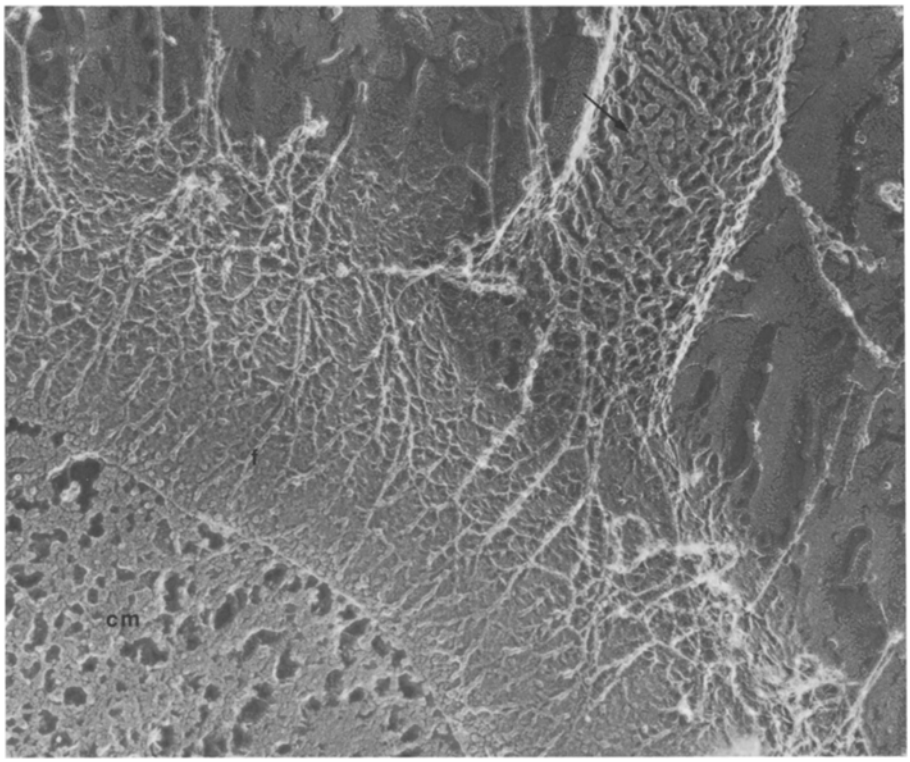

Fig. 3. Cell surface of wild-type *C. reinhardi* gamete treated with autolysin. The fracture plane passes through the cell membrane interior (*cm*), which is deeply pitted by the etching process. Etching exposes residual wall fibers (*f*) on the cell surface and projections (*arrow*) on the flagellar surface. The abrupt transition from the smooth cell membrane to the bumpy flagellar membrane occurs at the level of the flagellar bracelet (Fig. 1), although the bracelet has no detectable morphological manifestation at the cell surface. Micrograph provided by J.E. Heuser. ×99,000

glycoprotein moieties, and that they represent the major substrates for lactoperoxidase-catalyzed iodination of the flagellar surface.

Vectoral labeling

Two approaches were used to demonstrate that iodination is vectoral. In the first, flagella-less *bald-2* [22] cells were iodinated, extensively washed, and treated with a preparation of autolysin. As shown in Table 2, only 5.7% of the counts pellet with the whole cells at 3,000 *g*; the remaining counts are found in the 18,000-*g* pellet (wall fragments) and the 18,000-*g* supernatant (wall fibers). The radiolabel that pellets with the cell is most likely associated with the wall fibers that remain on the surface of autolysin-treated cells (Fig. 3). If, in the worst case, all of these counts are instead associated with components of the plasma membrane and-or the cell interior, they would presumably be distributed among a large number of polypeptides, any one species of which would be unlikely to contribute a detectable radiolabeled band in our autoradiograms.

In the second approach, flagella labeled in vivo and then isolated were compared with flagella that were isolated and then labeled in vitro, in one case after freeze-thawing to ensure membrane damage. As seen in Fig. 6, many more polypeptides are labeled in vitro (lanes III and IV) than in vivo (lane II), including species which comigrate with such identified axonemal components as the dyneins and tubulins (D and T, respectively, Coomassie-stained lane I). The tubulins are also labeled in the in-vitro iodination experiments of Musgrave et al. [42] using *C. eugamatos* flagella. It is clear, therefore, that when the membrane is damaged, the pattern of labeling changes such that many of the prominent labeled polypeptides in vivo become relatively minor labeled species in vitro. It is also clear that, were intra-flagellar components accessible to lactoperoxidase, they would act as substrates for iodination. Therefore, the flagellar membrane acts as an effective permeability barrier to lactoperoxidase in vivo, and the labeling protocol we have devised does not appear to compromise the integrity of this membrane.

Approaches used to identify polypeptides in isolated flagellar preparations and in cell walls

Figures 6–13 illustrate the variety of approaches taken to identify the polypeptides present in isolated flagellar preparations and in cell walls. In

