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GLYCOPHOSPHATIDYLINOSITOL-ANCHORED PROTEINS IN *PARAMECIUM TETRAURELIA*: POSSIBLE ROLE IN CHEMORESPONSE

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Summary

We have begun to characterize the glycophosphatidylinositol (GPI)-anchored proteins of the *Paramecium tetraurelia* cell body surface where receptors and binding sites for attractant stimuli are found. We demonstrate here (i) that inositol-specific exogenous phospholipase C (PLC) treatment of the cell body membranes (pellicles) removes proteins with GPI anchors, (ii) that, as in *P. primaurelia*, there is an endogenous lipase that responds differently to PLC inhibitors compared with its response to an exogenous PLC, (iii) that salt and ethanol treatment of cells removes GPI-anchored proteins from whole, intact cells, (iv) that Triton X-114 phase partitioning shows that many GPI-anchored proteins are cleaved from pellicles by the endogenous lipase and enter the aqueous phase, and (v) that integral membrane proteins are not among those cleaved with PLC or in the salt/ethanol wash.

Antisera against the proteins removed by the salt/ethanol washing procedure include antibodies against large surface antigens, which we confirm in this species to be GPI-anchored, and against an array of proteins of smaller molecular mass. These antisera specifically block the chemoresponse to some stimuli, such as folate, which we suggest are signaled through GPI-anchored receptors. Responses to cyclic AMP, which we believe involve an integral membrane protein receptor, and to NH_4Cl, which requires no receptor, are not affected by the antisera. Antiserum against a mammalian GPI-anchored folate-binding protein recognizes a single band among the GPI-anchored salt and ethanol wash proteins. The same antiserum specifically blocks the chemoresponse to folate.

Key words: receptor, chemosensory, *Paramecium tetraurelia*, GPI anchor, signal transduction, folate.

Introduction

There is a growing awareness that many surface membrane proteins are peripheral proteins, tethered to the membrane by a glycophosphatidylinositol (GPI) anchor (Lisanti et al., 1990; Cross, 1990). These surface proteins serve many diverse functions: transport of folate (Rothberg et al., 1990), protection from complement (Kooyman, et al., 1995), transfer of surface proteins from cell to cell (Ilangumaran et al., 1996), channel activation (Vallet et al., 1997), axon guidance (Faivre-Sarrailh and Rougon, 1997) and binding of ligand for signal transduction (Nosjean et al., 1997; Parolini et al., 1996; Casey, 1995). The catalog of functions for these surface proteins is increasing rapidly.

GPI-anchored proteins have been well characterized as coat proteins from parasitic protozoa such as trypanosomes (McConville and Ferguson, 1993), and their function in these cells may include protection and camouflage from the immune system of the host. Free-living protozoa, such as *Paramecium*, also have GPI-anchored surface proteins, but the functions of these proteins in the life history of the cells is not yet clear. On the surface of *Paramecium* spp. are extremely large and glycosylated proteins (‘immobilization’ antigens, referred to here as surface antigens). These make a coating on the cell that is so prevalent as to appear to be ‘fuzzy’ when seen in the transmission electron microscope (Ramanathan et al., 1981). Such a large amount of protein could perhaps be a form of metal-ion or pH buffering for cells that live in extremes of ionic strength in freshwater habitats. Regardless of their function, they are plentiful surface proteins and have been demonstrated to be GPI-anchored in *P. primaurelia* (Capdeville et al., 1987).

In our search for chemoreceptor proteins among the surface proteins of *P. tetraurelia*, we are exploring the GPI-anchored proteins in part because of their characteristic insolubility in vertebrate systems (Parton and Simons, 1995; Brown and London, 1997). Mammalian GPI-anchored proteins, such as folate receptors, are found in caveolae (Wu et al., 1997; Ying et al., 1992) or membrane domains (detergent-insoluble domains) (Futerman, 1995; Schnitzer et al., 1995; Parton and Simons, 1995; Brown and London, 1997), which are characterized as enriched in phosphoglycosphingolipids and cholesterol, and in many proteins that are associated with signal transduction (Nosjean et al., 1997). Our experience with *Paramecium tetraurelia* chemoreceptors is that some are highly insoluble in Triton X-100 (Sasner and Van Houten, 1989), indicating that they may be GPI-anchored proteins. It

was necessary to explore some aspects of the surface of *P. tetraurelia* before we could search for putative GPI-anchored chemoreceptors. As we describe below, we have demonstrated that the A and B large surface antigens of *P. tetraurelia*, like those of *P. primaurelia*, are GPI-anchored, since they can be released, together with the cleaved GPI anchor, using an exogenous phosphoinositol-specific phospholipase C (PI-PLC).

