A monoclonal antibody study of protein distribution in the membrane skeleton of the ciliate *Pseudomicrothorax*

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**Summary**

The membrane skeleton, or epiplasm, of the ciliated protozoan *Pseudomicrothorax dubius* is a chemically and structurally complex layer. It is responsible for the cell shape and the positioning of some cortical organelles. One may expect that its possible morphogenetic role can be achieved only via a regional differentiation of the protein distribution in the epiplasm. We have tried to demonstrate such differentiation by preparing an epiplasm extract, which consists predominantly of concanavalin A (ConA)-positive glycoproteins. This fraction, either untreated or deglycosylated, was used to raise monoclonal antibodies (mAbs), whose specificity was tested on western blots of either untreated or deglycosylated epiplasm. The recognized polypeptides were then localized *in situ* by fluorescence and electron microscopic immunocytochemistry. Six mAbs are presented here. Four of them are directed against ConA-positive glycoproteins and show a localization of the latter on the outer surface of the epiplasm. The two others are directed against other epiplasmic polypeptides: one is specific for a common epitope shared by most of the epiplasmic proteins, but not by the glycoproteins, and labels the entire membrane skeleton, whereas the other recognizes three minor polypeptides, which seem localized to the inner part of the epiplasm.

**Key words:** Ciliophora, cortical differentiation, cytoskeleton, epiplasm, glycoproteins, membrane skeleton, monoclonal antibodies, protein localization, protozoa, *Pseudomicrothorax*.

**Introduction**

The presence of a highly developed cytoskeleton, associated with the external membrane(s) of the cell, has been demonstrated in many cell types, including epithelial cells (Simmons and Fuller, 1985), erythrocytes (Marchesi, 1985) and ciliated protozoa (Grain, 1986; Cohen and Beisson, 1988; Peck et al., 1991). It is generally believed that these structures maintain cell shape and probably control the regional distribution of cell components.

In the cortex of most ciliates, the external membrane is called the plasma membrane, and it is subtended by an alveolus comprising inner and outer alveolar membranes enclosing an alveolar space. The membrane skeleton, called the epiplasm, lies immediately beneath the inner alveolar membrane in *Tetrahymena* (Allen, 1967), *Paramecium* (Allen, 1971) and *Pseudomicrothorax* (Peck, 1977). In flagellates like *Euglena*, it is in contact with the plasma membrane, since alveoli are not present (Dubreuil and Bouck, 1985; Bricheux and Brugerolle, 1986).

The epiplasm of the ciliate *Pseudomicrothorax* includes distinct regions, namely the terminal plates of the basal bodies and the docking sites of the trichocysts, that are the sites of attachment of these structures to the epiplasm (Peck, 1977; Peck et al., 1991). Moreover, in this cell, the epiplasm is a thick, continuous, proteinaceous layer, which is easily purified without microtubules or evident membrane contamination. In addition to its highly complex structure, it is composed of many polypeptides, most of which range from 11 to 80 kDa in molecular mass, and some of which are glycosylated (Peck et al., 1991; Huttenlauch and Peck, 1991). The major band of 75-80 kDa is among the main structural elements of the epiplasm, as previously demonstrated by the use of polyclonal antibodies (Huttenlauch and Peck, 1991).

Many cortical structures are inserted in this layer, such as basal bodies, extrusomes and microtubules. It thus presents opportunities for studying pattern formation mechanisms that may occur generally in membrane skeletons.

In order to elucidate the possible role of the epiplasm in morphogenetic events, its proteins have to be immunologically characterized and localized *in situ*.

In the present study, we have employed a fraction of the epiplasm soluble in dilute HCl and mainly composed of glycoproteins, to raise monoclonal antibodies (mAbs). The polypeptides recognized on western blots by these mAbs were then localized by immunofluorescence on the isolated epiplasm and by EM immunocytochemistry on thin sections of isolated epiplasm and of entire cells.

