

Properties and Topography of the Major Integral Plasma Membrane Protein of a Unicellular Organism

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Abstract. The cellular distribution, membrane orientation, and biochemical properties of the two major NaOH-insoluble (integral) plasma membrane proteins of *Euglena* are detailed. We present evidence which suggests that these two polypeptides (M_r 68 and 39 kD) are dimer and monomer of the same protein: (a) Antibodies directed against either the 68- or the 39-kD polypeptide bind to both 68- and 39-kD bands in Western blots. (b) Trypsin digests of the 68- and 39-kD polypeptides yield similar peptide fragments. (c) The 68- and 39-kD polypeptides interconvert during successive electrophoresis runs in the presence of SDS and β -mercaptoethanol. (d) The 39-kD band is the only major integral membrane protein evident after isoelectric focusing in acrylamide gels. The apparent shift from 68 to 39 kD in focusing gels has been duplicated in denaturing SDS gels by adding ampholyte solutions directly to the protein samples. The membrane orientation of the 39-kD protein and its 68-kD dimer has been assessed by radioiodination in

situ using intact cells or purified plasma membranes. Putative monomers and dimers are labeled only when the cytoplasmic side of the membrane is exposed. These results together with trypsin digestion data suggest that the 39-kD protein and its dimer have an asymmetric membrane orientation with a substantial cytoplasmic domain but with no detectable extracellular region. Immunolabeling of sectioned cells indicates that the plasma membrane is the only cellular membrane with significant amounts of 39-kD protein. No major 68- or 39-kD polypeptide bands are evident in SDS acrylamide gels or immunoblots of electrophoresed whole flagella or preparations enriched in flagellar membrane vesicles, nor is there a detectable shift in any flagellar polypeptide in the presence of ampholyte solutions. These findings are considered with respect to the well-known internal crystalline organization of the euglenoid plasma membrane and to the potential for these proteins to serve as anchors for membrane skeletal proteins.

THE surface complex of euglenoids is rigorously organized, easily isolated, and well suited for probing and dissecting the plasma membrane and the membrane skeleton. As in the human erythrocyte ghost (3), euglenoids lack a transcellular cytoskeleton, and cell form and surface topography are maintained by the membrane skeleton and its associated plasma membrane. Moreover, the euglenoid surface is patterned into readily identified structural and functional domains. For example: (a) the membrane skeleton consists of about 40 articulating strips (10), which are positioned to fashion the surface ridges and grooves diagnostic of these cells (23); (b) the grooves of the helically sculptured cell surface mark the sites at which surface expansion takes place in replicating cells (19); (c) the ridges are relatively immobile and the sliding associated with surface motility takes place between but not within the surface ridges (39).

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Similarly well-defined regions are also found in the interior of the plasma membrane which is remarkably well ordered (26), especially in those areas associated with the membrane skeleton (24). Furthermore, when the plasma membrane is stripped of all peripheral proteins by proteolysis, the undulating surface form is not lost and the paracrystalline interior remains unchanged (11). The biochemical composition of the paracrystalline domains in the plasma membrane, however, is not known nor is it clear how the tight association between the plasma membrane and the membrane skeleton is brought about and subsequently maintained. We have therefore undertaken a detailed study of the major integral plasma membrane proteins in order to assess their potential for binding to skeletal proteins as well to define some of their intrinsic properties that might help explain some of the unusual surface characteristics of euglenoids.

In the euglenoid *Distigma large* (540 kD), ordered "integral" membrane proteins have been mapped from Fourier transforms of electron diffraction patterns of negatively stained surface membranes (27). A substantial portion of

these molecules is exposed on the external membrane surface as revealed by iodination of whole cells and by negative staining of surface fragments. A surprisingly different pair of integral membrane proteins has been identified in the related euglenoid *Euglena gracilis* with apparent molecular masses of 68 and 39 kD (10). Since these latter proteins together comprise over two thirds of the total NaOH-insoluble (hence integral) plasma membrane proteins (10), it seems probable that they account for some of the plasma membrane properties of this organism. In this report we provide evidence that shows that these 68- and 39-kD proteins share immunologic determinants, have overlapping peptide maps, will interchange even under strong denaturing conditions and migrate with a similar M_r in the presence of ampholyte solutions, suggesting that they are in fact dimer and monomer of the same protein with strong aggregation properties. Evidence from iodination and peptide digestion experiments indicates that these proteins have a substantial cytoplasmic domain but are not iodinated at the external face of the plasma membrane. Consistent with a postulated role of these integral membrane proteins as binding sites for skeletal protein and/or other functions specific to the cell membrane, we were unable to identify a similar protein in the contiguous flagellar membrane or other cellular membranes that lack both associated membrane skeletons and ordered membrane interiors. These results are considered with respect to the differing previous interpretations of plasma membrane proteins in this and other euglenoids.

Materials and Methods

Cell Surface and Flagellar Membrane Isolation

About 5×10^8 cells (1 liter of log-phase cultures) of *Euglena gracilis* strain Z were harvested and cavitated with glass beads as previously described (10). The resulting surface fragments with associated surface (plasma) membranes were purified on sucrose gradients (10). Flagellar membranes were prepared by twofold extraction of isolated flagella (8, 32) with 10 mM NaOH followed by enrichment on a step gradient consisting of 60%, 85%, and 95% (wt/wt) sucrose in HKN buffer (10 mM Hepes, 25 mM KCl, 0.01% NaN_3 , pH 7.0). After centrifugation at 50,000 rpm for 90 min in a Beckman SW65 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, CA) at 4°C, a band of enriched flagellar membranes collected at the 60%/85% sucrose interface. Flagella from 8×10^9 cells were generally sufficient to yield a clearly visible "membrane band."

SDS Acrylamide Gel Electrophoresis

Single-dimension SDS acrylamide gel electrophoresis was carried out using the buffer system of Laemmli (21). Polypeptides were separated in two dimensions by two different methods: (a) To demonstrate the interconversions of 68- and 39-kD polypeptides, a 2-mm-wide section of a one-dimensional SDS slab gel (9%) containing the NaOH-insoluble membrane proteins was excised and equilibrated in a solution containing 125 mM Tris-HCl, pH 6.8, 0.1% SDS, and 1% β -mercaptoethanol. The gel slice was then photopolymerized with riboflavin phosphate (15) in a second-dimension stacking gel, and electrophoresed under conditions identical to those used in the first dimension. Gels were fixed (38) and stained with ammoniacal silver nitrate (43). (b) Polypeptides were also separated by isoelectric focusing followed by SDS electrophoresis as described (30) except that β -mercaptoethanol was omitted from the agarose overlay.