The large surface antigens of *Paramecium* spp. are known to be liberated from the surface by washing cells in high concentrations of salt and ethanol (Preer et al., 1981), which would provide an efficient method of removing and harvesting GPI-anchored receptors. We demonstrate here that a salt and ethanol wash of *P. tetraurelia* contains an enrichment of the A and B large surface antigens and an array of other GPI-anchored proteins and that there is an endogenous, probably constitutive, phospholipase that cleaves the GPI anchor. However, the salt/ethanol treatment does not seem to require a lipase to release some proteins from the cell surface because these proteins have no anchor or an intact one. Triton X-114 phase-partitioning has been useful in the solubilization of GPI-anchored proteins, and we discuss here the solubility of cell membrane proteins in Triton X-114. Two known integral membrane proteins, the plasma membrane Ca^{2+} pump and the cyclic AMP receptor, are not found among the salt/ethanol- or PLC-released proteins.

In this study, we also use antisera produced against salt/ethanol wash proteins to block a subset of chemoresponses, which we believe to be receptor-mediated. In addition, antiserum against a mammalian GPI-anchored folate-binding protein recognizes a GPI-anchored protein among the salt/ethanol wash proteins, and the same antiserum specifically blocks the chemoresponse to folate.

We anticipate that the results of these studies will generate interest in GPI-anchored proteins as receptors or partners of receptors in chemical sensing. The methods used for efficiently harvesting the proteins from the cell surface may also be more generally applicable.

Materials and methods

Cells and culturing

Wheat grass (Pines International Inc.) extract was used as a medium to culture bacteria, *Klebsiella pneumonia* (see Sasner and Van Houten, 1989). This extract, in turn, was used to culture stocks of *Paramecium tetraurelia*, strains 51-S (sensitive to killer) or d4-12-144, mutants that do not express the A or B surface antigens (courtesy of J. Forney; Scott et al., 1993). A richer culture medium was used for culturing large volumes of cells (for protocol, see Wright and Van Houten, 1990). Cells were grown at 28 °C.

Pellicle preparation

Surface membranes (pellicles) of cells were prepared from 6 l of culture using a modification of a protocol published previously (from Bilinski et al., 1981). The final pellet was resuspended in 2 ml of homogenization buffer and stored at −70 °C for up to 1 week (Van Houten et al., 1991).

Phospholipase treatments

Frozen pellicle membranes (9 mg) were thawed on ice and brought to 1 ml with 5 mmol l^{-1} KCl buffer (in mmol l^{-1}: 5 KCl, 1.3 Tris base, 1 $Ca(OH)_2$, 1 citric acid, pH 7.0). This low-ionic-strength buffer was used in anticipation of treating whole cells with PLC and because whole cells require a buffer comparable with this one. The sample was divided in two, one half for bacterial phosphoinositol-specific phospholipase C (PLC; Sigma) treatment and the other for sham treatments. A 500 μl sample was used to solubilize 5 units of lyophilized PLC and was incubated for 20 min at 37 °C (or just incubated at 37 °C for sham treatment) before centrifugation at 11 300 ***g*** in a Beckman microcentrifuge for 15 min at 4 °C. The supernatants were either collected and concentrated to 200 μl with Amicon Centricon 10 concentrators or precipitated with acetone and washed twice with acetone before resuspension in sample buffer for sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS–PAGE).

Salt/ethanol washes

Cultures (3 l) of cells grown in wheat extract medium (Wright et al., 1990) were filtered, centrifuged at 350 ***g*** in an IEC-HNSII centrifuge and resuspended in 3 ml of bacterium-free culture medium. Salt/ethanol stock solution (1.8 ml), consisting of 10 mmol l^{-1} Na_2HPO_4, 150 mmol l^{-1} NaCl and 30 % ethanol (Preer et al., 1981), was added to the sample, which was kept on ice for 1 h. The sample was then centrifuged at 551 ***g*** in a Beckman J2-21 centrifuge and JA-17 rotor for 5 min at 4 °C, and the supernatant was collected as described above.

Phospholipase inhibitor treatment

Small-scale salt/ethanol washes were carried out in the presence of PLC-specific inhibitors. A culture of cells (1 l) was centrifuged at 350 ***g***, and the pellet was resuspended in 1 ml of sterile culture fluid. To 200 μl samples of the resuspended cells were added 20 μl each of 10 mmol l^{-1} 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC; dissolved in ethanol) and 10 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF; dissolved in isopropanol), to give a final concentration of 1 mmol l^{-1} for each inhibitor, or 17 μl of a 100 mmol l^{-1} stock of *p*-chloromercuriphenylsulfonic acid (PCMPSA dissolved in water), to give a final concentration of 1 mmol l^{-1}. Neomycin, another reported PLC inhibitor, was also used at a final concentration of 1 mmol l^{-1}. (Results are not shown for the neomycin treatment, since this produced no change in the effects of PLC.) A stock of salt/ethanol (10 mmol l^{-1} Na_2HPO_4, 150 mmol l^{-1} NaCl and 30 % ethanol) was added to bring the volume to 300 μl and to a salt/ethanol concentration of 3–4 mmol l^{-1} Na_2HPO_4, 50–70 mmol l^{-1} NaCl and 10–14 % ethanol). Samples were incubated on ice for 1 h and centrifuged at 11 300 ***g*** in a Beckman microcentrifuge for 5 min at 4 °C. The supernatants were collected.

