Characterization of the Cilia and Ciliary Membrane Proteins of Wild-type *Paramecium tetraurelia* and a Pawn Mutant

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ABSTRACT Cilia and ciliary membranes were isolated from axenically grown, wild-type *Paramecium tetraurelia* strain 51s and from the extreme pawn mutant strain, $d_4$95, derived from this parental strain. Over 60 protein bands having molecular weights of 15 to >300 kdaltons were detected by Coomassie Blue staining of whole cilia proteins separated by one-dimensional SDS polyacrylamide gel electrophoresis. About 30 of these protein bands were visible in Coomassie Blue-stained membrane separations. About 60 bands were detected by silver staining of one-dimensional gels of membrane proteins. Differences between Coomassie Blue-stained separations of wild-type and pawn mutant strain $d_4$95 membrane proteins were seen in the quantity of a band present at 43 kdaltons. Radioiodination of cell surface proteins labeled ~15 protein bands in both wild-type and mutant cilia. The major axonemal proteins were unlabeled. Six membrane glycoproteins were identified by staining one-dimensional separations with iodinated concanavalin A and lentil lectin, two lectins that specifically bind both glucose and mannose residues. Two major neutral sugar species present in an acid hydrolysate of the cilia preparation were tentatively identified as glucose and mannose by gas chromatography of the alditol acetate derivatives.

The mechanism of force generation and motility in *Paramecium* as well as in other ciliated and flagellated eucaryotic organisms is associated with axonemal structures, while control of the direction and frequency of ciliary beat, or swimming behavior, is associated with ion fluxes through channels in the surface membrane (11, 34, 35). Several ultrastructural and biochemical studies have examined the axonemal components responsible for motility, but few have examined the membrane components that are responsible for its behavioral control. Electrophysiological studies have shown that backward swimming in *Paramecium* is associated with the influx of calcium ions down their electrochemical gradient into the cilia through voltage-sensitive membrane channels (12, 30). This active calcium current is eliminated by deciliation of the cell and returns with a time-course similar to that of ciliary regeneration, indicating that the voltage-sensitive Ca channels are localized in the ciliary membrane (10, 31, 37). Because methods are available for the isolation and purification of cilia and ciliary membrane, a large quantity of a relatively pure, excitable membrane preparation can be obtained for biochemical analysis.

A set of mutants possessing a defective calcium conductance has been isolated from nitrosoguanidine-treated cells on the basis of altered behavior (25, 26) as well as on their resistance to barium toxicity (47). These mutants possess a lesion in any of three independent loci and are known as pawn mutants because they are unable to reverse their forward swimming motion. Mutants whose membranes have been disrupted by treatment with the detergent Triton X-100 swim backward in the presence of sufficient calcium ions just as do wild-type cells, indicating that the calcium-sensitive axonemal component(s) responsible for backward swimming is intact in the mutant (27).

Paramecium, with its membrane mutants, provides a good model system for the electrophysiological and biochemical analysis of an excitable membrane. Thus, just as the electrophysiological properties of the calcium channel have been identified by comparing the responses of wild-type cells with those of pawn mutant cells that lack functional channels (36, 46), the biochemical properties of the channels may be determined by similar parallel analyses of the components of the wild-type and mutant membranes. In the past two years, such parallel analyses of the phospholipid and fatty acid compositions of wild-type and pawn mutant cells and cilia have failed to detect any difference which could be attributed to the loss of excitability characterizing the pawn mutation (4, 21, 45). A

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previous study also failed to detect a significant difference between wild-type and pawn mutant ciliary membrane proteins (18). In that study, one major and 12–15 minor components were detected in one-dimensional separations of ciliary membrane proteins. The major membrane component was shown to be immunologically related to the immobilization antigen (i-antigen), a large, peripheral surface protein (42, 43). Adoutte and his co-workers have recently reported the detection of up to 70 proteins in two-dimensional separations of wild-type ciliary membranes, which represents a considerable increase in the resolution of membrane components (3). No analyses of mutant membranes were reported.

In the present study, characteristics of the cilia and ciliary membrane proteins of wild-type *Paramecium tetraurelia* were examined after separation by one-dimensional SDS PAGE. Membrane proteins exposed on the exterior surface were identified by iodination with lactoperoxidase, and the glycoprotein composition of the preparations was determined by staining gels with iodinated lectins. Identification of the sugar moieties was confirmed by gas chromatography. Pawn mutant protein separations were examined in parallel with wild-type separations in an attempt to correlate the change in pawn membrane function with a specific alteration in the structure of a membrane protein.

MATERIALS AND METHODS

Cell Cultures

Paramecium tetraurelia strain 51s, pawn mutant strains $d_4$94 (pW A/pW A) and $d_4$95 (pW B/pW B), temperature-sensitive pawn strain $d_4$131 (pW C/pW C), and paranoic strain $d_4$149 (all mutants generously provided by Dr. C. Kung, University of Wisconsin) were grown at 25°C in the dark to early stationary phase in 2,800-ml Fernbach flasks containing 500 ml of medium. Cells were grown in an axenic crude medium (48) to a density of 2–3 × 10^4 cells/ml for wild-type and 0.5–2 × 10^4 cells/ml for the mutants.

Iodination of Surface Membrane Proteins

The ciliary surface membrane proteins were iodinated in vivo by a modification of the methods of Phillips and Morrison (39). Late log to early stationary phase cells were harvested, washed with buffer A (0.15 M sucrose, 4 mM KCl, 1 mM $CaCl_2$, 1 mM 3-(*N*-morpholino)propanesulfonic acid buffer [MOPS], pH 7.0), and resuspended in 30 ml of buffer A at 2–4 × 10^5 cells/ml. Lactoperoxidase and KI were added to final concentrations of 3 × 10^{-7} and 1 × 10^{-6} M, respectively. In a well-ventilated hood, 300–500 μCi ^{125}I-Na was added and the reaction was initiated with the addition of hydrogen peroxide to a final concentration of 2 μM. The reaction was maintained by the further addition of four more aliquots of peroxide at 5-min intervals. 5 min after the final addition, the cells were washed three times by centrifugation in buffer A containing 1 mM KI and 1 mM Na_2SO_3. Cilia and membranes were then isolated as described below.

Preparation of Cilia Fractions

Cilia preparations were obtained as previously described (21). Cells were routinely monitored by phase-contrast microscopy during the deciliation procedure to insure that no cell breakage was occurring.

Ciliary membranes were isolated according to the methods of Adoutte and co-workers (2, 3) with slight modifications. Washed cilia pellets were suspended in 25 ml of a solution containing 0.1 mM EDTA, 1.0 mM Tris buffer, pH 8.3, and agitated on a mixer (Vortex-Genie, Scientific Industries, Inc., Bohemia, N. Y.) at high speed for 2 min to disrupt the membrane. This suspension was centrifuged at 48,000 g_{max} for 30 min and the pellet was resuspended in 2 ml of a solution containing 0.1 mM $CaCl_2$, 10 mM MOPS buffer, pH 7.0 (Ca-MOPS). The suspension was layered over a discontinuous sucrose gradient containing 1 ml each of (wt/vol) 66, 55, and 44% sucrose in Ca-MOPS and centrifuged for 1 h at 243,000 g_{max} in a Beckman SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The white band of ciliary membrane was collected from just below the buffer-44% sucrose interface with a Pasteur pipet. Membrane material was washed in Ca-MOPS solution and collected by centrifugation at the same speed. Cilia and membrane preparations were used immediately or stored frozen at −70°C for up to several months with no apparent change in the protein profiles obtained upon one-dimensional electrophoretic analysis.

Immobilization antigen was prepared as described by Preer (42). Protein precipitated from the salt-ethanol solution in the 35–60% ammonium sulfate fraction was dissolved in Ca-MOPS, concentrated, and used for electrophoretic analysis without further purification.