Two-Dimensional Peptide Maps

Trypsin digestion was carried out as described by Elder et al. (12) with some modifications (17). 68- and 39-kD polypeptides were separately excised from a SDS 7.5% gel and each slice was rinsed exhaustively with 40% ethanol, lyophilized, and resuspended in phosphate buffer containing 300 μCi

of carrier-free Na^{125}I (Amersham Corp., Arlington, Heights, IL) with chloramine-T as catalyst. The reaction was quenched with tyrosine (12) after which free iodine and iodotyrosine were removed by dialysis. The gel slices with the now iodinated 68- and 39-kD polypeptides were lyophilized and then resuspended in 50 mM NH_4CO_3 buffer at pH 8.0 containing 65 $\mu\text{g}/\text{ml}$ trypsin-TPCK (Worthington Biochemical Corp., Freehold, NJ). After digestion for 16 h at 37°C the soluble tryptic digests were lyophilized, dissolved in 30 μl of 20% acetic acid, and separated on Polygram SIL G25 plates (Brinkmann Instruments, Inc., Westbury, NY). Chromatography was carried out in the first direction in solution I consisting of *n*-butanol/pyridine/acetic acid/ H_2O (44:25:6:25), and in the second direction in solution II consisting of *n*-butanol/acetic acid/ H_2O (4:1:1) until the solvent front was 15 cm from the origin. The chromatogram was air dried and autoradiographed using Kodak XRP-1 film and a Cronex (Dupont Co., Wilmington, DE) intensifying screen.

Surface Iodination

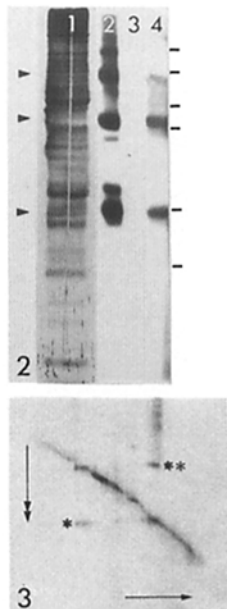
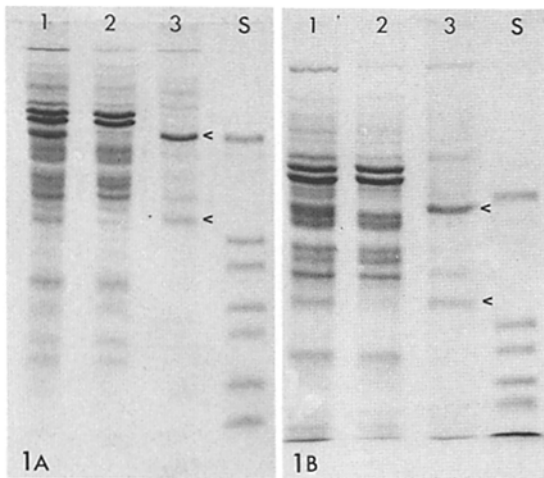
Deflagellated whole cells and surface isolates were radioiodinated with the water-insoluble catalyst Iodogen (Pierce Chemical Co., Rockford, IL [13]). 50 μg of Iodogen in 1 ml of chloroform was dried under a stream of nitrogen in a scintillation vial just before use. In a typical experiment surface isolates consisting of ~ 200 μg of protein or alternatively, $\sim 10^6$ whole deflagellated cells were suspended in 10 mM phosphate buffer, pH 7.4 (30 and 50 μl , respectively) and placed in the Iodogen-coated vials. 200–300 μCi of carrier-free Na^{125}I (Amersham Corp.) was added to the mixtures and allowed to react for 30 min at 4°C with frequent swirling. Unincorporated label was removed by rinsing in buffer followed by sedimentation by centrifugation three times, and then solubilization for SDS gel electrophoresis. NaOH-digested surface isolates were radioiodinated under identical conditions. Some surface isolates were iodinated followed by proteolysis with 0.1% trypsin in 60 mM borate buffer, pH 8.4, at room temperature. After 30 min the suspension was sedimented by centrifugation at 12,800 g for 10 min and rinsed twice with borate buffer containing 0.1% soybean trypsin inhibitor and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO).

Antibody Generation and Immunoblots

Female New Zealand white rabbits were separately immunized with either the 68- or the 39-kD polypeptide from homogenized gel slices essentially as described by Granger and Lazarides (16). Preimmune and immune sera were processed using standard procedures and the IgG flow-through fraction collected from a DE-52 (Whatman LabSales, Inc., Hillsboro, OR) column (42). For Western (immuno-) blots the polypeptides separated on one-dimensional or two-dimensional gels were transferred electrophoretically (41) to nitrocellulose (Trans-blot medium, BioRad Laboratories, Richmond, CA). Nitrocellulose sheets with the transferred polypeptides were stained with India ink and incubated with rabbit primary antibody essentially as described (17) except that the sheets were blocked with 2% BSA for 30 min at room temperature. Incubation with IgG and subsequent washes were carried out in a solution containing 15 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% gelatin, 0.1% Tween 20, 0.05% SDS, and 0.05% NaN_3 . The rabbit primary antibodies were used at a concentration of 100 $\mu\text{g}/\text{ml}$. Bound antibody was detected with ^{125}I -labeled donkey anti-rabbit F(ab')₂ (Amersham Corp.). After labeling and washing the nitrocellulose sheets were air dried and autoradiographed on Kodak XRP-1 film using a Cronex (Dupont Co.) intensifying screen.