Triton X-114 phase extraction

A sample of pellicle membranes (500 μl; 3–17 mg) was incubated with 5 μl of 10 % Triton X-114, which had been pre-concentrated with Tris-buffered saline (TBS, 20 mmol l^{-1} Tris, 137 mmol l^{-1} NaCl, pH 7.6) at 30 °C overnight to remove contaminants (Bordier, 1981). The Triton/pellicle mixture was incubated at 4 °C with gentle mixing for 2 h. The sample was then centrifuged at 11 300 ***g*** for 30 min at 4 °C. The supernatant was layered over 100 μl of 6 % sucrose in TBS and incubated at 37 °C for 4 min. The samples were then centrifuged for 2 min at 11 300 ***g*** at room temperature (21–22 °C), and the aqueous phase and detergent pellet were collected. The separate layers were precipitated with 1 ml of cold acetone (4 °C) and washed three times in cold acetone. The samples were then resuspended in 20 μl of sample buffer for loading onto 8 % acrylamide gels and eventual transfer to nitrocellulose.

SDS–PAGE and western blots

Homogeneous and gradient SDS/polyacrylamide gels (for details of the percentage of acrylamide used, see figure legends) with 3 % stacking gels were used to separate proteins electrophoretically in a Hoeffer Tall Mighty Small apparatus (Laemmli, 1970).

The proteins separated in the SDS gels were electroblotted onto nitrocellulose membrane (Gelman Sciences) (according to Towbin et al., 1979) with a modified transfer buffer (15.6 mmol l^{-1} Tris-HCl, 120 mmol l^{-1} glycine, 20 % methanol) (according to Gershoni and Palade, 1982). Membranes were either stained for total protein with 0.1 % Amido Black in 50 % methanol and 10 % acetic acid or probed with polyclonal antiserum and anti-rabbit secondary antibody conjugated with alkaline phosphatase (according to McGadey, 1970) after blocking with dried milk (Thean and Toh, 1989). The antisera used were anti-A and anti-B developed against salt/ethanol washes of cells expressing either the A or B surface antigen (kind gifts from J. Forney), anti-cross-reactive-determinant (CRD) of the GPI anchor (anti-CRD, Oxford GlycoSystems) or anti-folate-binding-protein (bovine, Biogenesis, Poole, England).

T-maze assays of chemoresponse behavior

D4-12-144 cells were assayed for chemoresponse using T-mazes, as described previously (Van Houten et al., 1982) with the following modifications. Cells are allowed to distribute between two arms of a glass stopcock, which is the T-maze. The two arms of the T-stopcock contain buffers that differ by one component, e.g. acetate in test solution and chloride in the control arm. After 30 min, the cells are counted, and the index of chemokinesis, I_{che}, is calculated as the number of cells in the test arm divided by the total number of cells in both arms. Indices above 0.5 indicate attraction to the test solution; indices below 0.5 indicate repulsion. A sample of cells (100 ml) was centrifuged at 350 ***g***, and the pellet was resuspended in chemokinesis buffer [1 mmol l^{-1} $Ca(OH)_2$, 1.3 mmol l^{-1} Tris base, 1 mmol l^{-1} citric acid, and the salt indicated, usually 5 mmol l^{-1} KCl or NaCl] and centrifuged again. The resulting pellet of cells was resuspended in 2 ml of buffer and divided into 1 ml samples. To one of the samples, 4 or 8 μl of each antiserum against salt/ethanol washes of A or B cells (anti-A and anti-B sera) was added to one of the cell samples; pre-immune serum from a different set of rabbits was added to the second sample as control. (Although this is not the best control, we had no other pre-immune serum to use. A pre-immune serum from different rabbits was used at the same dilution in a selection of T-mazes for a different purpose, and there was no difference between the results with pre-immune treatment and sham-treated controls; W. E. Bell, personal communication.) After 30 min, the cells were used in T-maze assays without further washing.

In other T-maze tests, D4-12-144 cells were concentrated as above, resuspended in 2×0.5 ml samples of 5 mmol l^{-1} NaCl buffer and incubated for 1 h with or without 10 μl of anti-folate-binding-protein antiserum. Cells were then immediately tested in T-mazes for 20 min.

Immobilization tests to verify the serotypes of the cells

Cells were transferred to Dryl's solution (Dryl, 1959) in a glass depression slide using a micropipette. Ten cells were then transferred to 300 μl of Dryl's solution with 0.6 μl of anti-A or anti-B serum. The cells were then observed for motility. Cells rendered immotile by anti-A serum, for example, were expected to be expressing only anti-A surface antigen (Preer, 1959). The majority of cells, which were grown at 28 °C, expressed antigen A, as expected for this condition. The remaining cells appeared to be expressing antigen B.

Antiserum against the C terminus of the plasma membrane Ca^{2+} pump was prepared in rabbits against a synthetic peptide based on the last 17 amino acid residues of the plasma membrane Ca^{2+} pump clone (Elwess and Van Houten, 1997). The peptide was conjugated to keyhole limpet hemocyanin and used as an immunogen in rabbits (Quality Controlled Biochemicals, Hopkinton, MA, USA). The antiserum was affinity-purified. The specificity of binding to western blots of the bacterially expressed C terminus (10 kDa) of the pump was determined by comparison of results with and without preabsorption of the antiserum with excess peptide (Yano et al., 1997). The antiserum is referred to as anti-cbd in Fig. 6 because the C terminus of the pump, used in the fusion protein as antigen, is the calmodulin-binding domain (CBD).