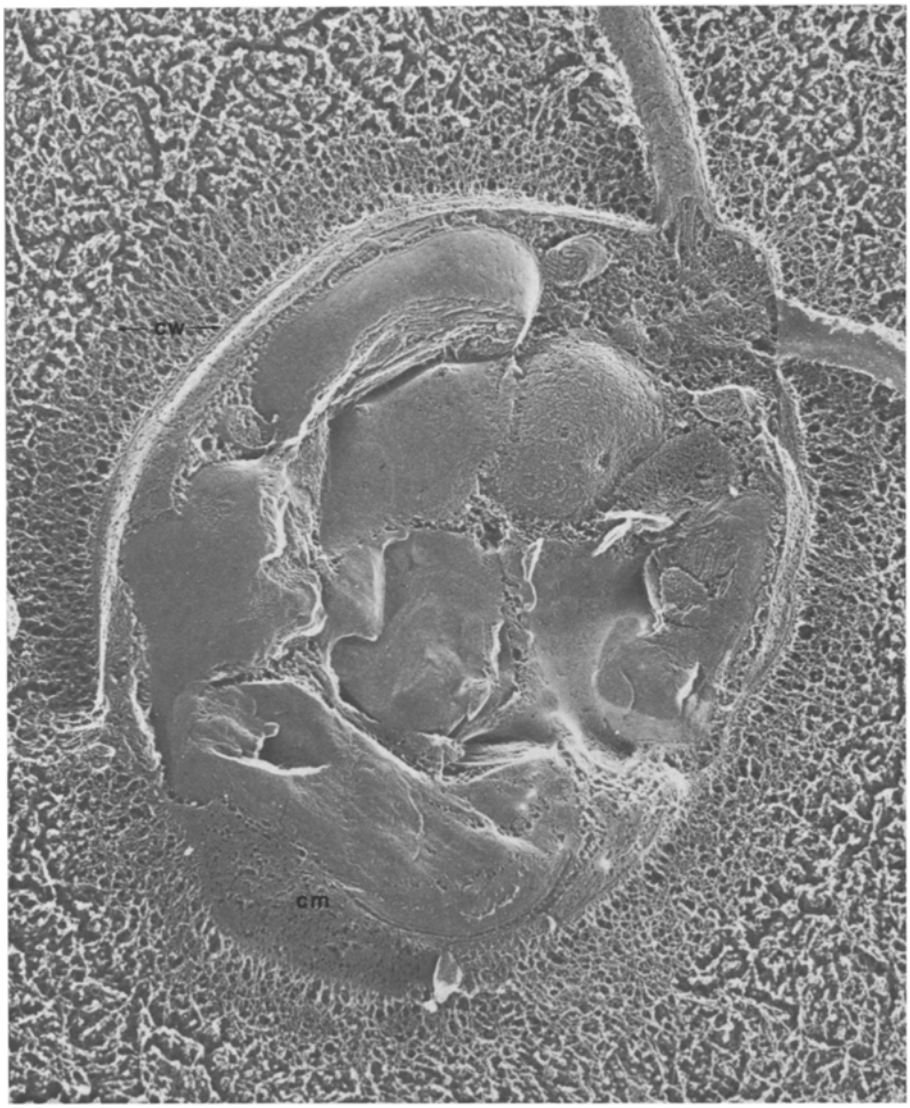

Fig. 4. Cell surface of the *cw-15* mutant of *C. reinhardi*. A layer of cell-wall fibers (*cw*) extends from the etched cell membrane (*cm*) and surrounds the flagellar bases. Absent are the flagellar collar and dense central layer of the wild-type (Fig. 1). The disposition and morphology of the *cw-15* fibers most resemble the outer layer of the wild-type wall (Fig. 1). Micrograph provided by J.E. Heuser. × 35,000

this section we describe the rationale and design of each experimental approach; in the following section we summarize the information obtained from these experiments.

Figure 6, lane II, Fig. 7, lane I, Fig. 8, lane II, and Fig. 9, lane I, and Fig. 11, lane II display the polypeptides present when isolated preparations of in-vivo vectorally labeled wt^+ flagella are subjected to one-dimensional SDS-PAGE and autoradiography. The Fig. 6 gel is heavily over-exposed for the polypeptides in the high-molecular-weight region; the Fig. 7, Fig. 8 and Fig. 11 gels show intermediate exposures, and the Fig. 9 gel is a composite, the upper half derived from a 12-h exposure and the lower half from a 48-h exposure. Such flexibility in exposure time is an important attribute of gel autoradiography for preparations such as these, where some polypeptides are far more heavily labelled than others.

Because electron microscopy indicates that the outer fibers of the cell wall appear to be sloughing into the medium (Figs. 1, 2), we suspected that flagellar preparations might be contaminated with radiolabeled wall material. Four approaches were therefore used to identify any wall polypeptides:

(1) Cells of the *bald-2* strain, which lacks flagella, were iodinated in vivo, and the cells were then subjected to SDS-PAGE and autoradiography (Fig. 10, lane I).

(2) The cell walls of iodinated *bald-2* cells were isolated by mechanical means, and the polypeptides visualized by SDS-PAGE and autoradiography (Fig. 7, lane IV; Fig. 10, lane V; Fig. 11, lane I).

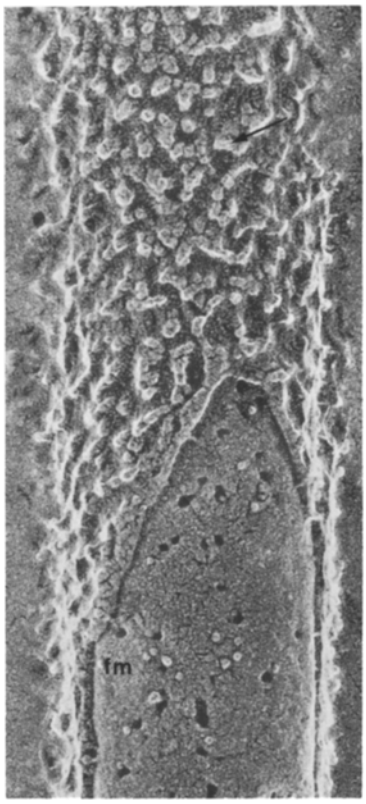

Fig. 5. Flagellar surface of wild-type *C. reinhardi* gamete. High magnification view of the etched flagellar membrane E face (*fm*) and the flagellar surface showing the projections (*arrow*) believed to represent replicas of glycoprotein moieties. Micrograph provided by J.E. Heuser. ×156,000

(3) Labeled *bald-2* cells were treated with autolysin, and the polypeptides released into the supernatant were analyzed by SDS-PAGE and autoradiography (Fig. 10, lane IV).

(4) "Mock-contamination" experiments were performed in which *bald-2* cells were radiolabeled, washed, and mixed with a sevenfold excess of cold wt^+ cells; the cells were then subjected to a standard flagellar isolation procedure, and the radiolabeled bands present in the resultant gels were determined by autoradiography (Fig. 11, lane III). (Unfortunately, it proves difficult in these experiments to wash out all traces of enzyme and ^{125}I, presumably because they become trapped in the wall-fiber interstices, and the two major proteins of the flagellum (asterisk) become lightly radiolabeled in the mock-contaminated sample. Nonetheless, the approach is useful in confirming our identification of the major wall polypeptides that contaminate flagellar preparations, e.g. W1–3, W6, W7, W9–12 and W17).