Cellular Localization

Attempts to permeabilize whole *Euglena* cells gave inconsistent results. Therefore frozen 0.5- μm sections were prepared for immunofluorescent localization and Lowicryl K4M-embedded cells were sectioned for immunogold localization. For immunofluorescence, cells were fixed in 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 2 h at room temperature. After several rinses in PBS, the cells were infiltrated stepwise with sucrose to a final concentration of 2.3 M (40). Packed cells were mounted on copper rods and frozen in liquid nitrogen. Sectioning was carried out at -90°C on the cryostage of a Sorvall MT-2 ultramicrotome (DuPont-Sorvall, Newtown, CT). Dry, frozen sections were transferred to poly-L-lysine coated glass slides and allowed to warm to room temperature. After rinsing sequentially in PBS, PBS plus 10 mM glycine, and PBS plus 0.1% BSA, the sections were incubated in primary antibody for 1 h at 37°C, rinsed, incubated with sheep anti-rabbit IgG conjugated to fluorescein, rinsed again, and mounted in an anti-bleed solution (20) before addition



Figures 1–3. (Figure 1.) Peripheral and integral proteins of surface isolates. (A) 9% acrylamide SDS gel of surface isolates. (B) 7% gel. (Lane 1) Whole-surface isolates; (lane 2) NaOH extracts of surface isolates; (lane 3) NaOH resistant (integral) membrane proteins of surface isolates. The running buffer for these gels contained 0.05% NP-40. Protein standards (S) are: BSA, 66 kD; ovalbumin, 45 kD; glucose-6-phosphatase, 36 kD; carbonic anhydrase, 29 kD; trypsinogen, 24 kD; trypsin inhibitor, 20 kD; α -lactoalbumin, 14 kD. (Figure 2.) Immunological cross-reactivity of the 68- and 39-kD antigens. Surface isolates were separated in SDS 9% gels without NP-40 in the running buffer, transferred to nitrocellulose and stained with India ink (lane 1). The left half of a stained lane was incubated with anti-39-kD antibody (lane 2) and the right half with preimmune IgG (lane 3), followed by ^{125}I -secondary label. The resulting autoradiograms show binding to 110-, 68-, and 39-kD polypeptides (arrowheads) as well as to several other less prominent bands. (Lane 4) Anti-68-kD antibody binding on a similar transfer. Standards (hashmarks) from top: myosin, 205 kD; β -galactosidase, 116 kD; phosphorylase *a*, 97.4 kD; bovine serum albumin, 66 kD; ovalbumin, 45 kD; carbonic anhydrase, 29 kD. (Figure 3.) The 68- and 39-kD polypeptides can interconvert in two-dimensional SDS gels. Proteins from surface isolates were separated on SDS 9% gels containing 1% β -mercaptoethanol (single arrow), and then rerun in a second dimension (double arrow) in an identical denaturing gel. Most polypeptides migrated with the same mobilities in both directions and fell along a diagonal equidistant from the ori-

of a cover glass. Viewing and photography were performed with incident illumination using a mercury lamp on a Zeiss Photomicroscope II equipped with appropriate FITC excitation and barrier filters. For electron microscopy, cells were fixed as above, dehydrated in dimethylformamide, and infiltrated with Lowicryl (1). Sections were mounted on Formvar-coated nickel grids, rinsed in PBS with glycine and BSA as above, and incubated with primary antibody followed by 10-nm gold conjugated, goat anti-rabbit IgG (SPI Supplies, West Chester, PA). Sections were stained with uranyl acetate and lead citrate and photographed in a JEOL 1200 EX transmission electron microscope.

Results

The Two Major Integral Membrane Proteins Cross-react Immunologically

Surface isolates of *Euglena* purified isopycally on sucrose gradients have only one membrane, the plasma membrane. Associated with the plasma membrane is the underlying membrane skeleton: microtubules and at least four sets of interconnecting bridges (10). Biochemically, surface isolates consist of two major polypeptides at 80 and 86 kD (Fig. 1, A and B; lane 1), which are associated with the membrane skeleton (10), and polypeptide bands at 53–55 kD which have been equated with microtubule proteins (tubulins). The 80-, 86-, and 55-kD proteins as well as a number of less abundant polypeptides can be removed from surface isolates with 10 mM NaOH (Fig. 1, A and B; lane 2), leaving only the insoluble plasma membrane with two prominent NaOH resistant (integral) membrane proteins (Fig. 1, A and B; lane 3). The latter or any surface fraction containing the membrane proteins often (inconsistently) produced unresolved smears under reducing conditions in SDS gels. Repeated efforts to eliminate smearing by adjusting pH or detergent concentrations, by alkylation of reduced samples, or by prolonged sample boiling were not helpful. This problem could be remedied, however, by adding small amounts of Nonidet P-40 to the running buffer prior to electrophoresis. The smaller of the two membrane proteins as well as several protein standards migrated anomalously (compare relative positions of bands in Fig. 1 A with those of Fig. 1 B, especially lanes 3 and S) under these conditions. Presumably there is a partial replacement of protein-bound anionic SDS with the neutral detergent NP-40 (9), which then may be carried into the gel as a mixed micelle (29). Our membrane proteins probably undergo “charge shift” effects similar to that described for other amphiphilic proteins separated in the presence of detergent mixtures (18). Molecular masses therefore were estimated in the absence of NP-40 but some polypeptides will appear to have differing M_r in those gels identified in this report as containing NP-40 in the running buffer.

The M_r 68- and 39-kD polypeptides (10) together accounted for ~67% of the total NaOH-resistant (integral) membrane proteins as determined by densitometric scans of Coomassie Blue-stained gels. In order to further characterize these proteins each was excised from a preparative (curtain) gel of stripped membranes, and each was used to immunize separate rabbits. In immunoblots of whole-surface isolates, the

gins. A portion, however, of the polypeptides at 68 kD in the first dimension migrated to 39 kD in the second dimension (asterisk) whereas some of the 39-kD polypeptides of the first dimension appeared at 68 kD in the second dimension (double asterisk).

immune IgG directed against the 39-kD antigen bound strongly to three polypeptides at 39, 68, and 110 kD, and it reacted less strongly with five additional polypeptide bands (Fig. 2, lane 2). Preimmune serum showed no binding in similar blots (Fig. 2, lane 3). Antibodies generated against the 68-kD antigen were more selective but still recognized both 68- and 39-kD polypeptides (Fig. 2, lane 4). As a further test of their immunosimilarity, the anti-68-kD antibody was extracted from the 39-kD band on a nitrocellulose blot, and this affinity-purified antibody was used to probe a second blot of cell surface proteins. Both 68- and 39-kD polypeptides still exhibited binding (see below), indicating that these two proteins share common determinants.