**Materials and methods**

**Cultures**

*Pseudomicrothorax dubius* strain N5b cells were cultivated...
and harvested as described elsewhere (Peck and Duborgel, 1985).

Epiplasm isolation and HCl extraction
Isolation and extraction procedures were performed as described previously (Peck et al. 1991).

Enzymatic deglycosylation
Epiplasmin proteins (400 µg) or HCl extract (150 µg) were dissolved in 0.2% (w/v) SDS containing 0.01% (v/v) β-mercaptoethanol and 0.375% (w/v) of n-octylglucoside. In order to remove all N-linked carbohydrate moieties, Endo-β-N-acetylglucosaminidase F (Endo F) and glycopeptidase-N-glycosidase (PNGase; Endoglycosidase F/N-glycosidase F; Boehringer Mannheim) was used at 2.5 units/ml in 40 mM sodium phosphate, pH 6.5, 20 mM EDTA, for 18 h at 37°C. The reaction was stopped by adding sample buffer and boiling for 5 min.

The glycoprotein ovalbumin was used as a control for the digestions.

Preparation of monoclonal antibodies
Balb/CJ strain mice were immunized with 30-50 µg of HCl extract either with or without previous deglycosylation. The first injection was intraperitoneal, in complete Freund’s adjuvant, while the second was with incomplete adjuvant, in the same location. Four days before fusion, the mouse was boosted intravenously without adjuvant. Blood was taken before and after immunization for negative and positive controls.

Monoclonal antibodies (mAbs) were produced according to the method of Kohler and Milstein (1975) as modified by Kennett et al. (1978). Spleen cells were fused with myeloma NS-1 strain cells in a tenfold excess of the former. Selection of hybridomas started 24 h after fusion. Screening of hybridomas was performed first on dot-blots of epiplasmin solubilized in reducing sample buffer, and then on western blots of epiplasmin. Positive hybridomas were then cloned. For some clones with low viability, the medium was supplemented with 2.5% Origen HCF (hybridoma cloning factor, purchased from IGEN, MD, USA). Cells of selected clones were then frozen with 10% dimethyl sulfoxide (DMSO) in foetal calf serum (FCS).

Antibodies were concentrated ten times from cell culture supernatants by precipitation with 50% (v/v) ammonium sulfate and dialysed extensively against PBS (10 mM sodium phosphate, pH 7.5, 150 mM NaCl).

Electrophoretic procedures
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) using 12% (w/v) polyacrylamide slab gels. The protein Mr standards used were (x10^3): myosin (200); β-galactosidase (116.2); phosphorylase B (97); bovine serum albumin (66.2); ovalbumin (45); carbonic anhydrase (31); soybean trypsin inhibitor (21.5) and lysozyme (14.4). Epiplasmin or HCl extract were dissolved in SDS-sample buffer containing 25 mM dithiothreitol. Gels were stained with 0.05% (w/v) Coomassie brilliant blue R in 25% (w/v) trichloroacetic acid (TCA).

Nonequilibrium pH gradient electrophoresis (NEPHGE) was performed as described elsewhere (Peck et al. 1991), and was followed by SDS-PAGE.

For immunoblotting, one-dimensional gels were made with 11% (w/v) polyacrylamide without a sample comb. Migration was only 5 cm into the separating gel.

One- or two-dimensional gels were electrophoretically transferred to nitrocellulose sheets as described by Towbin et al. (1979). Transfer buffer contained 25 mM Tris, 192 mM glycine and 25% (v/v) methanol. Electrophoretic transfer was carried out at 400 mA for 50 min. Transferred proteins were visualized by staining the nitrocellulose sheets with 0.2% (w/v) Ponceau S in 3% (w/v) TCA, followed by destaining in distilled water.