Table 2. Effect of autolysin treatment on *Chlamydomonas* cell walls. *Bald-2* cells (10^8, ^{125}I-labeled), suspended in 3 ml nitrogen-free, high-salt minimal medium, were treated at room temperature for 1 h with 2 ml of crude autolysin prepared as described in Methods. Microscopic observation showed that cell walls were quantitatively removed from the *bald-2* cells, and little cell lysis was observed. The cells were centrifuged to yield the fractions described above; the 18,000 *g*, 10 min, supernatant was dialyzed extensively against 5% sucrose in 10 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 7.4

Sample	Total cpm	% in fraction
Bald-2 cells	2,086,140	100
Autolysin-treated *bald-2* cells		
3,000 *g* × 5 min pellet	119,360	5.7
18,000 *g* × 10 min pellet	216,260	10.4
18,000 *g* × 10 min supernatant (dialyzed)	1,381,430	66,2
Dialysable material	248,000	11.9
Total recovered		94.2

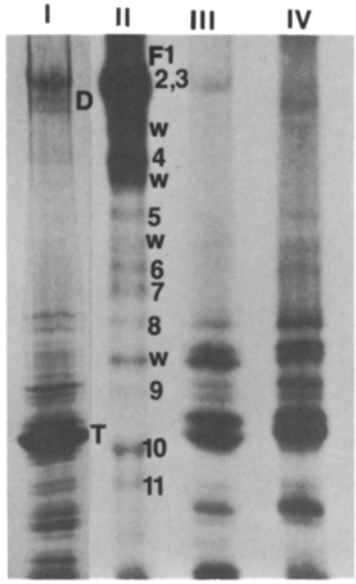

Fig. 6. In-vivo versus in-vitro surface labeling of flagella of *C. reinhardi*. Flagella were either labeled in vivo as described in Methods or labeled in vitro using the following protocol: 2 mg of isolated flagella were resuspended at 1 mg ml^{-1} in 5% sucrose in 5 mM Tris, pH 7.4, and a 1-mg aliquot was freeze-thawed five times (room temperature to −70° C). The two sets of flagella were then iodinated using equivalent amounts of reactants and catalysts as used in vivo. After 15 min iodination, the reaction was stopped by addition of 15 ml sucrose-Tris containing 0.375 mg ml^{-1} cold KI. The flagella were pelleted by centrifugation and washed four more times with sucrose-Tris prior to dissolution in SDS lysis buffer. Approximately 12,000 cpm were applied per lane. Lane I: Coomassie-stained, in-vivo-labeled flagella. *D*, dyneins; *T*, tubulins. Lane II: Autoradiogram, in-vivo-labeled flagella. *F*, flagellar polypeptides; *w* = wall contaminants. Lane III: Autoradiogram, in-vitro-labeled flagella. Lane IV: Autoradiogram, in-vitro-labeled freeze-thawed flagella

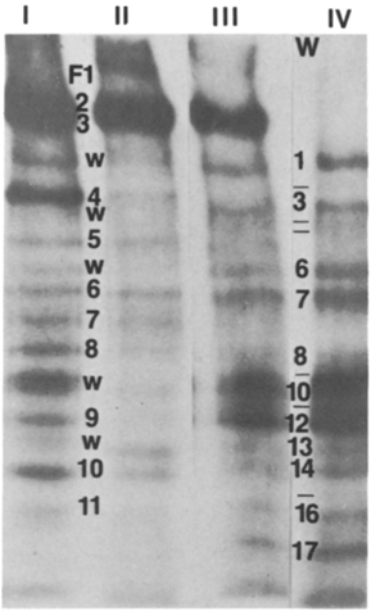

Fig. 7. Autoradiogram (SDS-PAGE) of ^{125}I-surface labeled flagella and cell walls of *C. reinhardi*. Aliquots (15 µl) of each surface-labeled sample (3,750 cpm) in SDS lysis buffer were loaded on 4–15% acrylamide slab gels and analyzed as described in Methods. Lane I: wt$^+$ flagella. *F*, flagellar polypeptides; *w*, wall contaminants. Lane II: *cw-15* flagella. Lane III: Equivalent labeled *cw-15* flagella and mechanically isolated cell walls. Lane IV: Mechanically isolated cell walls. *W*, cell-wall polypeptides; unnumbered polypeptides (*dashes*) indicate positions of bands that are faintly labeled or difficult to resolve in this gel system

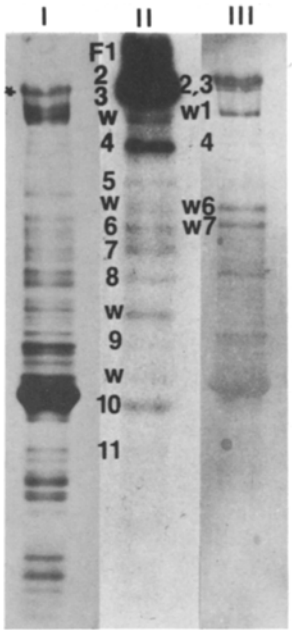

Fig. 8. Comparative staining patterns of flagellar preparations from *C. reinhardi*. Approximately 5 µg of in-vivo ^{125}I-labeled wt$^+$ flagella were separated in each lane. Lanes of the gel were stained with Coomassie blue and autoradiogrammed or else stained by the PAS procedure. Some nonspecific background PAS staining is evident (e.g. over the tubulins); therefore, only deeply pink bands are scored as PAS-positive. Lane I: Coomassie stained. Major membrane polypeptide. Lane II: Autoradiogram of Lane I. Lane III: PAS stain of companion lane

To ask whether the *cw-15* strain, whose endowment of wall fibers is less than that of wild type (Fig. 4), might yield flagella with a reduced and-or modified pattern of wall contamination, flagella were prepared from vectorally labeled *cw-15* cells (Fig. 7, lane II). A mixture of these flagella and *bald-2* cell walls was also examined (Fig. 7, lane III) to determine the extent to which it resembled a wt$^+$ flagellar preparation.

To learn which polypeptides in flagellar preparations carry carbohydrate residues, gels were stained with the PAS reagent (Fig. 8, lane III). In addition, Con A-binding species were identified in SDS gels of unlabeled flagellar preparations by ^{125}I-Con A overlay as described in Methods (Fig. 9, lane II). Many of these assignments were confirmed by Con A affinity chromatography (data not shown).