The 68- and 39-kD Proteins Can Interconvert

Because the 68- and 39-kD polypeptides seemed to share immunologic determinants, we tested whether one might be an oxidized or a degraded form of the other, or alternatively, whether they interconvert under denaturing conditions. NaOH-extracted membranes were solubilized in 1% SDS and 5% β -mercaptoethanol with (22) and without alkylation before separation on a SDS 9% gel. The two polypeptides migrated as two separate bands under either condition (not shown). In a second series of experiments the membrane proteins were solubilized as above without alkylation and separated on a 9% gel. The center region of the gel lane was excised, incubated in SDS and β -mercaptoethanol, polymerized in the stacking region of an SDS 9% slab gel (39), and electrophoresed under conditions identical to those used in the first direction. As expected, most of the proteins assumed a position extending along a diagonal from high to low molecular mass (Fig. 3), i.e., migrated with the same mobility in both directions. A portion of the polypeptides, however, which migrated at 68 kD in the first direction migrated at 39 kD in the second direction (Fig. 3, *asterisk*). Conversely, some of the polypeptides at 39 kD in the first direction migrated to 68 kD in the second direction (Fig. 3, *double asterisk*). These results demonstrated that the 39-kD protein is not a degradation product of the 68-kD protein.

Both 68- and 39-kD polypeptides stained prominently with silver procedures in one-dimensional gels (not shown),

but when surface isolates were silver stained after separation on two-dimensional O'Farrel gels (30), there was no significant staining at 68 kD (Fig. 4 *a*). The isoelectric points of the several apparent isoforms of 39-kD polypeptides ranged from 5.5 to 6.5. To confirm the identity of the 39-kD spots a two-dimensional separation parallel to that of Fig. 4 *a* was transferred to nitrocellulose and stained with India ink (Fig. 4 *b*) and then incubated with the anti-68-kD rabbit antibody followed by 125 I-donkey anti-rabbit IgG. The resulting autoradiogram (Fig. 4 *c*) revealed antibody binding to at least four isoforms ($M_r = 39$ kD) but no binding at 68 kD. The absence of 68-kD binding was unexpected (cf. Fig. 2, lane 4), but resulted from the apparent conversion of 68- to 39-kD polypeptides under the solution conditions of isoelectric focusing (see below). Unlike the strong silver staining of 39 kD in gels (Fig. 4 *a*), India ink was a poor stain of 39-kD polypeptides transferred to nitrocellulose (Fig. 4 *b*). By contrast many other polypeptides of surface isolates were more effectively stained with India ink on nitrocellulose blots than they were with silver in gels (compare Fig. 4 *a* with *b*).

Tryptic Digests of 68- and 39-kD Proteins Yield Overlapping Peptide Maps

To further assess the relationship between the 68- and 39-kD polypeptides each band was excised from a 7.5% acrylamide gel and iodinated using chloramine-T as a catalyst (12). Tryptic digests of the radioiodinated 39-kD polypeptides are shown in Fig. 5 *a* after two-dimensional chromatography on silica gel plates. It is evident that there is a close correspondence between the 39-kD tryptic maps and those of the 68-kD polypeptides (Fig. 5 *b*). There are, however, several additional radiolabeled peptides in the 68-kD map (Fig. 5 *b*, *arrows*) that appear to be quantitatively and/or qualitatively different from those of the 39-kD digests. Significantly, in a mixture of 39- and 68-kD peptides (Fig. 5 *c*) there are no radiolabeled tryptic fragments present that are not also represented in the 68-kD chromatogram, indicating an exact overlap of 39-kD peptides with those of 68 kD.

Topography of the 68/39 kD Polypeptides

The orientation of the 68- and 39-kD proteins relative to the

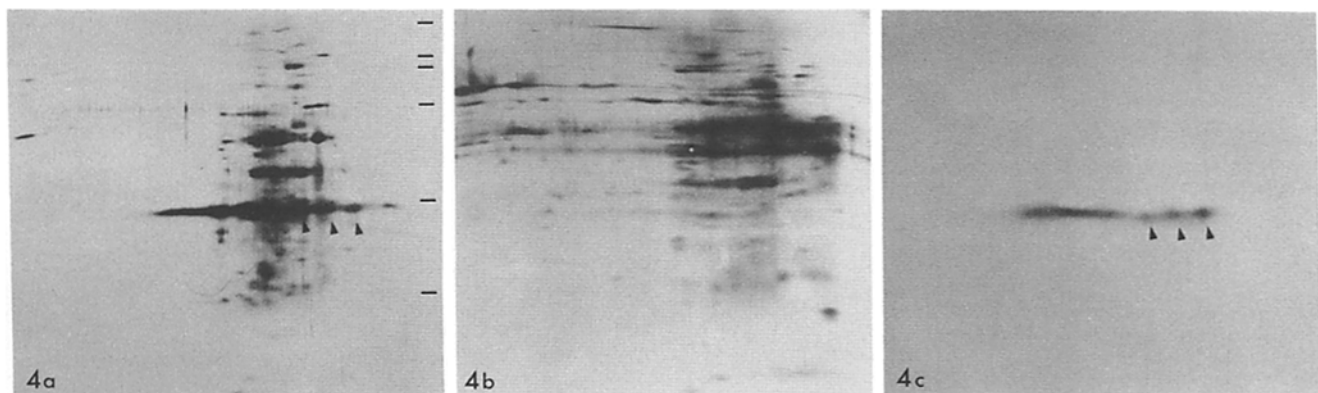


Figure 4. Two-dimensional gels and immunoblots of surface polypeptides show only 39-kD isoforms. Polypeptides of surface isolates were separated by isoelectric point (*horizontal axis*) and by molecular mass (*vertical axis*). Silver staining (*a*) revealed a major polypeptide at 39 kD with multiple isoforms (*arrowheads*), but no major 68-kD spots. Polypeptides from a similar gel were blotted to nitrocellulose and stained with India ink (*b*). The 39-kD band did not stain with India ink, but was identified in the autoradiograph (*c*) of the same blot after incubation with anti-68-kD antibody and iodinated secondary antibody. Molecular mass standards (*hashmarks* in *a*) same as in Fig. 2.