Immunoblots
Concentrated monoclonal antibodies were employed at a dilution of 1:100 in 3% (w/v) non-fat dry milk, 0.1% (w/v) Tween 20 in PBS. Incubations in primary antibodies were performed overnight on western blots first blocked for 2 h in incubation buffer. Blots were then washed five times, for 5 min each, in incubation buffer. Alkaline phosphatase-conjugated F(ab)2 goat anti-mouse IgG (Biogenzien Lemania, Switzerland), diluted 1:1000-1:2000 in the same buffer, was used as secondary antibody and applied for 2 h. Blots were washed three times, for 5 min each, first in the same buffer and then in PBS. Blots were developed in a freshly prepared mixture of equal volumes of 0.4 mg/ml Naphtol AS-MX phosphate (Sigma) in water and 6 mg/ml Fast red (Sigma) in 0.2 M Tris, pH 8.0, containing 2 mM MgCl2. The reaction was stopped by washing in water.

The rabbit polyclonal antiserum 015 (Peck et al. 1991) was diluted 1:1200 in the same incubation buffer, and peroxidase-conjugated goat anti-rabbit IgG (Nordic Immunology, Netherlands) was used as secondary antibody, diluted 1:500. The blot was developed in a solution of 0.2 mg/ml 3,3-diaminobenzidine tetrachloride, 0.045% (v/v) H2O2 in PBS. The reaction was stopped by washing in water.

All incubations were done at room temperature and with agitation.

Lectin staining
Blots of entire or deglycosylated epiplasmin were labeled with the lectin concanavalin A (ConA) as described elsewhere (Peck et al. 1991).

Indirect immunofluorescence microscopy
Extracted, unfixed epiplasmin was either dried directly on glass slides or, more generally, first fixed with 4% (v/v) paraformaldehyde for 1 h and then washed three times, 10 min each, in PBS, and then dried on slides coated with 0.1% (w/v) para-phenylenediamine as anti-fading medium.

Slides were blocked for 1 h in 1% (w/v) BSA, 0.1% (w/v) Tween 20 in PBS, or in the case of fixed epiplasmin, normal goat serum was added 1:1 to this solution to reduce non-specific labeling. Excess blocking solution was blotted from the slides, which were then incubated overnight in 1:1 dilution of mAb and 1% (w/v) poly-L-lysine.

Slides were blocked for 1 h in 1% (w/v) BSA, 0.1% (w/v) Tween 20 in PBS. Slides were subsequently rinsed in PBS four times for 5 min each, and incubated 1 h in FITC-conjugated goat anti-mouse total Ig (Nordic Immunology, Netherlands) diluted 1:100 in the same incubation buffer employed for the primary antibody. Finally, slides were rinsed five times for 5 min each in PBS, and then mounted in glycerol containing 0.1% (w/v) para-phenylenediamine as anti-fading medium.

Preparations were observed using an Olympus BH-2 epifluorescence microscope, and photographs were taken using Kodak Tri-X Pan film ASA 400.

In control experiments, immune and preimmune mouse sera were used at a dilution of 1:100-1:200.

All incubations were done at 4°C, without agitation.

Electron microscopy (EM)
For scanning EM, epiplasmin fractions were prepared as previously described (Peck, 1977). For immunolabeling, cells or epiplasmin were fixed, pre-embedded in agarose, dehydrated and finally embedded in Lowicryl K4M (Chemische Werke Lowi, Germany) as described elsewhere (Peck et al. 1991). Thin sections were cut with glass knives and then mounted on 300-mesh nickel grids without plastic film.
Each grid was incubated for 1.5 h at room temperature in a drop of 1% (w/v) BSA in PBS, transferred into a drop of mAb diluted 1:1 with PBT (0.2% (w/v) BSA and 0.05% (w/v) Tween 20 in PBS), and incubated overnight at 4°C. In control experiments, immune and preimmune sera were diluted 1:200. Grids were subsequently washed four times in PBT and incubated for 2 h in a drop of goat anti-mouse IgG and IgM coupled to 10 nm diameter colloidal gold particles (Biocell Research Laboratories, UK) diluted 1:50 with PBT. Grids were then washed three times in PBT, three times in distilled water and air dried. Finally, they were carbon-coated and stained with 2% (w/v) uranyl acetate for 10 min followed by lead acetate for 45 s (Millonig, 1961).