Finally, to aid in distinguishing flagellar and wall polypeptides, two-dimensional peptide mapping of isolated flagellar preparations was performed using a modification of the method of Bordier and Crettol-Jarvinen [9]. Vectorally labeled

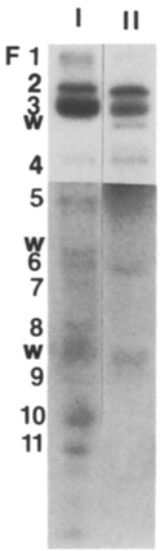

Fig. 9. Conconavalin A-binding polypeptides in flagellar preparations from *C. reinhardi*. Aliquots (5 µg) of flagellar polypeptides were displayed by SDS-PAGE. Con A binding was assessed in overlays as described in Methods. Lane I: wt$^+$ flagella in-vivo surface-labeled with ^{125}I by lactoperoxidase. *F*, flagellar polypeptides; *w*, wall contaminants. Lane II: Unlabeled wt$^+$ flagella incubated with ^{125}I-Con A

flagellar and cell-wall preparations were fractionated by SDS-PAGE (first dimension); the component polypeptides were then subjected to proteolytic digestion in situ, and the resultant proteolytic fragments were detected as off-diagonal spots fol-

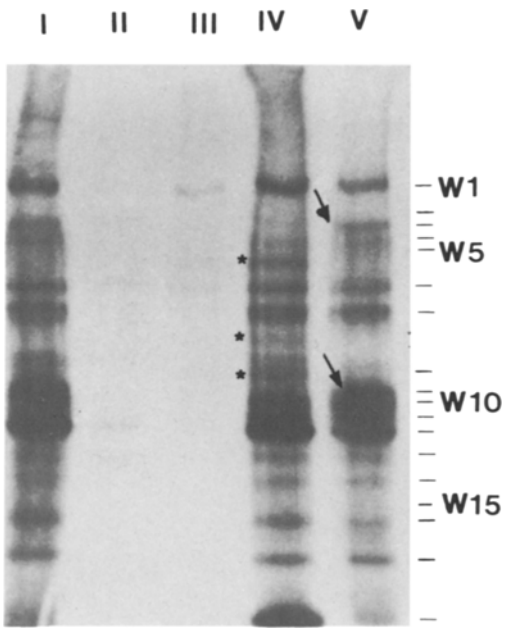

Fig. 10. Analysis by SDS-PAGE of the *Chlamydomonas* cell wall and the release of cell-wall polypeptides by autolysin. Samples prepared as described in Table 2 were dissolved in SDS-lysis buffer and analyzed by SDS-PAGE and autoradiography. Lanes I to IV were loaded relative to the percent recovery of each sample in the autolysin-treatment experiment (see Table 2) while the mechanically isolated wall (Lane V) sample was loaded to give an equivalent number of counts as the dialzyed autolysin-solubilized wall sample. Arrows denote wall polypeptides absent from autolysin-treated supernatants; asterisks denote new polypeptides present in these supernatants. Lane I: ^{125}I-*bald-2* cells (3,750 cpm). Lane II: Autolysin treated ^{125}I-*bald-2* cells (200 cpm). Lane III: Wall fragments; 18,000 $g \times$ 10 min pellet (390 cpm). Lane IV: Solubilized ^{125}I-*bald-2* walls; 18,000 $g \times 10$ min supernatant (2,480 cpm). Lane V: Mechanically isolated ^{125}I-*bald-2* cell walls (2,480 cpm); *W*, cell-wall polypeptides

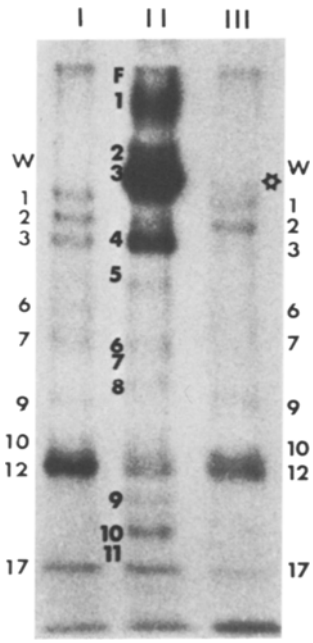

Fig. 11. Mock contamination. *Chlamydomonas reinhardi bald-2* cells were surface labeled, washed, and mixed with seven times the number of unlabeled wt$^+$ cells. The mixture was then subjected to deflagellation and the isolated "mock-contaminated" flagellar fraction was analyzed by SDS-PAGE and autoradiography. The F2-F3 bands from wt$^+$ were weakly labeled by residual lactoperoxidase and ^{125}I during their incubation with the washed *bald-2* cells. Lane I: In-vivo-labeled *bald-2* cells. Lane II: In-vivo-labeldd wt$^+$ flagella. Lane III: Cold wt$^+$ flagella "mock-contaminated" with wall components from in-vivo-labeled *bald-2* cells

lowing electrophoresis perpendicular to the first (second dimension). Figure 12 shows peptide maps of wt$^+$ samples exposed to V8 protease, chymotrypsin or pronase, plus an untreated control. Figure 13 shows, for comparison, peptide maps of isolated cell-wall components generated by the same three enzymes; also displayed are the V8 protease maps of *cw-15* flagella and of *CC-278* cells, a strain that is derived from *cw-15* also lacks flagella.

Flagellar polypeptides

Using the approaches described in the preceding section, 11 surface-labeled polypeptides, denoted F1–F11 in Figs. 6–9, have been identified as unique to the flagellar surface. The single most important criterion for a flagellar localization was that a polypeptide be present in the flagellated wt$^+$ and *cw-15* samples and absent from flagella-less *bald-2* and *CC-278* samples and from isolated *bald-2* walls. Ambiguity arose in cases where a wall polypeptide co-migrated with a polypeptide present in a flagellar sample. In such cases, additional criteria were used to judge whether the polypeptide in the flagellar sample represented a wall contaminant or a mixed band containing both flagellar and wall species.

Identified flagellar surface polypeptides are as follows:

F1 (M_r 500 k) is the slowest-migrating polypeptide to enter our separating gel, although additional radiolabeled bands are present in the stacker [3]. F1 binds Con A (Fig. 9, lane II) and is resistant to protease (Figs. 12, 13).