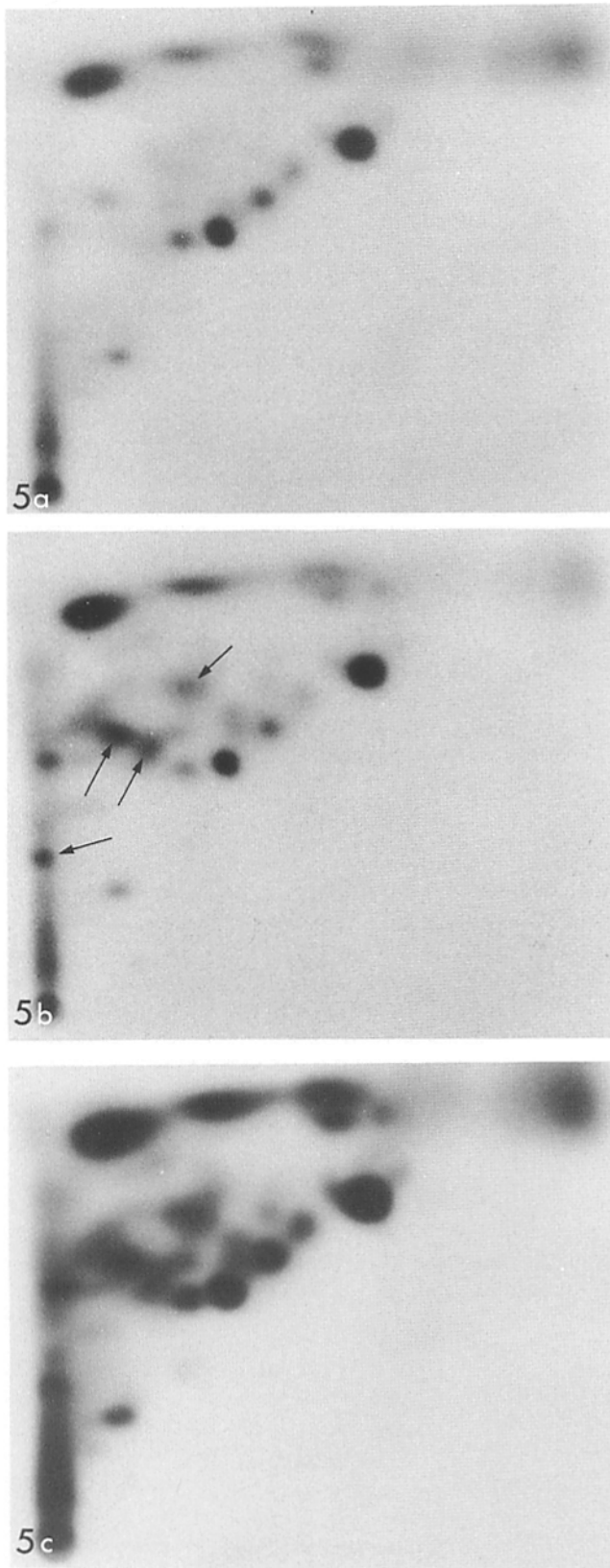


Figure 5. Two-dimensional tryptic peptide maps of 68- and 39-kD polypeptides are similar. Radiolabeled tryptic digests of 39- and 68-kD polypeptides were separated on silica gel plates using solvent system I in the vertical direction and solvent system II in the horizontal direction. Autoradiographs of the peptide maps for 39 kD (a) and 68 kD (b) were similar except for several fragments

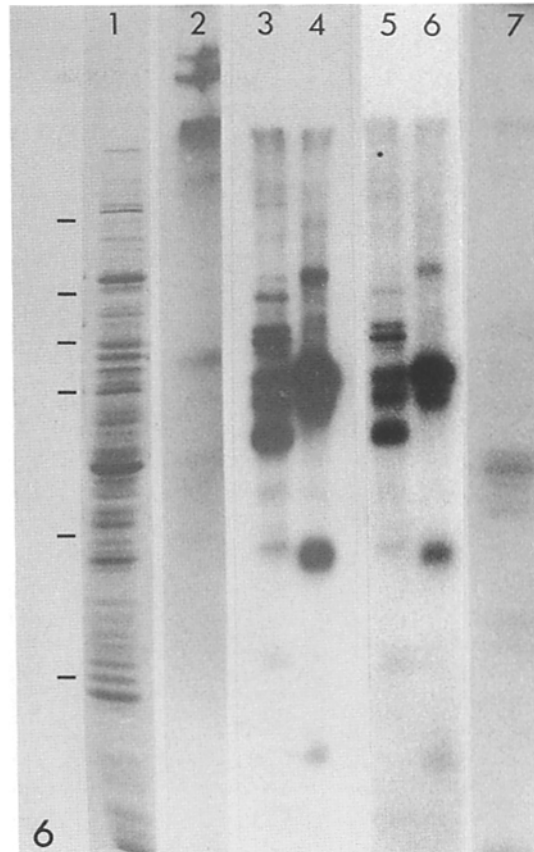


Figure 6. 68- and 39-kD proteins can be iodinated only on the cytoplasmic side of the plasma membrane. Whole cells or surface fractions were radioiodinated with Iodogen as the catalyst and autoradiographed after SDS gel electrophoresis (lanes 2-7). The Coomassie Blue-stained polypeptides from whole cells are shown in lane 1. (Lane 2) Iodinated intact deflagellated cells; (lane 3) iodinated cell surface isolates; (lane 4) iodinated NaOH-extracted plasma membranes; (lanes 5 and 6) shorter film exposure of lanes 3 and 4; (lane 7) iodinated surface isolates (as in lane 3) digested with trypsin in situ before electrophoresis. Counts per minute loaded per lane ($\times 10^{-5}$): lane 2, 2.9; lanes 3-6, 4.5; lane 7, 0.24. Protein standards (hashmarks) as in Fig. 2.

plasma membrane was determined by radiolabeling whole cells or various cell fractions in situ using the water-insoluble catalyst, Iodogen. Labeled cells and cell fractions were then solubilized and their respective proteins separated on SDS gels. Autoradiographs of gels of proteins from iodinated whole cells exhibited label in several high molecular mass bands which remained in the stacking gel or barely entered the running gel (Fig. 6, lane 2). Iodinated surface isolates, however, displayed five prominent (Fig. 6, lane 5) and a number of less well-labeled polypeptides (Fig. 6, lane 3). With prolonged exposure the high molecular mass, whole-cell labeled bands could also be identified in surface isolates (not shown). When the peripheral proteins of surface isolates were removed with NaOH and the remaining membrane-associated proteins were radioiodinated, a 68-kD polypep-

in the 68-kD digests (arrows) which appeared to be absent or reduced in amount in the 39-kD maps. The mixture of 68- and 39-kD peptides (c) closely matches the 68-kD pattern.