One-dimensional Gel Electrophoresis

Proteins were analyzed by one-dimensional polyacrylamide gel electrophoresis (PAGE) using the discontinuous SDS buffer system of Laemmli (28). Samples were solubilized at a concentration of 5 mg protein/ml in a solution containing 2.3% (wt/vol) SDS, 10% (wt/vol) glycerol, and 0.0625 M Tris base, pH 6.8, and heated at 100°C for 5 min. Proteins were reduced by adding 2-mercaptoethanol to a final concentration of 5% (vol/vol) and then were reheated. Sample aliquots containing 100–200 μg of protein were loaded into slots in slab gels (either 130 × 1.5 or 240 × 1.5 mm). A 5–12% acrylamide separating gel and a 4.5% acrylamide stacking gel were used. Proteins were separated by electrophoresis at 15 mA for 1 h and then at 20 mA until the tracking dye was within 1 cm of the bottom of the gel.

Gels were fixed overnight either in 7% (vol/vol) acetic acid or in acetic acid/isopropanol/water (2:5:13, vol/vol/vol), washed in acetic acid/ethanol/water (1:6:13, vol/vol/vol), and stained for 1 h in 0.1% (wt/vol) Coomassie Blue R-250 in acetic acid/ethanol/water (2:9:9, vol/vol/vol). Gels were destained overnight in acetic acid/ethanol/water (1:6:13, vol/vol/vol) and stored in 7% acetic acid. For determination of the relative amounts of the major proteins present, gels were stained with fast green after the procedure of Gorovsky and co-workers (17). Additional protein components in one-dimensional gels were visualized by the sensitive silver staining procedure of Switzer and co-workers (51).

The molecular weights of proteins were estimated by the method of Weber and Osborn (52). The standards used were myosin (200 kdaltons), phosphorylase A (94 kdaltons), bovine serum albumin (68 kdaltons), ovalbumin (43 kdaltons), DNase I (31 kdaltons), and hemoglobin (16 kdaltons). Protein concentrations were determined by the method of Lowry and co-workers (29).

The optical density profiles of Coomassie Blue-stained gels were obtained by scanning the gels at 560 nm using a Gilford model 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) with accompanying gel scanner. Gels stained with fast green were scanned at 600 nm and tracings of individual peaks were cut out and weighed to determine the relative amounts of individual proteins present.

The activity of iodinated proteins separated on one-dimensional gels was determined by slicing gels into 2-mm sections and counting the slices in a Packard Auto-gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Iodinated protein bands were also detected by exposing gels (dried on filter paper under vacuum) to Kodak XR-1 film for a period of 5–30 d.

Binding of Lectins

Concanavalin A (Con A) and lentil lectin were dissolved at 1–5 mg/ml in phosphate-buffered saline (PBS) containing (per liter) 0.1 g of $CaCl_2$, 0.2 g of KCl, 0.1 g of $MgCl_2$, 8.0 g of NaCl, 0.2 g of KH_2PO_4, 2.16 g of $Na_2HPO_4 \cdot 7H_2O$, pH 6.8, and iodinated according to the method of Phillips and Morrison (39) in the presence of 0.2 M α-methyl-D-mannoside to protect the sugar-binding site. The iodinated lectins were dialyzed against several changes of PBS at 4°C and repurified by affinity chromatography on Sephadex G-100. Lectin bound to the column was eluted with 0.2 M α-methyl-D-mannoside in PBS and dialyzed extensively against PBS at 4°C before use. Iodinated lectins had specific activities of 0.5–5.0 × 10^7 cpm/mg and retained their sugar-binding properties as determined by their ability to agglutinate human type O erythrocytes.

Fixed gels were washed in several changes of PBS at 4°C to remove the fixative solution and incubated overnight at room temperature in PBS solution containing 10^5–10^6 cpm/ml (20–100 μg/ml) of iodinated lectin with 1 mg/ml hemoglobin added as a carrier protein (6). Labeled gels were washed free of unbound lectin in several changes of PBS at 4°C over a 48-h period and stained for protein as described above. Stained gels were dried on filter paper under vacuum and subjected to autoradiography for 7–35 d. Control gels were treated in the same manner, except that 0.1 M α-methyl-D-mannoside was present in the lectin staining solution and in the PBS wash solutions after staining.

Analysis of Neutral Sugars

Analysis of the neutral sugars of the ciliary preparation was carried out according to the procedure of Kim and co-workers (22). Sugars were hydrolyzed in the presence of 0.25 N H_2SO_4 and Dowex 50 (H^+) (Sigma Chemical Co., St. Louis, Mo.), reduced to the corresponding alditols with $NaBH_4$, and acetylated using acetic anhydride in the presence of pyridine. A standard mixture containing

the acetylated sugar derivatives of 2-deoxyribose, fucose, ribose, xylose, 2-deoxyglucose, 2-deoxygalactose, mannose, galactose, and glucose was prepared in the same manner, except that the initial hydrolysis step was omitted. The acetylated derivatives were analyzed by gas chromatography (Hewlett-Packard model 1850 A, Hewlett-Packard Co., Avondale, Pa) on a 6 ft × 2 mm ID glass column packed with Gas Chrom Q, 60–80 mesh, precoated with 3% ECNSS-M (Applied Sciences Lab., Inc., State College, Pa.). Chromatography was carried out at 150°C for 10 min, after which the temperature was increased at 1°/min to 200°C. The injection port and detector temperatures were set at 270° and 310°C, respectively, and the flow rate of the nitrogen carrier gas was 25 ml/min. The neutral sugars present in the ciliary hydrolysate were identified on the basis of their retention times compared to standards.

Materials

Reagents used for electrophoresis and high molecular weight protein standards were obtained from Bio-Rad Laboratories (Richmond, Calif.). SDS was obtained from BDH Chemicals, Ltd. (Poole, England). ^{125}I-Na was obtained from Amersham Corp. (Arlington Heights, Ill.), and lactoperoxidase purified from milk was obtained as the lyophilized powder from Sigma Chemical Co. (St. Louis, Mo.). MOPS buffer was synthesized according to the method of Good and Izawa (16). Lentil lectin and Con A were obtained from Sigma Chemical Co. All other chemicals were obtained from Sigma Chemical Co., Fisher Scientific Co. (Pittsburgh, Pa.), and Scientific Products Div., American Hospital Supply Corp. (McGaw Park, Ill.), and were of at least reagent grade.

RESULTS

One-dimensional Separations

Separations of *Paramecium* cilia proteins by one-dimensional SDS PAGE gave a reproducible pattern of over 50 Coomassie Blue-staining bands having molecular weights of 15 to >300 kdaltons (Fig. 1). Approximately two-thirds of the Coomassie Blue-staining protein was localized in three regions of the gel which corresponded to: (*a*) tubulins having estimated molecular weights of ~50 and 53 kdaltons, (*b*) at least two distinct bands that migrated with i-antigens prepared by the method of Preer (42) and having estimated molecular weights of 200–250 kdaltons, and (*c*) high molecular weight bands of