In some cases, labeling was performed before embedding. Incubation of formol-fixed epiplasm with primary antibody was done as in the fluorescence experiments, except that epiplasm was not stuck on glass slides, but left in suspension in a microtest-tube. The secondary antibody was gold-conjugated and used at a 1:3 dilution in the same buffer as was used for FITC-conjugated antibody. Afterwards, epiplasm was fixed in 2% (v/v) glutaraldehyde in PBS and washed in the same buffer, then dehydrated and embedded in either Epon (Fluka, Switzerland) or Spurr (EMS, Washington, USA) resin. Thin sections were cut with a diamond knife, mounted on 200 mesh copper grids with a carbon-coated Formvar film, and stained with 2% (w/v) uranyl acetate for 10 min and lead citrate for 10 min (Reynolds, 1963).

Results

Monoclonal antibody (mAb) production

The membrane skeleton (epiplasm) of the ciliate *P. dubius* is a complex structure that includes ribs on its inner surface, running between the grooves of the ciliary rows (Fig. 1A, B).

Gentle treatment with 0.01 M HCl following epiplasm purification solubilizes the terminal plates of basal bodies and the trichocyst docking sites, as seen by scanning electron microscopy (SEM) (Fig. 1C).

The complexity of the epiplasm is also observed in its many polypeptides, most of which have a molecular mass range of 11-80 kDa (Fig. 2, lane b). The HCl-soluble fraction mainly contains four polypeptides (Fig. 2, lane c), which are almost totally extracted from the insoluble epiplasm (Fig. 2, lane a). These polypeptides are glycoproteins labeled with the lectin concanavalin A (Fig. 2, lane d), and are of 62, 53, 48 and 33 kDa. This HCl fraction, either with or without previous deglycosylation, was used to raise monoclonal antibodies. The fusion performed with the spleen of a mouse immunized with non-deglycosylated fractions produced antibodies of the F3 group: 4B5F3, 5C2F3, 2D4F3, 2F11F3 and 2A6F3, whereas antibody 2A11F7 was obtained using a deglycosylated fraction as antigen.

Specificity of these mAbs

Epiplasmic proteins with or without previous deglycosylation were separated on 11% SDS-PAGE. The efficiency of the enzymatic reaction was tested by the loss of ConA reactivity (Fig. 2, lane d) and the shift of apparent molecular mass (Fig. 3, lanes e and f).

On immunoblots (Fig. 3), we distinguish three classes of mAbs. The first class consists of antibodies which recognize most of the epiplasmic proteins, but not those of 24-28 kDa. This class of mAb, represented by 4B5F3 (Fig. 3, lane a), strongly labels the complex band of 75-80 kDa, but also labels many lower molecular mass bands. Since it is difficult to see in the labeling pattern whether ConA-positive glycoproteins are recognized by this mAb, we have also labeled a two-dimensional gel blot. From the absence of labeling in glycoprotein positions (arrows, Fig. 4), we conclude that the glycoproteins do not share this common epiplasmic epitope. Other mAbs of this sort were obtained (not shown), that exhibit small differences in bands labeled, indicating that they are directed against other common epiplasmic epitopes.

The second class is composed of one mAb, 2A6F3 (Fig. 3, lane b), which is directed against minor epiplasmic polypeptides of 45, 40 and 27 kDa, which are not labeled by ConA and do not appear to be glycosylated, as shown by labeling with other lectins (not shown). Nonetheless, the 27 and 40 kDa bands are not, or are less strongly, labeled following deglycosylation, probably because of epitope degradation during incubation.