Antiserum against 15 amino acid residues of the N terminus of the cyclic AMP receptor (Van Houten et al., 1991) was prepared by Lampire Biological Laboratories (Pipersville, PA, USA) in rabbits using a glutathione-*S*-transferase (GST) fusion protein expressed from pGEX (Strategene) vector in BL21 cells. To control for recognition of GST, we preabsorbed the antiserum with expressed bacterial GST both on nitrocellulose strips and in solution. There still remained some recognition of GST on western blots, as can be see in Fig. 6.

Cilia for phase contrast microscopy were prepared as described by Eisenbach et al. (Eisenbach et al., 1983).

The Pierce protein microassay was used for photometric analysis of protein using bovine serum albumin (BSA) as standard.

Results

PLC treatment of Paramecium *pellicles releases GPI-anchored proteins*

We treated pellicle membranes with buffer alone or with PLC and separated the preparation into pellet and supernatant. Proteins from the pellet and supernatant were separated on gels, electroblotted to nitrocellulose and visualized using anti-A and anti-B antisera, i.e. antisera to salt/ethanol washes of cells expressing the A or B surface antigen (sera provided by J. Forney) (Fig. 1A) or anti-cross-reacting-determinant (CRD) antiserum (Fig. 1B).

GPI-anchored proteins are recognized by an anti-CRD antibody only when the anchor is cleaved because only then is the epitope revealed. The anti-A and anti-B antisera were originally used to identify the serotype of *P. tetraurelia,* i.e. the surface antigen that is expressed on the cells. There are 12 surface antigens, and cells express exclusively only one of the genes (Preer et al., 1981; Schmidt, 1988). Historically, a simple wash of the whole cells with salt and ethanol (Preer et al., 1981) was used to prepare antisera to surface antigens and, since these surface proteins are very immunogenic, the antisera contained a high titer of anti-surface-antigen antibodies. Hence, the antisera could immobilize cells expressing that specific antigen. We will refer to these antisera as anti-A and anti-B because of the cell types that were washed with salt/ethanol to produce them, but they also contain antibodies to proteins other than the surface antigens.

Cells exclusively express only one surface antigen at a time, and we expected that most of the cells grown at 28 °C would express antigen A, the balance expressing antigen B (Beale, 1954; for a review, see Schmidt, 1988). Cells become immobilized when exposed to antiserum prepared against the salt/ethanol washes containing the antigen they are expressing and, using this immobilization test, we verified that most of the cells were expressing antigen A and the balance antigen B (results not shown).

In Fig. 1A, proteins from the pellet or supernatant of PLC-treated or sham-treated pellicle are visualized with a mixture of anti-A and anti-B antisera; in Fig. 1B, the same proteins are visualized with anti-CRD antiserum. In sham-treated pellicles, the large surface antigen remained in the pellet, as did many proteins in the 40–60 kDa range (Fig. 1A, lanes 1 and 2). Treatment with PLC shifted the surface antigen from the pellet to the supernatant, together with other proteins of lower molecular mass (Fig. 1A, lanes 3 and 4). In Fig. 1B, we see that only after PLC treatment is the GPI anchor recognized on proteins and that these reactive proteins are almost exclusively in the supernatant from the PLC treatment. Note that the filled arrows show the surface antigens, which are among the proteins in the supernatant and have a cleaved GPI anchor after PLC treatment. Thus, exogenous PLC does release GPI-anchored proteins from the pellicle. (To show the large surface antigen clearly, we used 8 % gels, which did not provide good

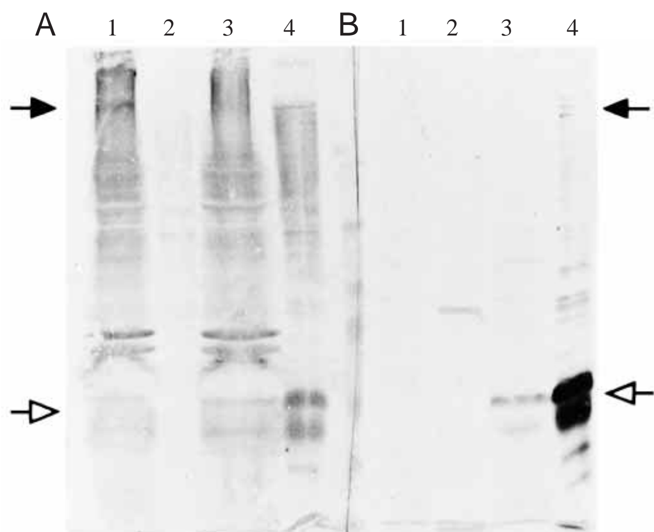

Fig. 1. Phospholipase treatment removes proteins with glycophosphatidylinositol (GPI) anchors from *Paramecium* pellicles. (A) Western blot probed with anti-A and anti-B antisera. Lane 1, pellet of sham-treated pellicle; lane 2, supernatant of sham-treated pellicle; lane 3, pellet of inositol-phosphate-specific phospholipase C (PLC)-treated pellicle; lane 4, supernatant of PLC-treated pellicle. The filled arrow points to surface antigens. The open arrow points to 40–60 kDa proteins. (B) Western blot probed with anti-cross-reacting-determinant (anti-CRD) antiserum, which binds to the cleaved GPI anchor. Lanes as in A. Gradient gels contained 8 % polyacrylamide.