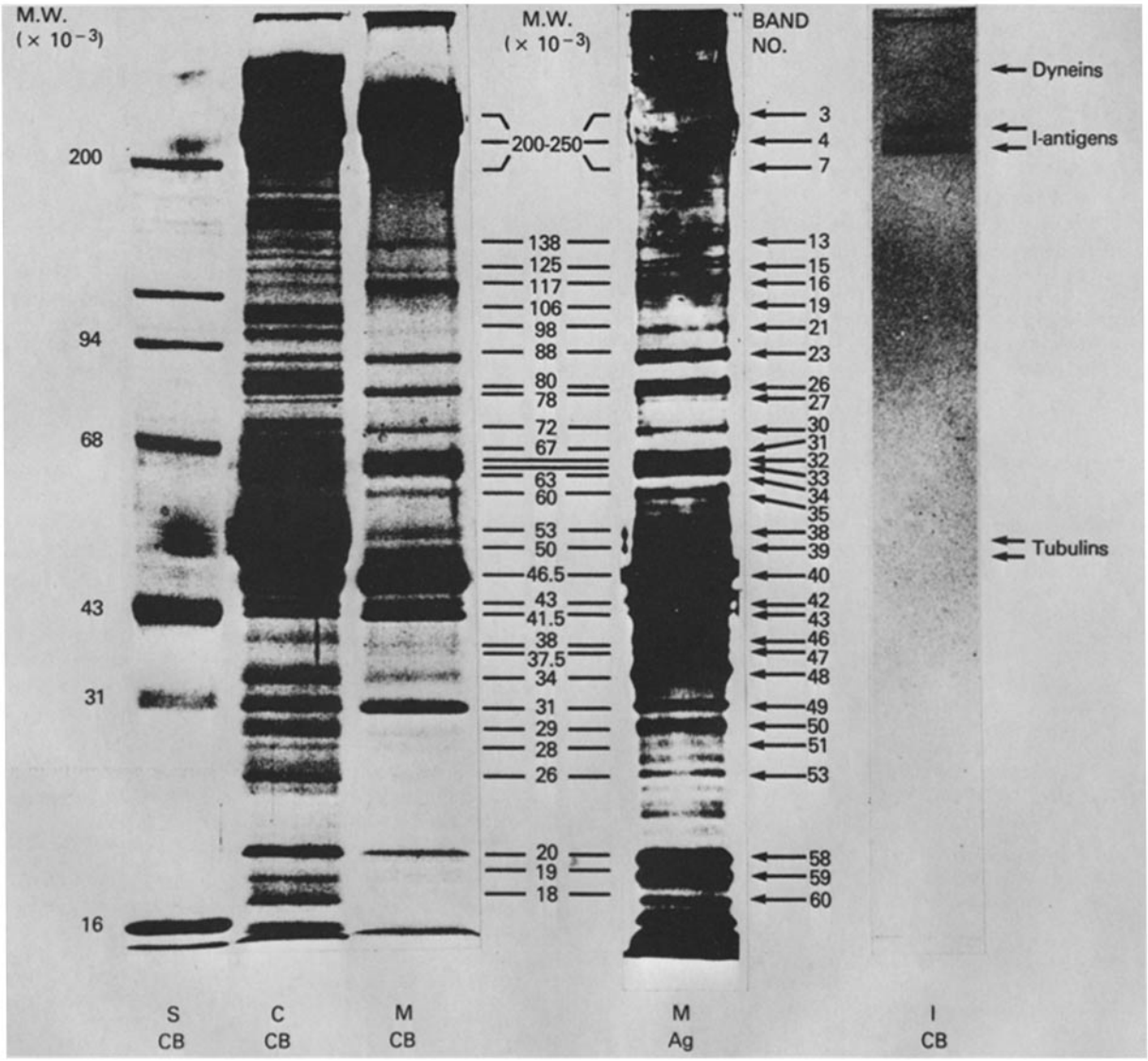


FIGURE 1 SDS PAGE of cilia (*C*), ciliary membrane (*M*), ciliary membrane stained with silver (M_{Ag}) and i-antigen (*I*) preparations. Lanes containing cilia and ciliary membrane proteins were heavily loaded with 200–250 μg protein to allow visualization of minor Coomassie Blue (*CB*)-staining bands. The high molecular weight major membrane proteins migrated the same distance as did purified i-antigens. The major axonemal proteins, tubulins and dyneins, were greatly reduced or absent in the membrane preparations. The approximate molecular weight of proteins was determined from the position of protein standards (*S*) separated on the same gel.

>280 kdaltons which appear as a single broad band above the i-antigen bands. The major bands of a dynein preparation, obtained by the method of Kincaid and co-workers (23) as modified by Doughty for *Paramecium* dyneins (9), migrated in this region of the gel (M. J. Doughty and E. S. Kaneshiro, unpublished observations). Quantitative densitometry of fast green-stained gels indicated that the tubulins contained approximately one-third, the i-antigens one-fourth, and the dyneins 8–9% of the total ciliary proteins. These approximations assume similar specific staining intensities of these proteins with fast green.

Separations of ciliary membrane proteins resolved at least 30 Coomassie Blue-staining bands (Fig. 1). The principal bands present in membrane separations were the i-antigens at 200–250 kdaltons which comprised approximately two-thirds of the total membrane protein. The next most prominent band had an apparent molecular weight of 46.5 kdaltons. Two bands that comigrated with tubulins were either absent or present in greatly reduced amounts of membrane separations as compared to separations of whole cilia. The large dynein band was absent.

Several one-dimensional separations of ciliary membrane were stained for protein by the silver staining procedure of Switzer and co-workers (51). This procedure, which stains proteins differently than Coomassie Blue, detected ~30 additional membrane protein bands (Fig. 1). Interestingly, it failed to stain the i-antigen bands, bands that readily stained with Coomassie Blue on the other gels.

Comparison of Wild-type and Mutant Protein Patterns

One-dimensional separations of pawn mutant strain $d_4$95 ciliary membrane proteins produced Coomassie Blue-staining patterns very similar to those of wild-type separations (Fig. 2). None of the proteins resolved in this study were present in one strain and not in the other. However, two consistent differences

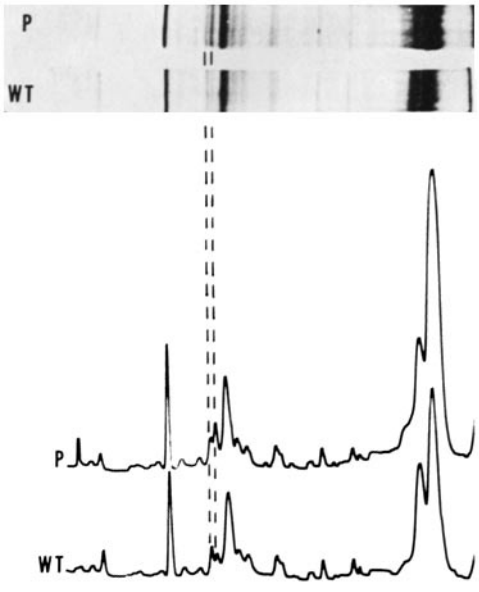


FIGURE 2 Comparison of the Coomassie Blue-staining profiles of wild-type (*WT*) and pawn mutant (*P*) membrane protein separations. Densitometer tracings of the Coomassie Blue-stained gels are shown. The 43-kdalton band consistently stained with a greater intensity in pawn mutant separations.

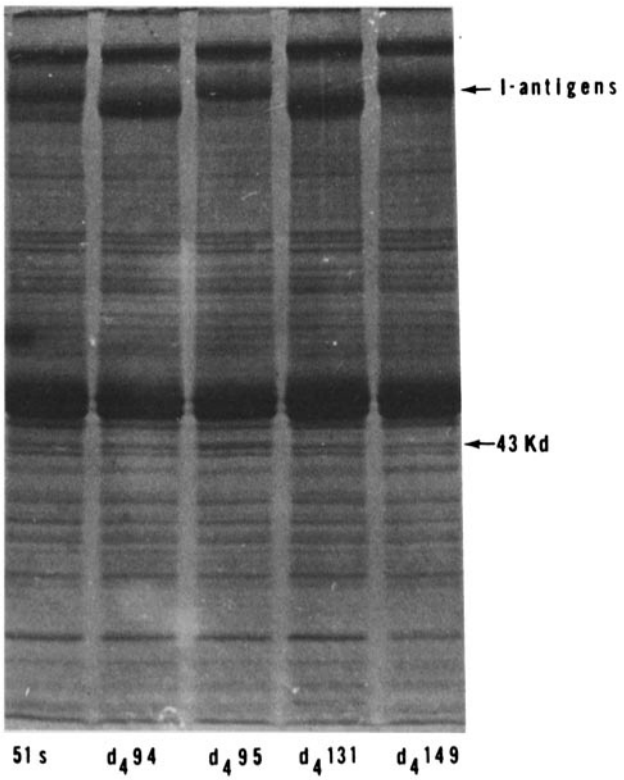


FIGURE 3 Comparison of the Coomassie Blue-staining profiles of ciliary membrane proteins from wild-type strain 51s and four behavioral mutants. Quantitative and qualitative differences were seen between strains in the banding pattern of the i-antigen-related protein bands. Only pawn mutant strain $d_4$95 showed the increased staining intensity of the 43-kdalton band as compared to the 41.5-kdalton band.

were noted in the banding patterns of wild-type and mutant membrane proteins. First, the slower migrating of the i-antigen-related bands in mutant protein separations was present in greater amounts than the corresponding band in the wild-type separations. Secondly, a quantitative difference was seen in the relative amounts of protein present in the two bands at 41.5 and 43 kdaltons. The 43-kdalton band consistently stained with a greater intensity than the 41.5 kdalton band in pawn mutant strain $d_4$95 separations while the opposite was generally true of wild-type separations (Fig. 2). While other small differences in the staining intensity of bands were sometimes found when comparing membrane protein separations, these two differences were the only two found to occur consistently in a large number of separations of independent ciliary membrane preparations.