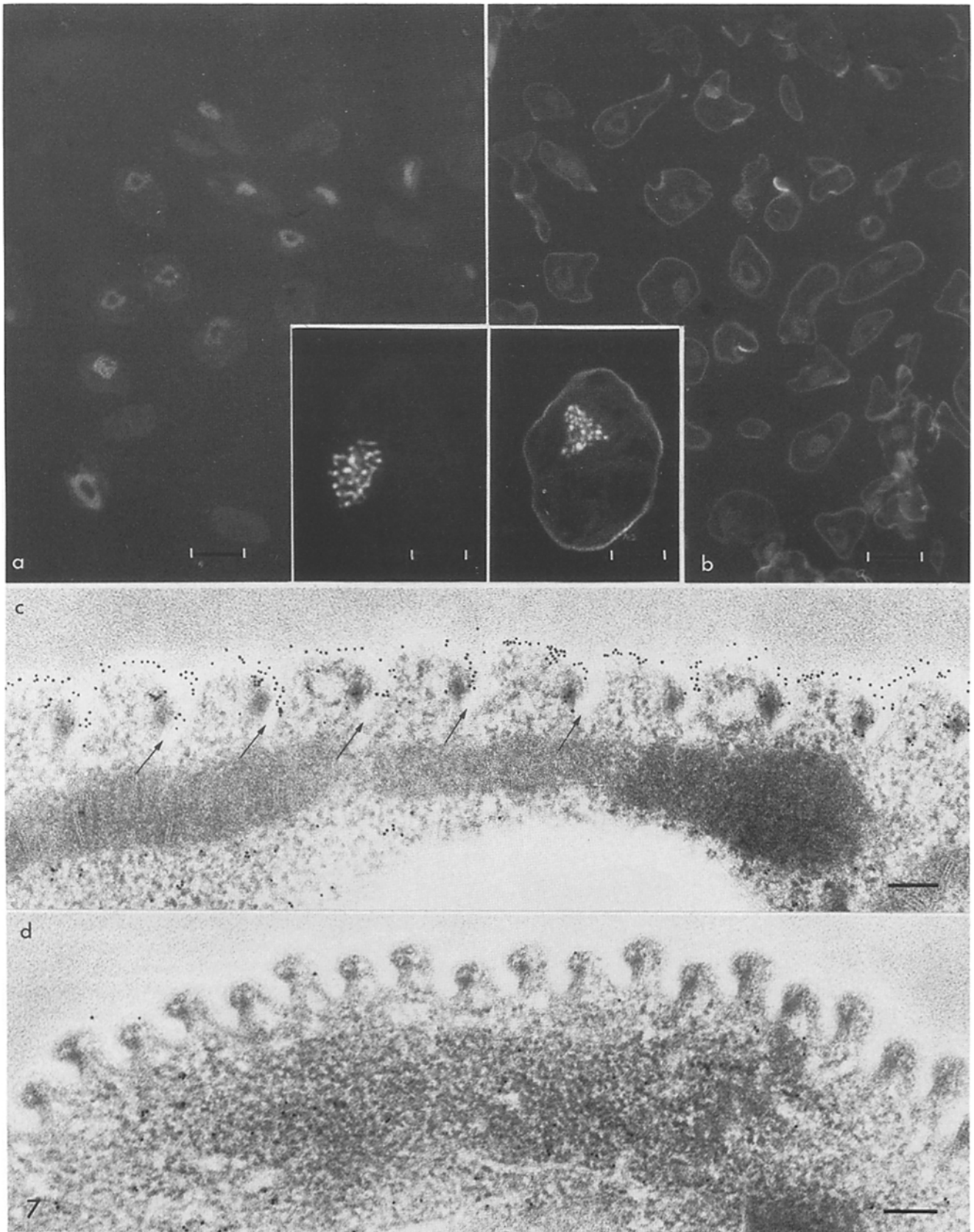


Figure 7. The 68- and 39-kD proteins are localized to the cell surface. Frozen-sectioned cells were transferred to polylysine-coated glass slides and incubated with anti-68-kD IgG followed by fluorescein-conjugated sheep anti-rabbit IgG. Preimmune serum (*a*) as well as immune serum (*b*) both produced nuclear staining, but only immune serum (*b*) showed fluorescence around the cell margins. Nuclear staining was due to the anti-quench solution which was used to reduce fluorescein fading. The same anti-68-kD antibody was applied to sections

tide was heavily labeled and bands at 39, 62, and 110 kD were also evident (Fig. 6, lanes 4 and 6). Most of the radiolabel incorporated into surface isolates was removed with trypsin (Fig. 6, lane 7), and after trypsin digestion the 68- and 39-kD polypeptides were reduced in mass to several new lightly labeled bands at 50 and 30 kD (cf. also reference 11). These results taken together suggest that the 68- and 39-kD integral membrane proteins have no iodinated regions exposed on the external cell surface (Fig. 6, lane 2), but do have cytoplasmic domains which are both iodinated and accessible to partial proteolysis.

Intracellular Localization of the 68- and 39-kD Proteins

In accord with the radiolabeling results, whole cells incubated with rabbit antibody directed against the 68-kD proteins showed no surface binding after incubation with a fluorescent secondary antibody (not shown). Similar treatments carried out on frozen and sectioned cells, however, produced a uniform fluorescence along the cell margins (Fig. 7 *b*). Little or no fluorescence was found in the cytoplasm, although the nucleus gave a strong fluorescent signal (Fig. 7 *b*, *inset*). The latter was also found in control cells treated with preimmune serum (Fig. 7 *a*), but not in preparations from which the anti-quench solution (20) had been omitted (not shown). The nonspecific nuclear staining provided a convenient marker for cell orientation, and an indicator of the relative intensity of secondary antibody fluorescence. Antibody-binding regions at the cell surface often appeared to be broader than the membrane or membrane-skeletal regions (Fig. 7 *b*), and we attribute this both to cell curvature and to section collapse which occurs during thawing, thereby exposing the full or partial width of these unembedded sections. In support of this interpretation when anti-68-kD antibodies were applied to sections of Lowicryl-embedded cells, the colloidal gold which marks antibody binding was closely associated with the plasma membrane (Fig. 7 *c*). Interestingly, the gold particles were largely absent from the plasma membrane of the deepest region of the groove (Fig. 7 *c*, *arrows*) between the surface ridges. This latter finding is consonant with previous reports of differences in stability (19), fluidity (37), and intramembrane organization (24) of the groove relative to the ridge. Preimmune serum produced only low levels of nonspecific background labeling (Fig. 7 *d*).