F2 (M_r 375 k) can be distinguished from the closely migrating F3 only when radiolabeled gels are exposed for short periods (Fig. 9). In two-dimensional gels, the F2–F3 region shows two overlap-

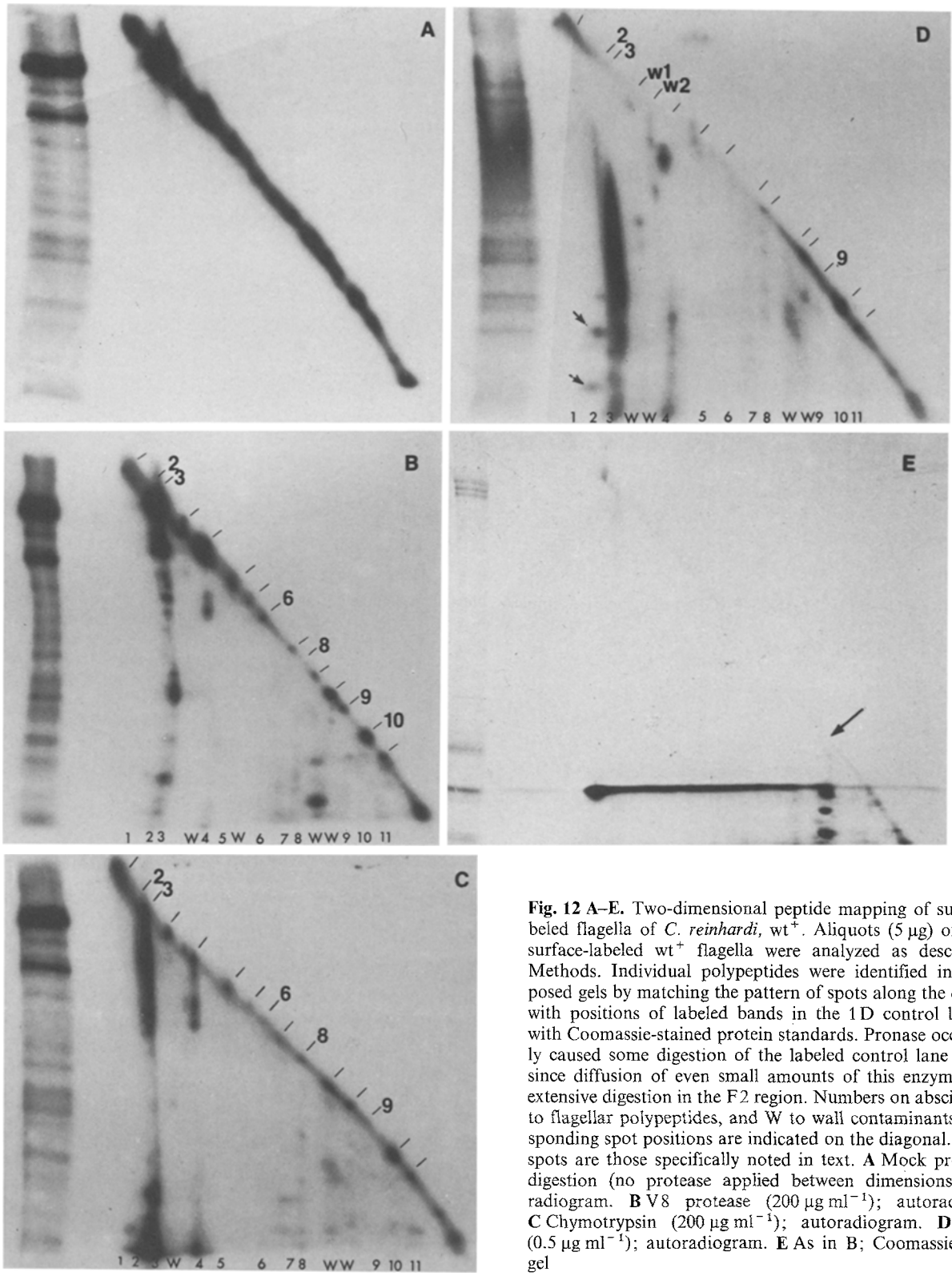

Fig. 12 A–E. Two-dimensional peptide mapping of surface-labeled flagella of *C. reinhardi*, wt^+. Aliquots (5 μg) of in-vivo surface-labeled wt^+ flagella were analyzed as described in Methods. Individual polypeptides were identified in less-exposed gels by matching the pattern of spots along the diagonal with positions of labeled bands in the 1D control lane and with Coomassie-stained protein standards. Pronase occasionally caused some digestion of the labeled control lane (e.g. **D**) since diffusion of even small amounts of this enzyme causes extensive digestion in the F2 region. Numbers on abscissa refer to flagellar polypeptides, and W to wall contaminants. Corresponding spot positions are indicated on the diagonal. Labeled spots are those specifically noted in text. **A** Mock proteolytic digestion (no protease applied between dimensions); autoradiogram. **B** V8 protease (200 μg ml^{-1}); autoradiogram. **C** Chymotrypsin (200 μg ml^{-1}); autoradiogram. **D** Pronase (0.5 μg ml^{-1}); autoradiogram. **E** As in B; Coomassie-stained gel