To determine if these two differences between wild-type and pawn strain $d_4$95 were unique or if similar differences could be detected in other mutants, one-dimensional separations of cilia proteins isolated from a number of additional behavioral mutants were examined. Fig. 3 shows the ciliary protein patterns of wild-type strain 51s, three pawn mutant strains having lesions at independent genetic loci ($d_4$94, pW A; $d_4$95, pW B; $d_4$131, pW C), and paranoiac mutant strain $d_4$149. The quantity and migration distance of the i-antigen-related proteins were found to differ from strain to strain, but no correlation was observed between the banding pattern and the behavioral phenotype. On the other hand, only pawn mutant strain $d_4$95 showed the increased Coomassie Blue-staining intensity of the 43-kdalton band. Thus, this difference does not appear to result

from some general, secondary effect of the mutation such as a reduction in growth rate.

Iodination of Membrane Proteins

The lactoperoxidase-catalyzed iodination of *Paramecium* proteins in vivo resulted in the labeling of 15–18 protein bands present in the membrane fraction. The major axonemal proteins, dyneins and tubulins, remained unlabeled, supporting the conclusion that only membrane proteins were labeled under these conditions. When the proteins of cilia isolated from iodinated cells were separated by one-dimensional SDS PAGE and exposed to film for 40 h (Fig. 4), three major iodinated bands were seen. These bands corresponded to the i-antigen-related protein and two membrane proteins at 46.5 and 43 kdaltons. Longer exposure for 110 h (Fig. 4) have additional labeled bands that corresponded to membrane proteins of 138, 117, 106, 98, 88, 80, 60, 38, 34, and 28 kdaltons. One iodinated band did not correspond precisely to any Coomassie Blue-staining membrane band (Fig. 4, see ×). The molecular weight of this band was between 72 and 78 kdaltons and corresponded to one of the two silver-staining membrane bands present in this region of the gel. Three additional faintly labeled bands could be distinguished on the original autoradiogram of the iodinated cilia proteins: one of these was between the Coomassie Blue-staining bands of 20 and 26 kdaltons, the other two

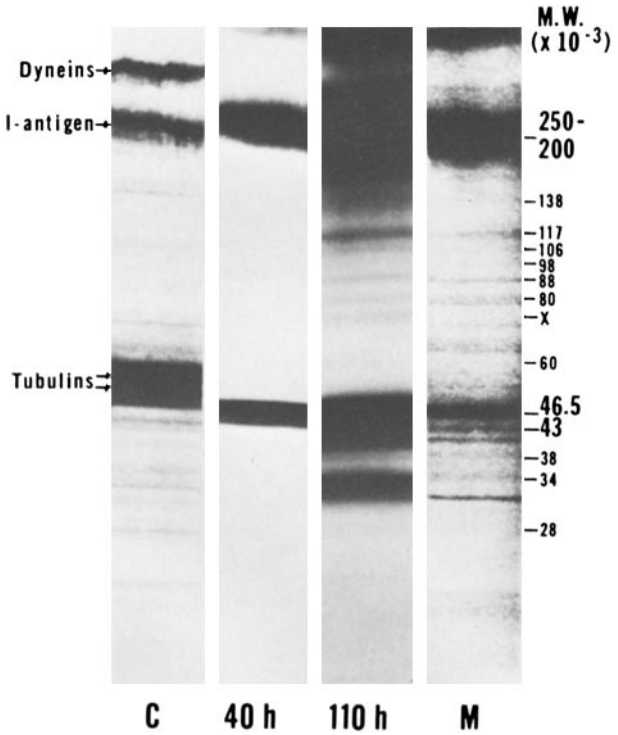


FIGURE 4 Identification of wild-type cilia proteins iodinated in vivo. One-dimensional separations of iodinated cilia proteins were stained with Coomassie Blue (C) and subjected to autoradiography. The major labeled proteins were apparent after autoradiography for a short time period (40 h) and are indicated next to the Coomassie Blue-stained membrane reference gel (M) by large numerals corresponding to their molecular weights. Additional labeled bands were evident after a longer time exposure (110 h). All but one of the labeled cilia protein bands (×) corresponded to a Coomassie Blue-staining membrane protein band. This band was detected by the silver stain.

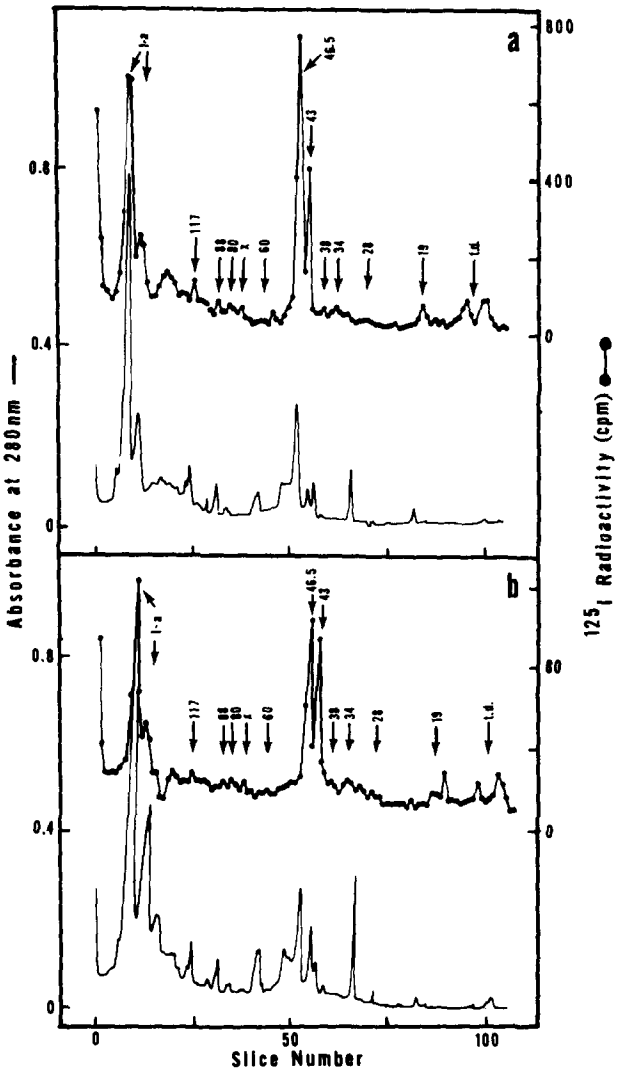


FIGURE 5 Iodination profiles of wild-type (*a*) and pawn mutant (*b*) ciliary membrane proteins iodinated in vivo. Densitometer tracings of Coomassie Blue-stained gels (—) and the corresponding ^{125}I activity determined by counting 2-mm gel slices (●—●) are shown. Arrows indicate the position of labeled bands. The iodination profiles were similar.

were at 19 kdaltons and at the dye front. The membrane protein bands at 125 and 78 kdaltons and the group of four proteins between 63 and 67 kdaltons were not detectably iodinated. It was not possible to determine from autoradiograms whether membrane protein bands at 41.5, 37.5, and 31 kdaltons were iodinated because of their proximity to heavily labeled proteins that mask the detection of minor labeled components. When control iodinations were performed in the absence of lactoperoxidase, no cilia protein bands were labeled.