Finally, the third class contains four mAbs, each of which recognizes one or more glycoproteins. The different labeling patterns indicate that they are specific for different epitopes; thus, they are different markers of these glycoproteins. Two of them, 5C2F3 and 2D4F3 (Fig. 3, lanes c, d), are directed against glycosidic epitopes, as shown by the absence of labeling on the blots of deglycosylated epiplasm. The mAb 5C2F3 recognizes the glycoproteins of 62, 53 and

Fig. 1. Nomarski microscopy of a living *P. dubius* cell (A) showing the grooves of the ciliary rows, the kineties (k), and an SEM view of the inner surface of the isolated epiplasm (B). The grooves contain the terminal plates of basal bodies (t) and the docking sites of trichocysts (d). Epiplasmic ribs (r) run on this inner surface, between adjacent grooves. After treatment of the epiplasm with 0.01 M HCl (C), terminal plates and docking sites are missing. A, ×400; B, ×11,000 and C, ×13,000.
The two other mAbs of this class are each specific for only one band, and both are directed against peptidic epitopes; 2F11F3 (Fig. 3, lane e) labels the 62 kDa glycoprotein and 2A11F7 (Fig. 3, lane f) the 48 kDa glycoprotein. Notice that both glycoproteins are highly glycosylated, as shown by the large shift in the apparent molecular mass following deglycosylation.

Microscopic localization of the proteins recognized by the mAbs

The localization of these polypeptides was studied on Lowicryl K4M thin sections of cells and of extracted epiplasm. Immunofluorescence was performed on extracted epiplasm following fixation for 1 h with 4% paraformaldehyde, except for labeling with 4B5F3, for which unfixed epiplasm was employed. The presence or absence of fixation before fluorescent labeling leads to the same results, but fixation induces a stronger response, principally for labeling with the anti-glycoprotein antibodies.

In all cases EM immunocytochemistry exhibits the same localization of reactivity as immunofluorescence labeling.

The anti-glycoprotein mAbs 5C2F3 (Fig. 5A-C), 2D4F3 (Fig. 5D-F), 2F11F3 (Fig. 5G-I) and 2A11F7 (Fig. 5J, K) always labeled only the outer surface of the epiplasm. The mAb 5C2F3, directed against a carbohydrate epitope, also appears to recognize the plasma membrane, as observed by EM immunolabeling of entire cells (Fig. 5C). These four mAbs never labeled the inside of the epiplasm layer or the ribs, or the terminal plates or docking sites.

Because of the differences in specimen fixation and preparation conditions between fluorescence and EM immunolabeling, we performed gold-labeling on extracted epiplasm with the same conditions of fixation employed for immunofluorescence, and then observed the distribution of gold particles by EM. This was done to ensure that the fluorescent labeling seen in Fig. 5A, D, G and J is really localized only to the outer surface of the epiplasm. As observed in Fig. 7, such pre-embedding label is localized to the outer surface of the epiplasm, with no label on the inner surface, clearly identifiable by the presence of ribs.

As it was impossible to label Lowicryl thin sections with 2A11F7, we also carried out pre-embedding labeling, which confirmed that labeling was localized to the external surface of the epiplasm (Fig. 5K).

The mAb 4B5F3, which recognizes most of the non-glycosylated proteins of the epiplasmic fraction, labels the
entire epiplasm, including the ribs (Fig. 6A-C). Sometimes, we obtained fluorescent labeling of terminal plates in unfixed epiplasm (not shown), but we observed no labeling of the terminal plates by EM. The cytoplasm is not labeled with this mAb, as is evident on cell sections (Fig. 6C), nor is the plasma membrane or ciliary membrane labeled, as observed by the absence of gold particles around the cilium.

Finally, the mAb 2A6F3, which is directed against non-glycosylated, minor epiplasmic polypeptides, preferentially labels the inner surface of the epiplasm and mainly the ribs (Fig. 6D-F).