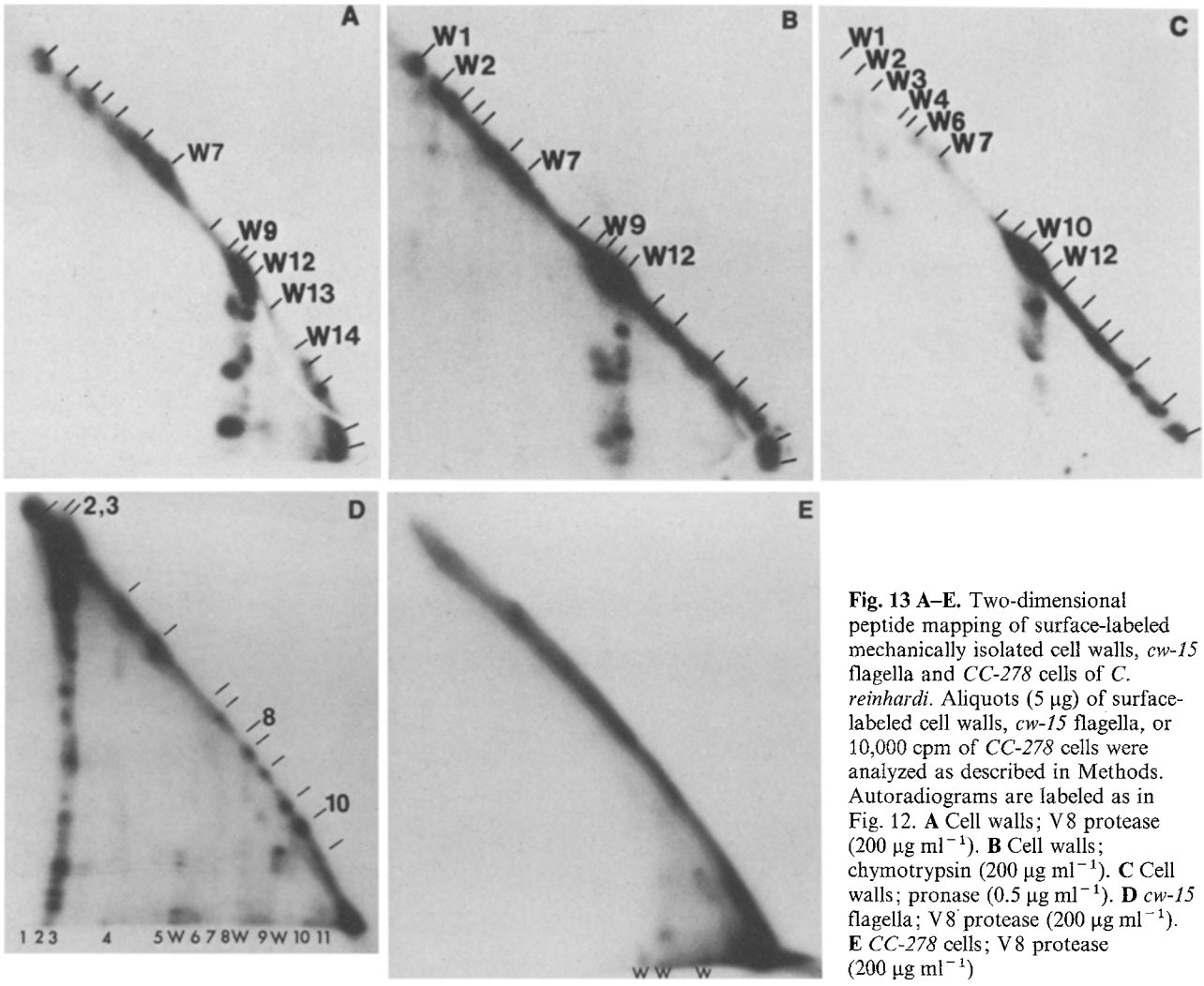

Fig. 13 A–E. Two-dimensional peptide mapping of surface-labeled mechanically isolated cell walls, *cw-15* flagella and *CC-278* cells of *C. reinhardi*. Aliquots (5 μg) of surface-labeled cell walls, *cw-15* flagella, or 10,000 cpm of *CC-278* cells were analyzed as described in Methods. Autoradiograms are labeled as in Fig. 12. **A** Cell walls; V8 protease (200 μg ml^{-1}). **B** Cell walls; chymotrypsin (200 μg ml^{-1}). **C** Cell walls; pronase (0.5 μg ml^{-1}). **D** *cw-15* flagella; V8 protease (200 μg ml^{-1}). **E** *CC-278* cells; V8 protease (200 μg ml^{-1})

ping tracts of digestion products (Figs. 12B–D, 13D). The presence of at least two unique pronase peptides in the F2 map (Fig. 12D, arrows) indicates that F2 and F3 are unique polypeptides. Moreover, F2 binds Con A more avidly than F3 (Fig. 9).

F3 (M_r 350 k) is more heavily surface-labeled than F2, but binds Con A proportionately less well (Fig. 9). In autoradiograms exposed to show lower-molecular-weight polypeptides, F2 and F3 appear to comprise a single band (Figs. 7, 8). This "band" corresponds to the major membrane component identified in earlier studies of *Chlamydomonas* flagella [6, 51, 60]; it carries at least 30% of the incorporated radiolabel, is PAS-positive (Fig. 8, lane III; see also [5]), and is the only flagellar surface component that can be readily identified in Coomassie-blue-stained gels of whole flagella (Fig. 8, lane I, asterisk). Polypeptides yielding F2–F3 peptide maps are absent from *bald-2* wall preparations (Fig. 13A–C) and from *CC-278* cells (Fig. 13E), indicating that F2 and F3 are unique to the flagellar membranes.

F4 (M_r 230 k) copurifies with mastigonemes during the isolation procedure of Witman et al. [60], is faintly PAS-positive (Fig. 8, lane III), and binds Con A (Fig. 9).

F5 (M_r 195 k) binds Con A (Fig. 9) and has no co-migrating cell-wall counterpart.

F6 (M_r 145 k) is a Con A binding polypeptide (Fig. 9) which is distinguished from the wall protein W7 (M_r 140 k) by its slightly slower electrophoretic mobility and by its sensitivity to chymotrypsin digestion (compare the F6 position in Fig. 12B) relative to W7 (compare the W7 position in Fig. 13A, B).

F7 (M_r 125 k) fails to bind Con A (Fig. 9) and has no comigrating cell-wall counterpart.

F8 (M_r 105 k) forms a sharper band than the comigrating W8, and is sensitive to V8 protease (Fig. 12B, 13D) whereas W8 is not (Fig. 13A). F8 also yields a pattern of chymotryptic partial digestion products (Fig. 12C) not observed for W8 (Fig. 13B). Neither bind significant levels of Con A (Fig. 9).

F9 (M_r 70 k) is the most troublesome polypeptide to characterize. It runs slightly ahead of a variable region of contaminating cell-wall polypeptides (W9–12; see next section) and is relatively protease-resistant compared with these wall species (Fig. 12B–D versus Fig. 13A–C). Because there is variable contamination by W9–12, however, the position of F9 is not easily related to its neighbors. It does not bind measurable levels of Con A (Fig. 9). We should note that the lactoperoxidase enzyme has a molecular weight of 70 k, and we have not ruled out the possibility that this band has been produced by lactoperoxidase autolabeling.

F10 (M_r 52 k) runs slightly ahead of the contaminating cell-wall polypeptide W14 in two-dimensional gels, and, unlike W14, is resistant to V8 protease (Figs. 12B, 13D versus 13A). A number of experimental observations led to the proposal from this laboratory [2] that F10 might represent a membrane-localized tubulin. We find, however, that the α- and β-axonemal tubulins are quantitatively degraded by V8 protease when viewed in Coomassie-stained gels (Fig. 12E, arrow) whereas, as noted above, F10 is resistant to V8. Moreover, the rich V8-protease peptide map given by the tubulins (Fig. 12E) is not mirrored by the complementary autoradiogram (Fig. 12B) which shows only surface-labeled material. A similar differentiation between F10 and the axonemal tubulins is observed in chymotrypsin maps. We therefore withdraw this polypeptide as a membrane-tubulin candidate.

F11 (M_r 40 k) binds no detectable Con A (Fig. 9) and has no obvious contaminating cell-wall counterpart.

Lower-molecular-weight species. A number of bands migrate ahead of F11 (Figs. 6–9) at least one of which binds Con A (Fig. 9). Since the peptide mapping procedure is not informative for small polypeptides, we are unable to identify these definitively; some, however, are likely to be of flagellar origin; others may represent degradation products; and still others may be derived from lactoperoxidase protein that has decomposed following autolabeling.