The iodination profiles of wild-type and pawn mutant strain $d_4$95 membrane separations were compared and no major differences were noted (Fig. 5). As in the analyses of whole cilia, the major peaks of radioactivity were associated with the i-antigens and proteins at 46.5 and 43 kdaltons. It was not possible to compare the activity of the 43-kdalton band in the two strains and the activity of the 41.5-kdalton band because the 41.5-kdalton band was not detectably labeled. However, comparison of the activity of the 43-kdalton band and the activity of the 46.5 kdalton band, which does iodinate, reflects the relatively greater amount of protein found in the 43-kdalton

band of pawn mutant separations (see Fig. 2). Minor peaks of activity were associated with bands at 117, 88, 80, 60, 34, 28 and 19 kdaltons. One peak of activity occurred on either side of the dye front which may correspond to labeled lipids or small peptides below 15 kdaltons. A broad peak of activity occurred in the region of the gels between 117 and 200 kdaltons which could not be resolved into its individual components, although it is likely that a portion of the activity was associated with the 138-kdalton band as seen as Fig. 4. In both wild-type and mutant separations, a small peak of activity also appeared to be associated with the 31-kdalton protein and with a protein between 20 and 26 kdaltons. In most cases, when wild-type and mutant cells were iodinated under the same conditions, the specific activity of the isolated wild-type cilia proteins was greater than the activity of the mutant cilia proteins (compare scales, Fig. 5); however, this difference in activities was no greater than that which could be obtained from consecutive iodinations of wild-type proteins.

Analyses of Glycoproteins

Analysis of the neutral sugars of the isolated cilia preparation by gas chromatography of the alditol acetate derivatives indicated the presence of two major and three minor sugar components (Fig. 6). By comparison with the retention times of derivatized sugar standards, the two major components were tentatively identified as mannose and glucose. Two of the minor components had the same retention times as ribose and galactose, while the third minor component had a longer retention time than any of the sugar standards used.

The glycoprotein composition of the cilia and ciliary membrane preparations was identified by staining one-dimensional separations with iodinated Con A and lentil lectin, two lectins that specifically bind to glucose and mannose residues. Lentil lectin was found to consistently bind to the two i-antigen-related protein bands and to membrane protein bands at 46.5, 38, and 19 kdaltons (Fig. 7). Weak binding was sometimes seen to bands at 31 and 34 kdaltons. The binding of labeled lentil lectin to the protein bands was carbohydrate specific, because staining in the presence of the competing sugar, α-methyl-D-mannoside, completely eliminated lectin binding (Fig. 7*c*). Binding of lentil lectin to separations of whole cilia and membrane proteins was the same (Fig. 7*b*).

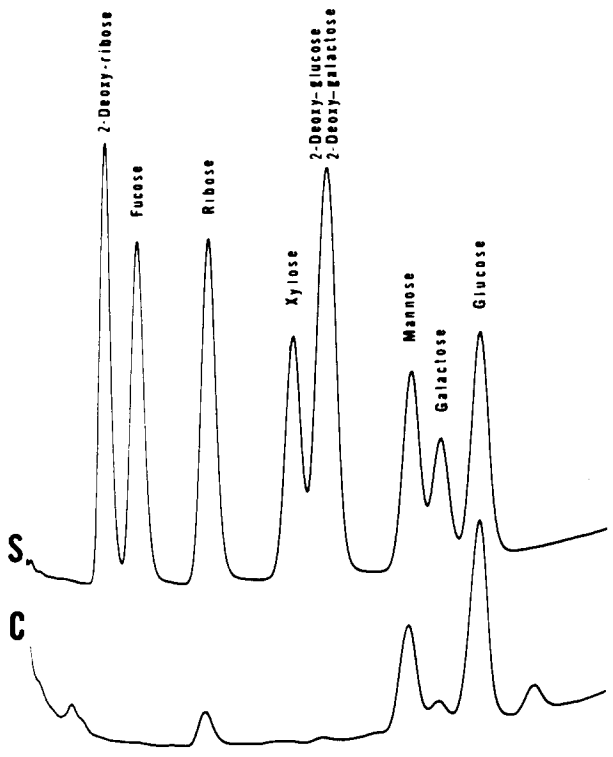


FIGURE 6 Gas chromatogram of the alditol acetate derivatives of a mixture of authentic standards of neutral sugars (*S*) and of neutral sugars contained in an acid hydrolysate of wild-type cilia (*C*) preparations. The major peaks of the cilia hydrolysate had the same retention times as mannose and glucose, while two of the minor peaks had the same retention times as ribose and galactose.

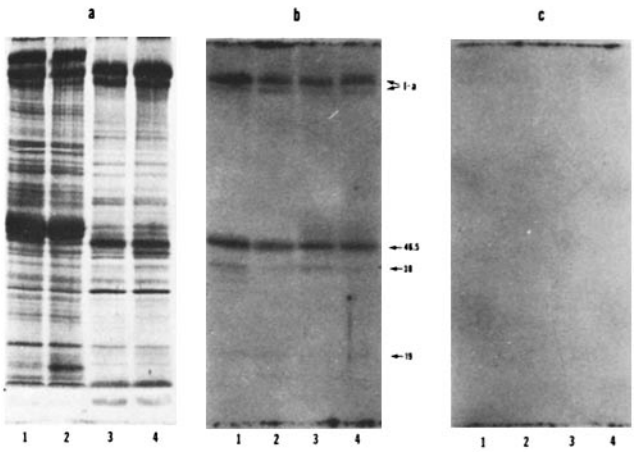


FIGURE 7 SDS PAGE of wild-type cilia (*1*), mutant (strain $d_4$95) cilia (*2*), wild-type membrane (*3*), and mutant membrane (*4*). Gels were stained with Coomassie Blue (*a*), ^{125}I-lentil lectin (*b*), and ^{125}I-lentil lectin in the presence of the specific blocking sugar, α-methyl-D-mannoside (*c*). Lentil lectin bound specifically to the i-antigen-related proteins (*1-a*) and to membrane proteins at 46.5 and 19 kdaltons.

Although Con A and lentil lectin have similar monosaccharide-binding specificities, Con A did not exhibit the same glycoprotein-binding properties as lentil lectin. However, it has previously been shown that monosaccharide binding specificity is not the only determinant of lectin-binding specificity in vivo. Feller and co-workers (14) reported that only 25% of the Con-A-binding sites on the cell surface of human fibroblasts were also lentil lectin-binding sites. In the present study, Con A consistently bound to a membrane protein band at 125 kdaltons and to a minor band above 250 kdaltons (Fig. 8). This higher molecular weight band corresponded to the faster migrating of two minor membrane bands that had approximately the same mobility as axonemal dynein proteins in one-dimensional separations.

Binding of Con A to the 125-kdalton band was unusual. The Coomassie Blue-staining intensity and the apparent molecular weight of this protein band were the same in wild-type and mutant cilia and ciliary membrane protein separations. On the other hand, Con A stained this band only weakly, if at all, in separations of whole cilia, very strongly in separations of wild-type membrane, and less intensely in separations of pawn mutant membrane. Because the protein component of this band is apparently not altered during the membrane preparation, it would appear that some modification of the carbohydrate moiety of the glycoprotein occurs to allow the greater degree of Con A binding to the membrane bands. The nature of this modification has not yet been determined.