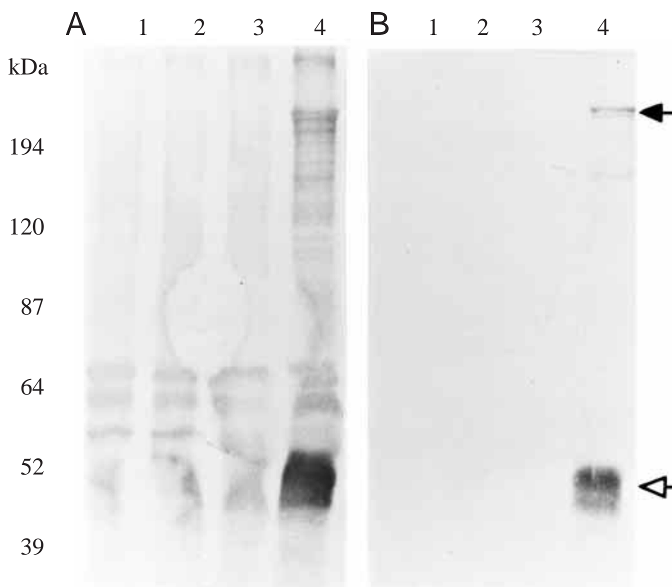

Fig. 2. Proteins removed from pellicle by phospholipase C (PLC) treatment at 37 and 0 °C. (A) Western blot probed with anti-A and anti-B antisera. Lane 1, supernatant of sham-treated pellicle at 0 °C; lane 2, supernatant of PLC-treated pellicle at 0 °C; lane 3, supernatant of sham-treated pellicle at 37 °C; lane 4, supernatant of PLC-treated pellicle at 37 °C. (B) Same supernatants as in A but probed with anti-cross-reacting-determinant (anti-CRD) antiserum. The filled arrow points to surface antigens; the open arrow points to proteins of 40–60 kDa in gradient gels ranging from 6 to 12 % polyacrylamide.

resolution of the lower-molecular-mass proteins. These are better displayed in other figures.)

To demonstrate in a different way that PLC was responsible for the protein release shown in Fig. 1, we incubated pellicles with and without lipase at 0 °C to inhibit any endogenous and exogenous enzymes, and compared this with incubations at 37 °C (Fig. 2). Note in Fig. 2A (lanes 1–3) that proteins are released from the pellicle at both 0 and 37 °C, but that only at 37 °C with PLC (lane 4) are the surface antigens and lower-molecular-mass proteins enriched. Also, only the PLC-cleaved proteins (Fig. 2B, lane 4) show a GPI anchor epitope recognized by the anti-CRD antibody.

Many of the proteins released by salt/ethanol washes are GPI-anchored

Whole cells in sterile culture fluid were washed in a salt/ethanol mixture, as described in Materials and methods. The proteins from the wash were compared with the profiles of proteins released from the cell surface pellicles with PLC using immunoblots with anti-A and anti-B sera.

We have focused on two sets of proteins among those released by PLC and salt/ethanol from the surface of *P. tetraurelia*, i.e. the surface antigens and the cluster of proteins ranging in mass from 40 to 60 kDa. PLC and salt/ethanol treatment release proteins of similar molecular masses and, like the proteins in the salt/ethanol washes, the proteins released by PLC are recognized by anti-A and anti-B antisera (Fig. 3A, lanes 2 and 5) and many are detected with the anti-CRD antiserum that identifies GPI-anchored proteins (Fig. 3B, lanes 2 and 5).

Because some GPI-anchored proteins are liberated with a cleaved anchor epitope, a lipase rather than a protease seems to be active during the release of proteins with the salt/ethanol treatment. Furthermore, inhibition of PLC and the lipases that are active in salt/ethanol reduces the quantities of proteins recognized by anti-CRD antiserum (see below). Therefore, GPI-anchored proteins are among those released by PLC and salt/ethanol treatment, including the surface antigens of *P. tetraurelia*.

To examine whether salt/ethanol treatment solubilized proteins had been cleaved by an endogenous lipase, we included PLC inhibitors in both the exogenous PLC treatment (as control) (Fig. 3A, lanes 3 and 4) and salt/ethanol washes (Fig. 3A, lanes 6 and 7). The PLC inhibitors neomycin (Lipsky and Lietman, 1982), PMSF (Ohia and Jumblatt, 1993), NCDC (Jino et al., 1994; Clark and Garland, 1993) and PCMPSA (Capdeville and Benwakrim, 1996; Low, 1987) were used, but the results with neomycin are not shown because it had no inhibitory effect on protein release. These results are not unexpected since it has been reported that neomycin can actually activate PI-PLC in other cell types (Morris et al., 1996) and has unpredictable effects on the *Tetrahymena* GPI system (Kovács and Csaba, 1996).