Cell-wall polypeptides and their flagellar contamination

When isolated cell walls from surface-labeled *bald-2* cells are analyzed by SDS-PAGE, 17 polypeptides (W 1–W 17) are visualized (Fig. 7, lane IV); their apparent M_r's are given in Table 3. There is some variability in the proportions of labeled wall polypeptides from one experiment to the next, particularly in the W 8–W 12 region (compare Fig. 11, lane I, with Fig. 7, lane IV); this could be because of variability in the accessibility of the wall layers to the enzyme system and-or to differential success in solubilizing walls for electrophoresis.

Any band in flagellar preparations with a comigrating counterpart in the cell-wall preparation was considered a potential cell-wall contaminant. The tendency of cell-wall polypeptides to contaminate flagellar samples is documented in the "mock-contamination" experiment of Fig. 11: many of the major wall species (Lane I) can be identified in the in-vivo-labeled flagellar preparation (lane II) and in the mock-contaminated preparation (lane III). Identification of specific contaminants is tabulated below.

W1 is identified in two-dimensional flagellar gels by its characteristic pronase peptide map (Figs. 12D, 13C). It forms a conspicuous band between F3 and F4 (Fig. 7, lane I). For unknown reasons it escaped surface labeling in the experiment illustrated in Fig. 9, lane I; however, its presence in flagellar preparations is indicated by its intense Con A binding (lane II). It also gives an intense PAS reaction in Fig. 8, lane III. W1 is reduced or absent from the *cw-15* strain (Fig. 7, lane II).

W2 also yields characteristic pronase peptide maps in two-dimensional flagellar gels (Figs. 12D, 13C), indicating that it contaminates the region just behind the mastigoneme protein F 4. It is very faint in most cell-wall gels (e.g., Fig. 7, lane IV, Fig. 10, lane V), where the dithiothreitol (DTT) concentration of the lysis buffer is high (40 mM), but when the effective DTT concentration is lower in aged SDS lysis buffer (Fig. 11, lane I), the intensity of this band increases considerably. Two-dimensional gel electrophoresis demonstrates that unreduced W2 is converted to W5 and W6 by DTT (data not shown).

W3 forms a faint contaminating band at the leading edge of F4 (Fig. 7, lanes I, II and IV, Fig. 11).

W6 forms a conspicuous contaminating band in flagellar gels between F5 and F6. It appears in PAS gels as a strongly staining band (Fig. 8, lane III) but does not appear to bind Con A (Fig. 9, lane II). It is reduced or absent from *cw-15* (Fig. 7, lane II).

W7, also strongly PAS-positive (Fig. 8, lane III), comigrates with flagellar polypeptide F6 (see preceding section).

W8–W12. As noted above, this group of labelled wall polypeptides contaminates flagellar preparations to a varying extent. All except W10 appear to be absent from *cw-15* flagellar preparations (Fig. 13E) and CC-278 (Fig. 13F). Since *cw-15* lacks the dense central layer of the *Chlamydomonas* wall (Fig. 4), it is possible that these polypeptides reside in the central layer. The known resistance of this layer to solubilization [29] may account for their variable contamination of flagellar preparations. Several bind to Con A (Fig. 9, lane II).

W13 forms a contaminating band between F9 and F10.

W14 comigrates with polypeptide F10 in flagellar gels (see preceding section).

Autolysin treatment of cell walls

Because autolysin causes the dissolution of cell walls ([12]; Fig. 3), we explored the possibility that autolysin might specifically digest cell-wall polypeptides and thereby rid flagellar preparations of cell-wall contaminants. Electron-microscopic observation of cells exposed to autolysin for 2 min was not encouraging: although the walls indeed "dissolve", the medium becomes filled with wall fibers, as though the walls fall apart rather than being digested. This impression was supported by the experiment summarized in Fig. 10: after 1 h in autolysin, an 18 k supernatant (Fig. 10, lane V) contains virtually all the polypeptides present in the intact walls (Fig. 11, lane I). The only species that are conspicuously altered in abundance, and therefore potentially degraded, are W 3 and W 9 (Fig. 10, compare lanes IV and V, arrows); the new polypeptides marked with asterisks in lane IV may represent their degradation products. These results indicate that autolysin acts on only a few cell-wall components that are necessary for its structural integrity; when these are modified or degraded, the whole assemblage falls apart. These results also indicate that during the mating reaction of *Chlamydomonas,* release of cell-wall fibers may lead to *increased* contamination of isolated flagella, a possibility that must be born in mind while analyzing flagellar polypeptide profiles during the mating process.

Discussion

Methodology. Surface-labeling is a highly sensitive approach to the identification of exposed membrane polypeptides, but is reliable only if it can be shown that the labeling is vectoral and that bona-fide membrane components are being labeled and not, for example, adsorbed or secreted extracellular materials [31]. We show here that lactoperoxidase-dependent vectoral iodination of *Chlamydomonas* flagellar membranes can be reproducibly achieved, but that contamination by extra-membrane components, in this case cell-wall glycopolypeptides, is indeed a problem [see also 33, 42]. The problem cannot be circumvented using the "cell-wall-less" mutant strain *cw-15*, nor by "removal" of walls with autolysin, since contaminating fibers persist in both cases (Figs. 3, 4). Therefore, we have catalogued both the iodinable wall proteins and the flagellar proteins, and have documented the extent to which the first contaminate the second.

When complex polypeptide patterns are being analyzed, maximal information is usually obtained by two-dimensional gel electrophoresis in the fashion of O'Farrel [43]. In preliminary chromatofocusing experiments, however, we found that the surface-labeled flagellar polypeptides have a narrow range of isoelectric points around pH 4.25, indicating that isoelectric focusing would be of limited usefulness. The large size and probable charge heterogeneity of many of the surface-labeled glycopolypeptides further discouraged the use of the O'Farrel approach. We therefore utilized the two-dimensional peptide mapping procedure as a sensitive alternative to isoelectric focusing, and demonstrate here that the technique can detect the presence of co-migrating polypeptides in the first dimension of SDS gels.