Fig. 8*b* shows that Con A also bound weakly and diffusely

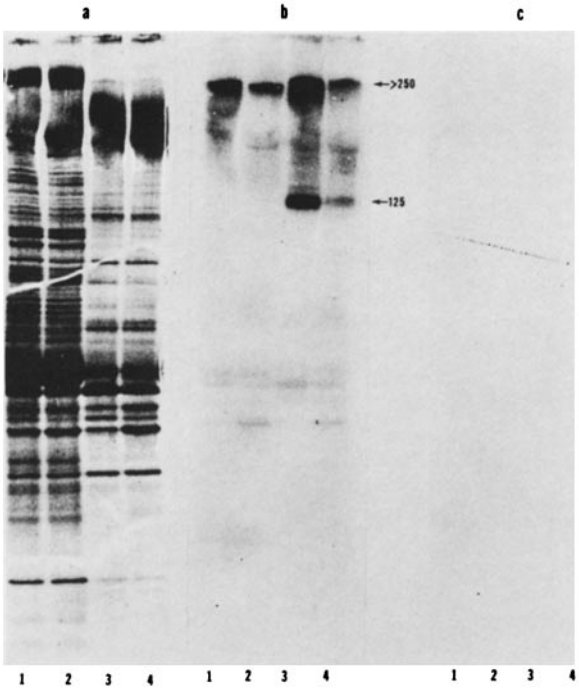


FIGURE 8 SDS PAGE of wild-type cilia (*1*), mutant (strain $d_4$95) cilia (*2*), wild-type membrane (*3*), and mutant membrane (*4*). Gels were stained with Coomassie Blue (*a*), ^{125}I-Con A (*b*), and ^{125}I-Con A in the presence of the specific blocking sugar, α-methyl-D-mannoside (*c*). Con A bound specifically to a minor protein band above 250 kdaltons and to a band at 125 kdaltons in membrane separations. Con A also bound somewhat diffusely in the region of the i-antigen-related proteins and to bands in the 31- to 55-kdalton region of the gel.

in the i-antigen region of the gel and to protein bands at 46.5 and 38 kdaltons, bands that also bound lentil lectin. Con A also bound weakly in the region of the gel just above the 46.5-kdalton band and above the 125-kdalton band. All Con A binding could be eliminated by incubating gels in the presence of the competing sugar, α-methyl-D-mannoside (Fig. 8*c*). Although Con A consistently bound the 125-kdalton band and the band above 250 kdaltons, binding to other bands was not always observed, and, when present, the binding was not always totally eliminated by incubation in the presence of the competing sugar. Thus, Con A appears to have a low specificity for these bands, and Con A binding may involve protein-protein as well as protein-carbohydrate interactions. In addition to the obvious difference in the binding of Con A to the 125-kdalton band of wild-type and mutant membrane separations, smaller quantitative differences in the binding of Con A to other bands can be seen in Fig. 8*b*. However, because of the variability of the binding to these bands, no consistent differences in binding could be determined.

Lectins were examined for their ability to produce behavioral changes in wild-type and pawn mutant cells. When cells were incubated with a lectin at the same concentration and for the same time period that gave strong agglutination of human erythrocytes, no noticeable change occurred either in the swimming behavior of wild-type or mutant cells or in the potassium-induced reversal behavior of wild-type cells.

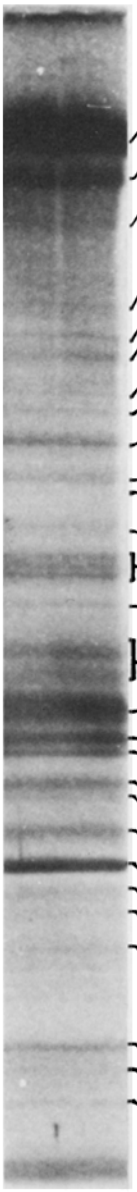
DISCUSSION

The ciliary membrane of *Paramecium* is functionally complex. In addition to exhibiting the voltage-dependent calcium conductance that regulates the ciliate's behavior, the membrane has also been implicated in the mating reactivity (7, 24) and in the immobilization reaction in the presence of specific antisera (see reference 15). Thus, it is not surprising that, by use of more sensitive techniques, a more heterogeneous membrane protein composition was found in this study than had been indicated in earlier studies of this membrane. In the present investigation, ~30 Coomassie Blue-staining membrane protein bands were reproducibly resolved on one-dimensional polyacrylamide gels containing SDS, and ~30 additional bands were identified by the silver-staining procedure. Two earlier studies of the *Paramecium* ciliary membrane reported only 3 (38) and 12–15 (19) protein bands present in one-dimensional separations. However, recent studies (3, 5) have achieved a resolution of ciliary membrane proteins (detected by Coomassie Blue staining) similar to that reported here. Despite differences in culture conditions, fractionation methods, and composition of acrylamide gels used, the protein patterns obtained for *Paramecium* cilia and ciliary membrane proteins are in general agreement (this paper; 3, 5). However, in light of a previous report (3), we were unable to detect any changes in ciliary protein profiles for cilia isolated from cells at different stages in the culture growth cycle.

Comparison of Wild-type and Mutant Ciliary Membranes

Behavioral and electrophysiologic studies have shown that the pawn mutant that we have studied has a defect in the mechanism responsible for the voltage-dependent increase in ciliary membrane calcium conductance exhibited by wild-type paramecia (27, 34). We consider it unlikely that this defect is directly associated with quantitative and qualitative changes in the i-antigen proteins, which is in general agreement with other investigators (18, 35). No obvious correlation can be made between the banding pattern of these proteins and the mutant behavioral phenotypes (three pawns and one paranoiac) representing modifications in different genetic loci (35). It is well known that a single strain of wild-type *Paramecium* may express different serotypes when grown under different environmental conditions (44), and it has previously been shown that cells of different serotypes express different i-antigens that can be distinguished on polyacrylamide gels (18, 19). The wild-type strain, but not pawn mutant strain $d_4$95, used in this study was immobilized by antisera specific for either serotype A or B (Dr. J. Preer, Jr., Indiana University, personal communication). Thus, the differences seen between the i-antigen proteins of wild-type and mutant strains probably reflect serotypic differences.

The quantitative difference in the relative staining intensities of the 43- and 41.5-kdalton bands between wild-type and pawn mutant strain $d_4$95 was found consistently over a large number of preparations. This difference was not found in other mutant strains examined and thus appears to be specific for strain $d_4$95. However, we do not yet know whether this difference is associated directly with the pawn mutation or whether it is a secondary effect. Because the pawn mutant used has a nearly undetectable excitable calcium current, it was expected that either one or more protein components would exhibit a change in molecular weight and/or isoelectric point in the mutant, or that one or more protein components would be totally absent in the mutant, as occurs in axonemal mutants of *Chlamydomonas* (20, 40, 41). No consistent difference of this type was seen between wild-type and pawn strain $d_4$95 cilia and ciliary



CHARACTERIZATION OF MEMBRANE PROTEIN BANDS

Band #	M.W. (Kd)	Exposed to outer Membrane Surface ‡	Glycoprotein §	Comments
1-2‖				Band 2: binds Con A, does not bind lentil lectin
3	200-250	+	+	I-antigen: more intense band in pawn; binds lentil lectin
4	200-250	+	+	I-antigen: binds lentil lectin
5-6‖				
7	200-250	?	-	
8-12‖				
13	138	+	-	
14‖				
15	125	-	+	Binds Con A, does not bind lentil lectin
16	117	+	-	
17-18‖				
19	106	+	-	
20‖				
21	98	+	-	
22‖				
23	88	+	-	
24-25‖				
26	80	+	-	
27	78	-	-	
28-29‖				A protein band in this region is iodinated (see x, Fig. 4)
30	72	-	-	
31-34	63-67	-	-	
35	60	+	-	
36-37‖				
38-39	53-50	-	-	Comigrate with tubulins
40	46.5	+	+	Binds lentil lectin
41‖				
42	43	+	-	More intense band in pawn
43	41.5	?	-	
44-45‖				
46	38	+	+	Binds lentil lectin
47	37.5	?	-	
48	34	+	±	Binds lentil lectin weakly
49	31	±	±	Weakly iodinates and also binds lentil lectin weakly
50	29	-	-	
51	28	+	-	
52‖				
53	26	-	-	
54-57‖				
58	20	-	-	
59	19	+	+	Binds lentil lectin
60	18	-	-	
61-62‖				Band 62 runs at tracking dye front

‡ Determined by in vivo iodination using lactoperoxidase (figures 4, 5).
§ Determined by Con A and lentil lectin binding (figures 7, 8).
‖ Visualized by silver staining procedure, these bands are not consistently seen with coomassie blue staining.