Discussion

The glycoproteins

In the present study, four distinct mAbs have been produced that react with ConA-positive glycoproteins. They are directed against either glycosidic or peptidic epitopes, and all show the same distribution of these glycoproteins, confined to the outer part of the epiplasm which underlies the inner alveolar membrane.

On the other hand, the inner surface of the epiplasm is not labeled, nor are the ribs. Since both surfaces were exposed in the same manner to fixation and mAbs, glycoproteins are apparently limited in distribution to the outer surface of the epiplasm.

However, a previously published study was performed with polyclonal antibodies, affinity-purified from antisem 015 by their adsorption on the different, individual ConA-positive bands cut from a gel blot, and then desorption of the bound antibodies: the latter were used to probe other blots to test their specificity, and to label Lowicryl sections for EM (Huttenlauch and Peck, 1991). This study concluded that the ConA-positive glycoproteins are present throughout the epiplasm, principally because these affinity-purified antibodies labeled the entire epiplasm including the ribs, as shown by EM. The discrepancy between these results and our mAb results may be explained in the following manner.

The polyclonal serum 018 (Peck et al., 1991) and our mAb 4B5F3 indicate on immunoblots that several minor polypeptides are present immediately adjacent to the glycoproteins. As we have shown with 4B5F3, many epiplasmic polypeptides have common determinants, which are distributed throughout the epiplasmic layer. We suggest that serum 015 recognizes one or more of these determinants. In effect, immunoblots made with serum 015 employing a more sensitive labeling procedure (secondary antibody conjugated to alkaline phosphatase instead of peroxidase) reveal bands adjacent to the glycoproteins (unpublished results). The 015 antibodies that bind to such bands could easily be co-purified with the anti-glycoprotein antibodies during affinity purification. The low titer of the antibodies directed against the common determinant(s), and the wide M₀ distribution of the polypeptides sharing these determinants render their immunological detection difficult on western blots. However, these anti-common epitope antibodies may well produce labeling on Lowicryl sections: first, because higher concentrations of the affinity-purified antibodies are employed; and second, since individual gold particles representing one antibody molecule are detectable, even a weak reaction may be visualized on sections but may not be evident on transfers.

In situ labeling with ConA (Peck et al. 1991) has shown that the glycoproteins are not located uniformly throughout the epiplasm, or in the ribs. Although it was concluded that the glycoproteins are integral components of the epiplasm, since the labeling was aligned along what were interpreted as internal regions of the epiplasm, the complex morphology of the epiplasm and the lack of a clearly defined external epiplasm surface in many tangential sections render localization difficult. Recent unpublished results (Curtenaz) with GNA (Galanthus nivalis agglutinin), which like ConA recognizes N-linked, high-mannose sugars, show that this lectin labels the 62 and 48 kDa bands. On sections oriented perpendicularly to the epiplasm surface, the GNA labels uniquely the outer surface of the epiplasm.

The sugar moieties of the glycoproteins of the epiplasmic fraction are N-linked because they bind ConA and are digested by endoglycosidase F / N-glycosidase F. N-linked, high-mannose sugars are common membrane constituents; however, it is other sugars that have been found associated with non-membrane cellular constituents. Glycosylation has been demonstrated on the cytosolic and nucleoplasmic faces of nuclear pore proteins of higher organisms (Holt et al. 1987b; Davis and Blobel, 1987; Snow et al. 1987). Band 4.1 of erythrocytes also exhibits cytoplasmic glycosylation (Holt et al. 1987a). It is likely to mediate the anchoring of cytoskeletal proteins, as it has also been implicated in the proper assembly of multimeric nuclear pore complexes. Glycosylation of cytokeratin 13, an intermediate filament protein, was also reported (King and Hounsell, 1989). In all of these cases the glycosylation consists solely of a monosaccharide, an O-linked N-acetylglucosamine, and occurs through an unknown cellular pathway (for a review, see Hart et al. 1988). Complex sugar moieties, like high-mannose groups recognized by ConA, have never been found.