Topography of the cell wall. The cell wall of *C. reinhardi* has been shown by Roberts and his collaborators [10, 29, 46] to be a highly ordered structure that can be disassembled and re-crystallized in vitro. The deep-etch electron micrographs presented here (Figs. 1, 2) give a particularly detailed view of the crystalline central triplet layer, and define the outer layer as an array of fibers that appear to extend from the outer zone of the triplet. The replicas also indicate that the *Chlamydomonas* wall

Table 3. Iodinated cell-wall polypeptides in mechanically isolated preparations from *bald-2* cells of *C. reinhardi*

Cell-wall polypeptide No.	M_r	Relative intensity
W1	280 k	+++
W2	240 k	+
W3	220 k	++
W4	210 k	+
W5	195 k	+
W6	165 k	+++
W7	140 k	+++
W8	105 k	+
W9	94 k	++++
W10	85 k	++++
W11	80 k	++
W12	70 k	++++
W13	68 k	+
W14	52 k	++
W15	38 k	++
W16	34 k	+
W17	28 k	+++

is composed of five layers: an inner layer, a central triplet, and an outer layer. Two additional layers, designated W2 and W6, have been designated in the earlier studies of Roberts [46]; these, however, appear to represent the edges of the inner and outer triplet layers rather than discrete layers.

In our studies we have identified 17 surface-labeled polypeptides associated with the cell walls (Table 3); many of these polypeptides are glycosylated. Catt and coworkers [10] have analyzed isolated cell walls from *C. reinhardi,* and find eight PAS-positive polypeptides in the 100–300 k region of SDS gels. Many, if not all, of these undoubtedly correspond to W1–W8 in the present study; a direct comparison is not feasible, however, because different gel and detection systems were used.

Some but not all of the surface-labeled wall polypeptides persist on the surface of the "cell-wall-less" strain *cw-15*. Since this mutant fails to assemble the central triplet layer of the wild-type wall (Fig. 4), some of the missing polypeptides may represent components of this layer. It is likely that the *cw-15* strain is sensitive to detergent lysis because it lacks the central triplet; by extrapolation, the central triplet probably accounts for the relative resistance to detergent lysis exhibited by the wild-type.

Our results show that autolysin, the factor released during the mating reaction in response to flagellar agglutination [12], causes breakdown of the cell wall without degrading most of its component polypeptides; this same conclusion has been reached independently by Snell [52]. The only polypeptides that appear to be altered by exposure to autolysin are W3 and W9. Therefore, it would appear that W3 and W9 play a critical role in maintaining the wall in its assembled state, and that when they are altered by autolysin activity, the wall falls apart into its component fibers.

Cell-wall contamination of flagellar preparations, suspected for many years by students of *Chlamydomonas* flagella, is documented in the present report. Nine cell-wall polypeptides are identified in flagellar gels; some form discrete bands, while others co-migrate with true flagellar bands. Use of the *cw-15* mutant to "solve" the contamination problem is not a useful alternative, since its residual wall fibers also contaminate flagellar samples. Moreover, this mutant grows and mates less well than the wild-type and is prone to cell lysis; it therefore cannot substitute for wild-type for many experimental purposes. Autolysin treatment could potentially help to solve the contamination problem if a sufficient number of washes are included to rid the flagella of released fibers. New cell-wall fibers are immediately laid down after autolysin releases the old cell wall, however, so that contamination continues to occur during the ensuing stages of the experiment. The best solution, therefore, appears to be the one taken here, namely to identify the potential contaminants and to recognize them as such in flagellar gels.

Topography of the flagellar surface. Previous studies on the flagellar surface of *C. reinhardi* indicated that the membrane carries mastigoneme appendages and a single major polypeptide, a glycosylated species of very high apparent molecular weight [6, 51, 60]. To rationalize how this lone membrane component might carry out the diverse functions ascribed to the flagellar surface, it was suggested that the single band might in fact be comprised of several distinct polypeptides (the microheterogeneity hypothesis) and-or that Coomassie-blue stain failed to detect additional polypeptides (the minor-component hypothesis) [19]. We show here that both hypotheses are applicable. The major membrane polypeptide band is found to include two related polypeptides, F2 and F3; and the flagellar surface carries, in addition to the mastigoneme and F2–F3 polypeptides, eight minor components, some but not all of which bind the lectin Con A. We have not included in our tabulation those polypeptides that migrate in the stacker region of the gel, since these have been analyzed in a separate report [3]. We have also not included those polypeptides that reside in the flagellar transition region, the site of the "necklace arrays" [17, 57] and putative ion channels [15, 30], since defla-

gellation occurs distal to this region [45]. Finally, our analysis excludes low-molecular-weight polypeptides and, by definition, any species whose labeling is too faint to yield a definitive band. Therefore, we stress that our estimate of 11 flagellar-specific polypeptides is a minimum estimate.

The *Chlamydomonas* flagellar membrane must be under distinct biosynthetic control from the plasma membrane since the prominent surface projections on the flagellar membrane are absent from the plasma membrane (Fig. 3). Nonetheless, the two membrane systems are physically contiguous. Nothing is known about how these distinct membrane domains are established; once established, however, the intramembranous "bracelet" that surrounds the flagellar membrane as it everts from the plasma membrane ([57]; Fig. 1) may serve as a physical barricade to diffusion and thereby maintain the two domains.

Polypeptide profiles of ciliary-flagellar membranes have been described for several other organisms, and a considerable diversity is found. Thus, the *Euglena* membrane is dominated by a high-molecular-weight glycoprotein and mastigonemes [47]. *Paramecium* cilia [5, 39] and scallop sperm-tail flagella [55] also display a prominent large glycoprotein as well as a number of minor polypeptides. *Ochromonas* flagella [11] and scallop gill [55] and *Tetrahymena* [14] cilia, on the other hand, are dominated by lower-molecular-weight components. The last two membrane types, in particular, reportedly contain abundant membrane-localized tubulin. We show in this study that F10, a suspected *Chlamydomonas* membrane-tubulin candidate [2], is not tubulin-like in its peptide maps. The *Chlamydomonas* membrane is therefore more similar to the sperm-tail/*Euglena* version in its general composition, but the component polypeptides of all of these ciliary surfaces are likely to be as cell-type specific as are the polypeptides of plasma membranes. This diversity stands in striking contrast to the internal axoneme, whose structure, major polypeptide components (tubulins and dyneins), and beat patterns are very similar throughout the eukaryotic kingdom [18, 50]. Presumably the membrane diversity reflects the specialized kinds of environmental interactions entrusted to these organelles [48, 58].

Our warmest thanks to Dr. John Heuser, Dept. of Physiology & Biophysics, Washington University, for the electron micrographs, to Dr. D. Stuart for improvements in the cell-wall isolation protocols, and to Carol Hwang for pouring and inoculating countless plates of gametes and for photography. Supported by grants GM-26117, GM-26150 and GM-29647 from the National Institutes of Health.

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