FIGURE 9 Summary of the characteristics of Coomassie Blue-staining protein bands.

membrane proteins separated in one or two dimensions (32). It has recently been reported that the pawn strain $d_4$95 can exhibit the wild-type behavioral phenotype when cells are grown under certain nutritional conditions (12). Thus, rather than being an altered membrane component, the mutant gene product may function in the synthesis or insertion of a membrane component necessary for excitability.

Characterization of the Topography of the Ciliary Membrane

A summary of the characteristics of the Coomassie Blue-staining ciliary membrane protein bands is given in Fig. 9. Protein bands were classified as membrane proteins exposed to the outer surface if they were iodinated in vivo (Figs. 4 and 5) and as glycoproteins if they bound either Con A or lentil lectin (Figs. 7 and 8).

As was originally shown for the erythrocyte membrane (39), lactoperoxidase-catalyzed iodination appears to be specific for the surface membrane proteins of *Paramecium*. Dyneins, tubulins, and other major axonemal components were unlabeled by this iodination procedure, while the i-antigens, components of the surface coat (33), were heavily labeled. The bands at 43 and 46.5 kdaltons were also heavily labeled and thus are also probably components of the surface coat. The fact that prominent Coomassie Blue-staining membrane protein bands at 41.5

and 31 kdaltons were either not iodinated or only weakly iodinated argues that only exposed membrane proteins were labeled. Because both of these bands were readily iodinated after disruption of the membrane (32), these two proteins are probably exposed on the cytoplasmic surface of the ciliary membrane. The low activity associated with these two protein bands when cells were iodinated in vivo could indicate that these bands correspond to transmembrane proteins that have only a small number of reactive amino acid residues exposed to the external surface. Alternatively, these bands could be composed of more than a single protein, with only a minor protein component exposed to the external surface of the membrane.

While there are no previous reports in the literature on the number of enzymatically iodinated surface components of *Paramecium* cilia, there are several reports on the iodinated surface components of the ciliary and flagella membranes of other organisms. About 20 iodinated surface proteins were identified in *Tetrahymena* cilia and pellicle preparations (53). The major iodinated protein identified had a molecular weight of ~48 kdaltons, migrating slightly ahead of tubulins on one-dimensional SDS polyacrylamide gels. *Tetrahymena* ciliary tubulins were apparently not iodinated in that study (53). In contrast to these results, tubulin-like proteins have been identified as membrane components in both *Chlamydomonas* flagella (1) and molluscan gill cilia (49) as well as in mammalian neurite tissue culture cells (13) on the basis of lactoperoxidase-catalyzed iodination. Stephens (50) also found that periodic acid-Schiff (PAS)-positive bands having the same apparent molecular weights as tubulins on SDS polyacrylamide gels were the major components of molluscan gill ciliary membrane, and Dentler (8) reported similar findings using *Tetrahymena* ciliary membranes. In these two studies, the membrane-associated tubulin-like proteins were differentiated from axonemal tubulins by their PAS-positive staining and by slight differences in the tryptic peptide maps between axonemal and membrane-associated proteins. Because minor Coomassie Blue-staining bands which have the same apparent molecular weight as tubulins appeared in membrane separations, it is possible that tubulins are also minor components of the *Paramecium* ciliary membrane. However, because the tubulins found in the *Paramecium* ciliary membrane preparations do not have the carbohydrate-staining characteristics of the membrane-associated tubulins identified in the cilia or flagella of other organisms, and because they do not iodinate in vivo, it appears more probable that these tubulins represent minor contamination of the preparation by axonemal components.

The only glycoprotein identified in previous studies of *Paramecium* cilia was the i-antigen-related major membrane protein (18, 19). The identification of additional membrane glycoprotein bands in this study can be attributed to the increased sensitivity obtained by staining gels with iodinated lectins as compared to the PAS staining technique previously used. In addition to the two i-antigen bands, lentil lectin consistently stained bands at 46.5, 38, and 19 kdaltons. This staining was easily blocked by the addition of the competing sugar, α-methyl-D-mannoside, to the staining and wash solutions, indicating the glycoprotein nature of these bands. In contrast to lentil lectin, Con-A-stained gels had to be extensively washed in a solution containing a carrier protein (hemoglobin or bovine serum albumin) to reduce nonspecific staining. However, Con A did bind specifically to two bands at 125 and >250 kdaltons not bound by lentil lectin, as well as weakly to bands that did bind lentil lectin.

To summarize, we have (*a*) detected ~30 and 60 ciliary membrane protein bands on one-dimensional gels by Coomassie Blue and silver staining, respectively, (*b*) identified membrane components exposed to the external surface of the ciliary membrane by radioiodination techniques, (*c*) detected at least six glycoproteins in ciliary membranes by lentil lectin and Con-A-binding methods, (*d*) confirmed the lectin-binding studies by identifying glucose and mannose in acid hydrolysates of cilia, and (*e*) detected a quantitative difference in the 43-kdalton membrane protein band in a pawn B mutant. These studies establish characteristics of the ciliary membrane from axenically grown paramecia and confirm and extend observations from other laboratories. Further studies of specific individual components herein described are in progress employing selective isolation, immunochemical and enzymatic approaches.