No glycoproteins have been described so far in the epiplasm of other ciliates. However, since epiplasm has not
Fig. 5. Fluorescence and EM immunolabeling with the anti-glycoprotein mAbs 5C2F3 (A-C), 2D4F3 (D-F), 2F11F3 (G-I) and 2A11F7 (J, K). Immunofluorescence (A, D, G and J) of isolated epiplasm, following fixation with 4% formaldehyde for 1 h. Immunogold labeling was performed on Lowicryl K4M thin sections of either isolated epiplasm (B, E and H) or entire cells (C, F and I), except for 2A11F7, which failed to label thin sections. For the latter mAb, gold labeling (K) was obtained by labeling prior to embedding in Epon resin. These glycoproteins appear to be localized only on the outer surface of the epiplasm. Cilia (c) are indicated, as well as the epiplasm (e), epiplasmic ribs (r) and terminal plates of basal bodies (t). Int and ext, interior and exterior surfaces of the epiplasm, respectively. Fluorescence micrographs, ×720; electron micrographs, ×30,000.
been purified without membrane, from ciliates other than Pseudomicrothorax, lectin labeling on western blots (the principal method sensitive enough to detect low quantities of sugars in specific bands) is not applicable. Fluorescent labeling experiments with lectins were performed on permeabilized Paramecium and showed no glycosylation of the epiplasm (Allen et al. 1988). Polyclonal and monoclonal antibodies have been raised against epiplasm of different ciliates (Paramecium: Kéryer et al. 1990; Nahon, personal communication; Tetrahymena: Williams et al. 1987, 1990; entodiniomorphid ciliates: David et al. 1991; Viguès and David, 1989), but none of the proteins recognized have been shown to be glycosylated, although this aspect of the epiplasm has not been specifically studied in all cases.

In the flagellate Euglena acus, the cortical fraction contains glycoproteins, the two main ones being of 140 and 64 kDa. By radio-iodination, they were shown to emanate from the plasma membrane, which is in direct contact with the epiplasm, since there are no alveoli. This localization was confirmed by gold-labeling with antibodies produced against the 140 kDa polypeptide (Bricheux and Brugerolle, 1986). These membrane glycoproteins are co-extracted with the epiplasm, since the plasma membrane is not solubilized after treatment with Triton X-100. The plasma membrane of E. acus is reported to be solubilized only with a mixture of LIS (lithium 3,5-diiodosalicylate) and NP-40 (Dubreuil and Bouck, 1985).

Examples of integral membrane glycoproteins co-extracted with the cytoskeleton, in spite of the use of Triton X-100, are numerous in higher eukaryotes. In erythrocytes, both the minor sialoglycoproteins, glycophorin C and glycoprotein γ, remain predominantly associated with the Triton X-100-insoluble membrane skeleton (Bloy et al. 1987). Likewise, an integral membrane protein of 28 kDa and its glycosylated form of higher molecular mass, found in red...
blood cells and renal tubules, remain associated with the Triton-insoluble cytoskeletons of these cells (Denker et al. 1988). GP130, renamed contactin, is also enriched in the neuronal NP-40-insoluble membrane skeleton (Moss and White, 1992). A 240 kDa glycoprotein exposed at the flagellar surface of the eukaryotic green algae Chlamydomonas moewusii remains tightly associated with the axoneme, in spite of extraction with many different detergents (Reinhart and Bloodgood, 1988). A glycoprotein-containing transmembrane complex has been described in association with ascites tumor cell microvillar microfilament cores, following Triton treatment. This is thought to be the association site for microfilaments at the plasma membrane (Carruway et al. 1991).

The only polypeptide known to date to be of membrane origin and firmly bound to the epiplasm is a polypeptide of 39 kDa, apparently non-glycosylated, shown in Euglena gracilis to anchor the plasma membrane to the membrane skeleton (Rosiere et al. 1990).