Flagellar Membranes Lack 68 and 39 kD Proteins

A NaOH-resistant flagellar pellet was resuspended in buffer and layered over a sucrose gradient (Materials and Methods). The visible band at the 60/85% interface consisted largely of flagellar membranes with some contamination by flagellar mastigonemes, as determined by electron microscopy. This membrane enriched fraction together with whole, untreated flagella were separately solubilized in SDS and β -mercaptoethanol, their respective polypeptides separated on SDS acrylamide gels, blotted to nitrocellulose, and probed with the anti-68-kD antibody. Neither whole flagellar preparations (Fig. 8 *A*) or flagellar membrane vesicles yielded major polypeptide bands at 68 or 39 kD (Fig. 8 *A*,

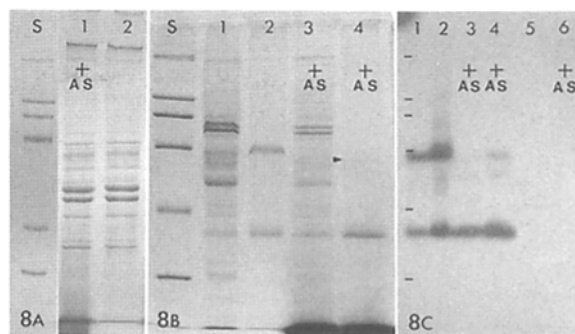


Figure 8. Ampholyte solutions affect the migration of some cell surface but not flagellar polypeptides. (A) Purified flagella were electrophoresed with (lane 1) or without (lane 2) ampholyte solution in the sample buffer. The running buffer contained 0.05% NP-40. (B) NaOH extracts of surface isolates (lanes 1 and 3) or NaOH-resistant pellets of surface isolates (lanes 2 and 4) were electrophoresed without (lanes 1 and 2) or with (lanes 3 and 4) ampholyte solution (AS) in the sample buffer. No NP-40 was added to the running buffer. (C) Autoradiographs of nitrocellulose blots probed with anti-68-kD antibody affinity purified with 39-kD polypeptides. (Lanes 1 and 3) Cell surface isolates; (lanes 2 and 4) NaOH resistant pellets of cell surface isolates; (lanes 5 and 6) purified flagella. Ampholyte solution was added to the sample buffer in lanes 3, 4, and 6. Protein standards (S and hashmarks) as in Fig. 2.

lanes 1 and 2), although these polypeptides were clearly recognized in cell surface fractions electrophoresed under the same conditions (cf. Fig. 1, lane 1). An assay for 68-kD polypeptides in flagella was carried out using the apparent conversion of 68- to 39-kD polypeptides in the presence of ampholyte solutions (cf. Fig. 4). In this assay cell surface or flagellar proteins were pretreated with ampholyte solutions before addition of sample buffer. In one-dimensional SDS gels of surface isolates most of the 68-kD polypeptides were absent, and there was a corresponding increase in 39-kD polypeptides as seen in Coomassie Blue-stained bands (Fig. 8 *B*, lanes 3 and 4) as well as in the corresponding nitrocellulose blots probed with anti-68-kD antibody (Fig. 8 *C*, lanes 3 and 4). Note that the minor 62-kD membrane band does not shift (Fig. 8 *B*, lane 4, *arrowhead*). When flagellar proteins were similarly treated with ampholyte solutions no changes in any polypeptide bands were evident (Fig. 8 *A*, lane 1). Immunoblots of whole flagella showed no binding by anti-68-kD IgG (Fig. 8 *C*, lanes 5 and 6), and no immunofluorescent signal was detected when anti-68-kD IgG was used to probe isolated flagella (not shown).

Discussion

The euglenoid cell surface has a number of extraordinary properties which include localized surface sliding (37), patterned surface replication (19), a paracrystalline plasma membrane interior (24, 26), and an unusually well-defined peripheral membrane skeleton (10). In order to provide additional understanding of the biochemical bases of some of these surface properties, we have further characterized the major integral plasma membrane proteins of *Euglena*. We conclude

of whole cells embedded in Lowicryl K4M, and antibody binding was visualized with colloidal gold conjugated to sheep anti-rabbit IgG (7 *c*). The control preparation using preimmune serum is shown in 7 *d*. Bars: (a and b) 25 μ m; (*insets*) 12 μ m; (c and d) 175 mm.

from these studies that: (a) the two major integral, plasma membrane proteins are in fact a single species which self-associates even under strong denaturing conditions; (b) the major integral membrane proteins are asymmetrically oriented in the plasma membrane as judged by the presence of a substantial cytoplasmic but no detectable external domain; (c) the cellular distribution of the 68- and 39-kD integral membrane proteins appears to be restricted to specific surface regions which coincide with the distribution of the membrane skeleton.

The 68- and 39-kD Polypeptides Are Related

The NaOH-resistant plasma membrane proteins separated on SDS gels into two major polypeptides at 68 and 39 kD. We suggest that these are related proteins based on their similar peptide maps, their immunologic cross-reactivity, and their ability to interconvert during separation on SDS gels. Similar behavior has been documented for the erythrocyte glycoprotein, glycophorin A, an integral membrane protein consisting of two subunits which are in "association-dissociation equilibrium" in the presence of detergents (14). This interaction is modulated by a hydrophobic region as judged by the inhibition of association with a transmembrane peptide derived from glycophorin A (14). Interactions between the euglenoid 68- and 39-kD proteins may depend on membrane protected (hydrophobic) regions as well since proteolytic digestion reduces the size of the 68- and 39-kD proteins by about one third, but two distinct bands still persist in denaturing gels (11). In isoelectric focusing gels, however, the equilibrium between 68- and 39-kD proteins is shifted towards 39 kD, presumably because ampholyte solutions interfere in some way with association—an assertion supported by the fact similar shifts can be generated by direct addition of such solutions to surface proteins prior to electrophoresis under denaturing conditions. We have found recently that high concentrations of NP-40 such as those found in the ampholyte solution are sufficient to mimic this effect (Rosiere and Bouck, manuscript in preparation). These results indicate that 68 and 39 kD are different forms of the same protein, i.e., 68 kD is presumably a dimer of 39 kD. Small differences in the peptide maps of 68- and 39-kD polypeptides may be due to a contaminating protein in the 68-kD region of the gel or possibly dimerization renders some regions of the polypeptide inaccessible to trypsin. The 39-kD monomers can probably also assemble to a limited extent under denaturing conditions into higher-order polymers (e.g., 110 kD, Fig. 2, lane 2) in addition to the more prevalent dimers.