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REFERENCES

1. Adair, W. C., and U. W. Goodenough. 1978. Identification of membrane tubulins in *Chlamydomonas* flagella. *J. Cell Biol.* 79:54*a* (Abstr.).
2. Adoutte, A., R. R. Dute, C. Kung, R. M. Lewis, K.-Y. Ling, D. L. Nelson, and R. Ramanathan. 1978. Proteins of cilia and ciliary membranes of *Paramecium tetraurelia*. *J. Cell Biol.* 79:224*a* (Abstr.).
3. Adoutte, A., R. Ramanathan, R. M. Lewis, R. R. Dute, K.-Y. Ling, C. Kung, and D. L. Nelson. 1980. Biochemical studies of the excitable membrane of *Paramecium tetraurelia*. III. Proteins of cilia and ciliary membranes. *J. Cell Biol.* 84:717–738.
4. Andrews, D., and D. L. Nelson. 1979. Biochemical studies of the excitable membrane of *Paramecium tetraurelia*. II. Phospholipids of ciliary and other membranes. *Biochim. Biophys. Acta.* 550:174–187.
5. Brugerolle, G., C. Andrivon, and J. Bohatier. 1980. Isolation, protein pattern and enzymatic characterization of the ciliary membrane of *Paramecium tetraurelia*. *Biol. Cell.* 37:251–260.
6. Burridge, K. 1976. Changes in cellular glycoprotein after transformation: identification of specific glycoproteins and antigens in sodium dodecyl sulfate gels. *Proc. Natl. Acad. Sci. U. S. A.* 73:4457–4461.
7. Butzel, H. M., Jr. 1974. Mating type determination and development in *Paramecium aurelia*. *In* Paramecium—A Current Survey. W. J. Van Wagtendonk, editor. Elsevier Publishing Co., New York. 91–130.
8. Dentler, W. L. 1980. Microtubule-membrane interactions in cilia. I. Isolation and characterization of ciliary membrane from *Tetrahymena pyriformis*. *J. Cell Biol.* 84:364–380.
9. Doughty, M. J. 1979. Control of ciliary activity in *Paramecium*. IV. Ca ion modification of Mg-dependent dynein ATPase activity. *Comp. Biochem. Physiol.* 64B:255–266.
10. Dunlap, K. 1977. Localization of calcium channels in *Paramecium caudatum*. *J. Physiol.* 271:119–133.
11. Eckert, R., and H. Machemer. 1975. Regulation of ciliary beating frequency by the surface membrane. *In* Molecules and Cell Movement. S. Inoue and R. E. Stephens, editors. Raven Press, New York. 151–164.
12. Eckert, R., and P. Brehm. 1979. Ionic mechanisms of excitation in *Paramecium*. *Annu. Rev. Biophys. Bioeng.* 8:353–383.
13. Estridge, M. 1977. Polypeptides similar to α and β subunits of tubulin are exposed on the neuronal surface. *Nature (Lond.).* 268:60–63.
14. Feller, M., W. D. Behnke, and E. I. Gruenstein. 1979. Relationship between lectin monosaccharide specificity and binding of lectin to the plasma membrane of human fibroblasts. *Biochim. Biophys. Acta.* 586:315–329.
15. Finger, I. 1974. Surface antigens of *Paramecium aurelia*. *In* Paramecium—A Current Survey. W. J. Van Wagtendonk, editor. Elsevier Scientific Publishing Co., New York. 131–164.
16. Good, N. E., and S. Izawa. 1972. Hydrogen ion buffers. *Methods Enzymol.* 24:53–68.
17. Gorovsky, M. A., K. Carlson, and J. L. Rosenbaum. 1970. Simple method for quantitative densitometry of polyacrylamide gels using fast green. *Anal. Biochem.* 35:359–370.
18. Hansma, H. 1974. Biochemical studies on the behavioral mutants of *Paramecium aurelia*: ion fluxes and ciliary membrane proteins. Ph.D dissertation. University of California at Santa Barbara, Santa Barbara, Calif.
19. Hansma, H., and C. Kung. 1975. Studies of the cell surface of *Paramecium*. Ciliary membrane proteins and immobilization antigen. *Biochem. J. (Tokyo).* 152:523–528.
20. Huang, B., G. Piperno, and D. J. L. Luck. 1979. Paralyzed flagella mutants of *Chlamydomonas reinhardtii* defective for axonemal doublet microtubule arms. *J. Biol. Chem.* 254: 3091–3099.
21. Kaneshiro, E. S., L. S. Beischel, S. J. Merkel, and D. E. Rhoads. 1979. The fatty acid composition of *Paramecium aurelia* cells and cilia: changes with culture age. *J. Protozool.* 26:147–158.
22. Kim, J. H., B. Shome, T.-H. Liao, and J. G. Pierce. 1967. Analysis of neutral sugars by gas-liquid chromatography of alditol acetates: application to thyrotropic hormone and other glycoproteins. *Anal. Biochem.* 20:258–274.
23. Kincaid, H. L., Jr. B. H. Gibbons, and I. R. Gibbons. 1973. The salt-extractable fraction of dynein from sea urchin sperm flagella: an analysis by gel electrophoresis and by adenosine triphosphatase activity. *J. Supramol. Struct.* 1:461–470.
24. Kitamura, A., and K. Hiwatashi. 1976. Mating reactive membrane vesicles from cilia of *Paramecium caudatum*. *J. Cell Biol.* 69:736–740.
25. Kung, C. 1971. Genic mutants with altered system of excitation in *Paramecium aurelia*. I. Phenotypes of the behavioral mutants. *Z. Vergl. Physiol.* 71:142–164.
26. Kung, C. 1971. Genic mutants with altered system of excitation in *Paramecium aurelia*. II. Mutagenesis, screening and genetic analysis of the mutants. *Genetics.* 69:29–45.
27. Kung, C., and Y. Naitoh. 1973. Calcium-induced ciliary reversal in the extracted models

of 'pawn', a behavioral mutant of *Paramecium*. *Science* (*Wash. D. C.*). 179:195–196.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (*Lond.*). 227:680–685.
29. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
30. Machemer, H., and R. Eckert. 1973. Electrophysiological control of reversed ciliary beating in *Paramecium*. *J. Gen. Physiol.* 61:572–587.
31. Machemer, H., and A. Ogura. 1979. Ionic conductances of membranes in ciliated and deciliated *Paramecium*. *J. Physiol.* (*Lond.*). 296:49–60.
32. Merkel, S. J. 1980. Protein composition of the cilia and ciliary membrane of wild-type *Paramecium tetraurelia* and a pawn mutant. Ph.D. dissertation. University of Cincinnati. Cincinnati, Ohio.
33. Mott, M. R. 1965. Electron microscopy studies on the immobilization antigens of *Paramecium aurelia*. *J. Gen. Microbiol.* 41:251–261.
34. Naitoh, Y., and R. Eckert. 1974. The control of ciliary activity in protozoa. *In* Cilia and Flagella. M. A. Sleigh, editor, Academic Press, Inc., New York. 305–352.
35. Nelson, D. L., and C. Kung. 1978. Behavior of *Paramecium*: chemical, physiological and genetic studies. *In* Taxis and Behavior. G. L. Hazelbauer, editor. Chapman and Hall, London. 77–100.
36. Oertel, D., S. J. Schein, and C. Kung. 1977. Separation of membrane currents using a *Paramecium* mutant. *Nature* (*Lond.*). 268:120–124.
37. Ogura, A., and K. Takahashi. 1976. Artificial deciliation causes loss of calcium-dependent responses in *Paramecium*. *Nature* (*Lond.*). 264:170–172.
38. Otter, T. 1978. Comparison of ciliary and flagellar membrane composition. *Biol. Bull.* 155: 461 (Abstr.).
39. Phillips, D. R., and M. Morrison. 1971. Exposed protein on the intact human erythrocyte. *Biochemistry*. 10:1766–1771.
40. Piperno, G., B. Huang, and D. J. L. Luck. 1977. Two-dimensional analysis of flagellar proteins from wild-type and paralyzed mutants of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U. S. A.* 74:1600–1604.
41. Piperno, G., and D. J. L. Luck. 1979. Axonemal adenosine triphosphatases from flagella of *Chlamydomonas reinhardtii*. Purification of two dyneins. *J. Biol. Chem.* 254:3084–3090.
42. Preer, J. R., Jr. 1959. Studies on the immobilization antigens of *Paramecium*. II. Isolation. *J. Immunol.* 83:378–384.
43. Preer, J. R., Jr. 1959. Studies on the immobilization antigens of *Paramecium*. III. Properties. *J. Immunol.* 83:385–391.
44. Preer, J. R., Jr. 1969. Genetics of protozoa. *In* Research in Protozoology. T. Chen, editor. Pergamon Press, Oxford. 3:129–278.
45. Rhoads, D. E., and E. S. Kaneshiro. 1979. Characterization of phospholipids from *Paramecium tetraurelia* cells and cilia. *J. Protozool.* 26:329–338.
46. Satow, Y., and C. Kung. 1980. Membrane currents of pawn mutants of the pW A group in *Paramecium tetraurelia*. *J. Exp. Biol.* 84:57–71.
47. Schein, S. J. 1976. Nonbehavioral selection for pawns, mutants of *Paramecium aurelia* with decreased excitability. *Genetics*. 84:453–468.
48. Soldo, A. T., G. A. Godoy, and W. J. Van Wagtendonk. 1966. Growth of particle-bearing and particle-free *Paramecium aurelia* in axenic culture. *J. Protozool.* 13:492–497.
49. Stephens, R. E. 1977. Vectoral labeling of ciliary membrane tubulin. *J. Cell Biol.* 75 (2, Pt. 2):223*a* (Abstr.).
50. Stephens, R. E. 1977. Major membrane protein differences in cilia and flagella: evidence for a membrane-associated tubulin. *Biochemistry*. 16:2047–2058.
51. Switzer, R. C., III, C. R. Merril, and S. Shifrin. 1979. A highly sensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 98:231–237.
52. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecylsulfate polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406–4412.
53. Williams, N. E., P. V. Subbaiah, and G. A. Thompson, Jr. 1980. Studies of membrane formation in *Tetrahymena*. *J. Biol. Chem.* 255:296–303.