Individually, PMSF and NCDC produce no inhibition of cleavage by exogenous PLC (results of individual tests are not shown), but NCDC and PMSF in combination reduce levels of the surface antigen and other proteins released by bacterial PLC treatment of *Paramecium* pellicle (Fig. 3A, lane 2 *versus* lane 3; also see Fig. 5A, lane 1 *versus* lane 2). The effect of

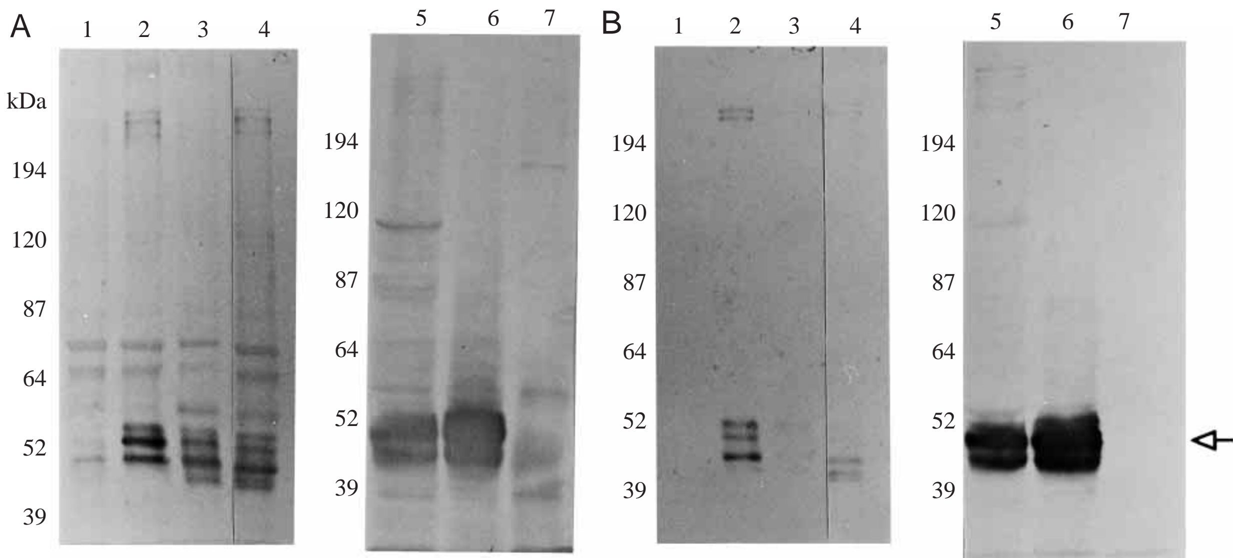

Fig. 3. Different phospholipase C (PLC) inhibitors affect exogenous and endogenous lipase activities. (A) Western blot probed with anti-A and anti-B antisera. Lane 1, supernatant from sham-treated pellicle; lane 2, supernatant from PLC-treated pellicle; lane 3, supernatant from phospholipase C (PLC)-treated pellicle with 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (NCDC) and phenylmethanesulfonyl fluoride (PMSF); lane 4, supernatant of PLC-treated pellicle with *p*-chloromercuriphenylsulfonic acid (PCMPSA); lane 5, salt/ethanol wash proteins; lane 6, proteins from salt/ethanol wash in the presence of NCDC and PMSF; lane 7, proteins from salt/ethanol wash in the presence of PCMPSA. (B) Same as A but probed with anti-cross-reacting-determinant (anti-CRD) antiserum. The open arrow points to 40–60 kDa proteins. Surface antigens (>200 kDa) are best seen in lanes A2, A4, A5 and B2. Other proteins of interest are between 40 and 60 kDa. Gels were 8 % polyacrylamide.

inhibitor on this exogenous PLC is more evident when examining the proteins released with cleaved GPI anchors and, therefore, recognized by the anti-CRD antibodies (Fig. 3B, lane 2 *versus* lane 3). The combination of NCDC and PMSF has little effect on salt/ethanol wash profiles (Fig. 3A,B, lane 5 *versus* lane 6). Thus, the conventional PLC inhibitors affect the exogenously added PLC but not the endogenous lipase of *P. tetraurelia*.