The Major Integral Protein is Asymmetrically Oriented in the Membrane

Since iodination of surface isolates but not intact cells labels a trypsin-sensitive region of the 68- and 39-kD proteins, we conclude that these proteins have a cytoplasmic but not a detectable external domain. Using the same cells Bré et al. (4) find lipophilic (Sudan black B staining) polypeptides at 64 and 38 kD (presumably the 68- and 39-kD proteins of the present report). The 38-kD polypeptide, but not the 64-kD polypeptide is periodic acid-Schiff (PAS)¹ positive and thus

38 kD is a "glycolipoprotein." After extraction of surface isolates with chloroform/methanol, however, neither the 64- or the 38-kD polypeptide present in the extract is PAS positive (Fig. 10, lane c' of reference 4), suggesting to us that perhaps the 38-kD, PAS-positive band found when whole-surface isolates are separated on SDS gels may be a different polypeptide with a mobility similar to that of the membrane-associated 38-kD polypeptide. We have treated gels containing 68- and 39-kD polypeptides with Sudan black B (31) as well as with the PAS procedure, and were able to confirm the lipophilic nature of the two polypeptides but were unable to demonstrate the presence of carbohydrates (data not shown). In *E. acus* a pepsin-insensitive glycoprotein of 64 kD has been identified in surface isolates but it appeared to be only a minor component of the membrane fraction (5). No 39-kD polypeptide was reported although a 140-kD glycoprotein was evident. These findings in *E. acus* are difficult to reconcile with the present work or with those of Bré et al. (4), and may be due to differences in the extraction methods used, real species differences or both. We suggest that at least in *E. gracilis* the major 68- and 39-kD polypeptides have no external domain which can be detected by iodination, proteolysis, or PAS staining, whereas there is clearly a iodinateable, trypsin-sensitive cytoplasmic region. Thus we tentatively conclude that the 68- and 39-kD proteins are oriented asymmetrically with a hydrophilic domain exposed in the cytoplasm.

This model of the *Euglena* integral plasma membrane proteins differs from that proposed for the related euglenoid *Distigma*. Using three-dimensional Fourier syntheses of the electron diffraction pattern obtained from negatively stained plasma membranes, Murray suggested that the integral membrane protein was large (540 kD) and could be iodinated from the cell exterior (27). Earlier shadow-cast (p. 102 in reference 23) and more recent negative staining of surface fragments (27) both showed that the outer surface of these cells consisted of well-ordered arrays of large particles, but whether these *Distigma* particles are indeed integral or, alternatively, are constituents of a surface coat closely associated with a plasma membrane anchor has not been fully resolved. It is not clear, for example, that these particles are resistant to extraction at high pH (i.e., integral, [36]), or that they extend appreciably into the membrane. *Distigma* surfaces exposed to pH above 9.0 began to lose particle orientation (27), but whether the particles continued to remain associated with the membrane was not reported. The unit cell calculated for the surface particles has a Z-axis dimension (depth) of 95 Å; high-resolution micrographs of sections through the surface membrane indicate much of that 95 Å could be attributed to the electron-dense outer membrane leaflet and the external coat (Fig. 1 in reference 28). It seems possible from the available evidence that the surface particles are only partially embedded in the membrane, and the question of whether they are integral remains open.

This nature of the integral membrane protein assumes additional importance in the light of recent evidence (6) which suggests different euglenoids may have similar membrane skeletal proteins, and thus would presumably have similar requirements for plasma membrane anchors. The 39-kD integral membrane protein of this report fulfills several requirements of such a membrane anchor: (a) The 39-kD protein has a large cytoplasmic domain available for protein/protein

1. Abbreviations used in this paper: PAS, periodic acid-Schiff (stain).

interaction. (b) It is the only integral membrane protein positioned in the appropriate surface regions and available in sufficient quantities to provide a reasonable number of skeletal binding sites. By quantitating Coomassie Blue binding to polypeptides in SDS gels, we estimate that there is in fact about one 39-kD protein molecule available for every two molecules of the major 80- and 86-kD skeletal proteins. (c) The 39-kD integral membrane protein is not found in flagellar membranes (present report) and flagellar membranes cannot bind skeletal proteins as judged both by the absence of 80- and 86-kD membrane skeletal proteins in flagella (Fig. 8 A) and by the inability of flagellar membranes to bind in vitro membrane skeletal proteins extracted from the cell surface (33). The 39 kD protein is relatively small for a membrane anchor but it is larger in molecular mass than glycoprotein A (3) and the actin-binding 20 kD plasma membrane protein of *Dictyostelium* (7).

Do the Major Integral Membrane Proteins of Euglena Correspond to the Intramembrane Particles?

In a number of cases intramembrane particles (IMPs) have been correlated with specific integral proteins or glycoproteins (2). In euglenoids the images of fractured plasma membranes have been difficult to interpret because the two fracture faces appear to be noncomplementary. One face (EF) consists of parallel striations; the opposing face (PF) contains $\sim 110\text{-\AA}$ particles apparently distributed randomly, although optical diffraction of the PF does reveal a two-dimensional lattice reciprocal to the E face (24). The visually noncomplementary images were interpreted to be the result of a postfracture deformation in one of the two faces—probably the P face (26). Noncomplementarity, however, may also be the result of asymmetry of some membrane component (34, 35). Since 67% of the integral membrane proteins (68 and 39 kD) in *E. gracilis* are asymmetrically oriented and self-aggregate strongly at least in vitro, they are good candidates for explaining the P face particles. Significantly, the flagellar membrane which lacks 68- and 39-kD proteins (present report) also lacks a striated membrane interior and has a conventional PF and EF (25).

In summary, we have provided evidence for a single major 39-kD integral membrane protein in plasma membranes of *Euglena* and have provided some details of its topography in the membrane. We would propose that the position of this protein is favorable for mediating membrane skeletal protein binding, and that its asymmetric orientation might provide a rationale for understanding the anomalous freeze-fracture images of the plasma membrane in these cells.

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