On the basis of our evidence and what is known of glycoprotein distribution in cells, we suggest that the ConA-positive glycoproteins, at the outer surface of the P. dubius epiplasm, are integral membrane glycoproteins that remain tightly bound to the epiplasm, despite the detergent solubilization leading to the absence of evident membrane contamination on TEM views of isolated epiplasm (Peck et al. 1991). They may play a role in anchoring the epiplasm to the inner alveolar membrane.

Although the terminal plates of basal bodies and the docking sites of trichocysts are also extracted with HCl, they were not labeled with our mAbs, suggesting that they do not contain glycoproteins, unless the epitopes cannot be recognized in these structures.

Because the epiplasm of P. dubius underlies the inner alveolar membrane and not the plasma membrane, it is impossible to perform radio-iodination experiments to determine whether the glycoproteins are of membrane origin. It will be interesting to examine the molecular organization of these glycoproteins to learn if transmembrane amino acid sequences occur. In this way, a definitive answer as to the origin of the glycoproteins of the epiplasm fraction may be possible.

The other proteins

The common epiplasmic polypeptide epitopes

The mAb 4B5F3, of broad specificity, recognizes nearly all the epiplasmic polypeptides, especially the thick band of 75-80 kDa that is resolved into many spots on two-dimensional gels and is the main structural component of the epiplasm (Peck et al. 1991; Huttenlauch and Peck, 1991). But it does not label the proteins of 24-28 kDa that are probably glycosylated, since they are labeled with the lectin Dolichos biflorus agglutinin (DBA) (Huttenlauch and Peck, 1991), and are also recognized by the antiserum 015, which is mainly directed against glycoproteins (Fig. 3, lane g). It shows that many epiplasmic proteins of P. dubius share at least one common determinant. The same finding has been described with mAbs in Paramecium tetraurelia ( Nahon, personal communication), and in the genus Euglena with polyclonal antibodies (Brichex and Brugerolle, 1987). In the latter case, the common epiplasmic determinants are distinct from those of the membrane glycoproteins associated with the epiplasm. It should be noted that the common epitope recognized by 4B5F3 is completely specific for the epiplasm, since it was never found in the cytoplasm, the cilia or the trichocysts (immunoblot results not presented).

The minor epiplasmic polypeptides

The mAb 2A6F3 has permitted localization of the 45, 40 and 27 kDa minor polypeptides to the epiplasmic ribs and the inner surface of the epiplasm. Specific localization of some proteins has also been reported for the epiplasm of Tetrahymena (Williams et al. 1990), where mAbs recognize either the epiplasm immediately surrounding the basal bodies, or the rest of the epiplasm. These results are consistent with the hypothesis that the epiplasm plays a morphogenetic role in the positioning of cortical structures, which can be carried out only if unique chemical differences exist at different sites in this layer.

Two polyclonal antisera were produced against different preparations of P. dubius epiplasm, and called 015 and 018 (Peck et al. 1991). The antiserum 018, which recognizes many epiplasmic polypeptides (as does the mAb 4B5F3), was used in cross-reaction studies among epiplasms, or cortical fractions, of different protozoa (Viguès et al. 1987). It strongly cross-reacts with representatives of other genera, while antiserum 015, directed mainly against the glycoproteins, is less cross-reactive (Peck et al. 1991). Our mAbs were also employed in this kind of study; the mAbs directed against glycoproteins did not cross-react, while the other mAbs (4B5F3 and 2A6F3) were found to cross-react (unpublished results).

The mAbs produced in different laboratories are suitable and precise tools for the screening of genomic libraries, in order to isolate the genes coding for epiplasmic proteins. The sequencing will determine whether the polypeptides sharing common epitopes (within or between species) have a common gene ancestor, or whether one or a few genes produce the numerous epiplasmic polypeptides via post-transcriptional processing. Alternatively, the amino acid sequences may differ totally, except for the common epitopes, which may represent an evolutionarily convergent component that is needed for epiplasmic function or formation.

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