The opposite effect is shown by PCMPSA, which inhibits the endogenous but not the exogenous lipase. This inhibitor affects exogenous PLC treatment profiles of pellicle proteins only slightly (Fig. 3, lane 2 *versus* lane 4). Pellicle proteins released by PLC even in the presence of this inhibitor retain the epitope recognized by anti-CRD antibody, i.e. they have a cleaved GPI anchor (Fig. 3A,B, lanes 4). The inhibitor PCMPSA more effectively reduces, but does not eliminate, the release of proteins from whole cells with a salt/ethanol wash (Fig. 3A, lane 5 *versus* lane 7). Some proteins that are released by a salt/ethanol treatment are recognized by the anti-CRD antiserum, but when the inhibitor PCMPSA is included in the salt/ethanol wash fewer proteins are released (Fig. 3A, lane 5 *versus* lane 7) and they are not recognized by the anti-CRD antibody, i.e. they either have intact GPI anchors or never had one (Fig. 3B, lane 5 *versus* lane 7). Thus, PCMPSA inhibits the endogenous lipase of *P. tetraurelia*, but has little effect on exogenously added bacterial PLC.

Ethanol treatment is known to affect membrane protein function (Schultz et al., 1997), perhaps by solubilization of some membrane proteins. We do not believe solubilization to be the major contributor to the appearance of proteins in the salt/ethanol wash because of the differences between the proteins in Fig. 3A (lanes 6 and 7) and Fig. 3B (lanes 6 and 7). If solubilization and not lipase activity were responsible for the liberation of the membrane proteins, the proteins in lanes 6 and 7 should be the same in quantity and pattern, but they are not. Solubilization would not provide an explanation for the loss of the anti-CRD epitope in Fig. 3B (lane 7). We consider the proteins in Fig. 3A (lane 7) to be those solubilized by the salt/ethanol treatment and to consist of GPI-anchored proteins with the intact anchor attached and peripheral proteins that never had a GPI anchor (Fig. 3B, lane 7). These same proteins will probably be included in lanes 5 and 6 of Fig. 3 as well.

A trivial explanation for the appearance of the surface antigens and 40–60 kDa proteins in the salt/ethanol wash could be the inadvertent deciliation and collection of cilia instead of cleaved proteins in the wash. Ethanol (5 %) is commonly used to deciliate cells, making this a concern. However, the high salt concentration and significantly higher ethanol concentration (15–20 %) used in the salt/ethanol wash appears to fix the cells, and cilia remain attached (Fig. 4). We have also examined our pellicle preparation for contamination with cilia using phase-contrast microscopy. Although we can identify cilia in preparations designed to harvest cilia (Eisenbach et al., 1983), we see only an occasional structure that could be a cilium in the pellicle preparation (data not shown). This result is expected from the low speeds used to pellet sheets of pellicle surface membranes (3000 ***g***).

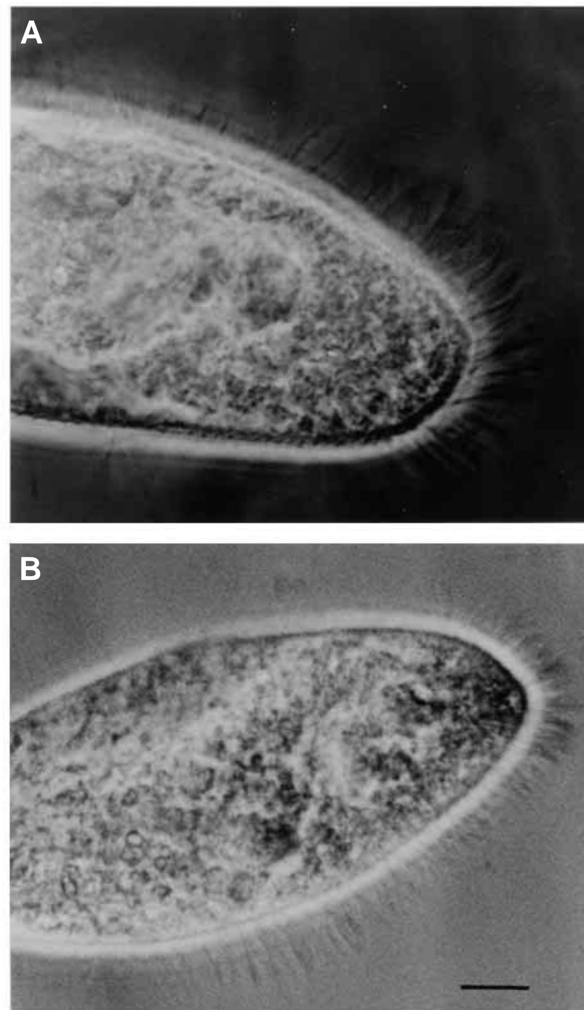

Fig. 4. Phase-contrast micrographs of cells treated with salt/ethanol wash. (A) Typical cell treated with Dryl’s solution before fixation and microscopy. (B) Typical cell treated with salt/ethanol before fixation and microscopy showing no loss of cilia. Scale bar, 10 μm.

Triton X-114 treatment to solubilize GPI-anchored proteins

Glycophosphatidylinositol (GPI)-anchored proteins are notoriously insoluble in Triton X-100, but can be solubilized and analyzed by phase partitioning in Triton X-114 (Ko and Thompson, 1995). Proteins with an intact GPI anchor would be enriched in the detergent phase (approximately 11 %), while those with no anchor or a cleaved anchor would be enriched in the aqueous phase, which still retains some detergent (approximately 0.06 %; Bordier, 1981). *P. tetraurelia* pellicles were extracted with Triton X-114, the extracts were separated into detergent and relative aqueous phases, and the proteins were analyzed by SDS–PAGE and western blotting using anti-A and anti-B antisera (Fig. 5A) and anti-CRD antiserum (Fig. 5B). Fig. 5 shows the proteins released by PLC treatment of pellicle membranes without (Fig. 5A,B, lanes 1) and with (Fig. 5A,B,

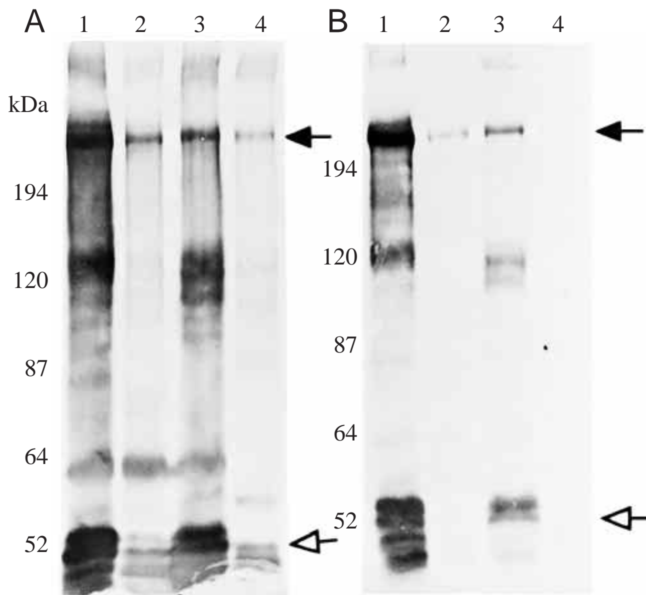

Fig. 5. Triton X-114 extraction solubilizes glycophosphatidylinositol (GPI)-anchored proteins. (A) Western blot probed with anti-A and anti-B antisera. Lane 1, supernatant from phospholipase C (PLC)-treated pellicle; lane 2, supernatant from PLC-treated pellicle in the presence of 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate and phenylmethanesulfonyl fluoride; lane 3, aqueous phase of Triton X-114 extraction from phase separation of pellicle proteins; lane 4, proteins from the detergent phase of Triton X-114 extraction and phase separation of pellicle. (B) Anti-cross-reactive-determinant developed electroblot of the same samples as in A. The filled arrow points to surface antigens, and the open arrow to 40–60 kDa proteins. 8 % polyacrylamide gels were used.

lanes 2) PLC inhibitors for comparison with the aqueous and detergent phases of the Triton X-114 extraction and phase separation without PLC treatment (Fig. 5A,B, lanes 3 aqueous, lanes 4 detergent phase, respectively). Note that the profile of proteins released by PLC is similar to that (focusing on surface antigens and the 40–60 kDa range) of the relative aqueous phase of the Triton X-114 extract whether probed with anti-A and anti-B or anti-CRD antiserum (Fig. 5A,B, lanes 1 *versus* lanes 3). Much less protein is typically found in the detergent phase than in the aqueous phase (Fig. 5A, lane 3 *versus* lane 4). Notably, there is less surface antigen in the detergent phase.

Anti-CRD antiserum recognizes only the cleaved GPI-anchored proteins and, therefore, it was expected that hydrophobic proteins with intact GPI anchors would be found in the detergent phase and would not react with the anti-CRD antiserum. However, we found little of the surface antigen in the detergent phase as detected by the anti-A and anti-B antisera (Fig. 5A, lane 4), and none of it appeared to react with the anti-CRD antibody (Fig. 5B, lane 4). It was unexpected that so many of the GPI-anchored proteins should be found in the aqueous phase, with GPI anchors cleaved (Fig. 5A,B, lanes 3). These Triton X-114 extraction studies provide new information about the surface proteins of *Paramecium*, and of *P. tetraurelia* in particular.

Integral membrane proteins are not among the GPI-anchored proteins

A surface membrane protein, the Ca^{2+} pump, should be an integral membrane protein (Wright and Van Houten, 1990; Elwess and Van Houten, 1997) and, therefore, not among the GPI-anchored proteins liberated by PLC, by a salt/ethanol wash or in the Triton X-114 aqueous phase. The cyclic AMP chemoreceptor is readily soluble in Triton X-100 and is also likely to be an integral membrane protein (Van Houten et al., 1991). We examined the supernatant from PLC treatment of pellicle and the salt/ethanol wash for these proteins using antibodies to the C terminus of the Ca^{2+} pump and the N-terminal sequence of the cyclic AMP receptor (see Materials and methods). A bacterially expressed protein of approximately 10 kDa of the C terminus of the Ca^{2+} pump fused with GST and the GST fusion protein from the N terminus of the cyclic AMP receptor were used as positive controls for the antisera in western blots (Fig. 6). The anti-A and anti-B antisera reacted with an array of bands in the PLC supernatant, but not with the fusion proteins (Fig. 6A, lane 1 *versus* lanes 2 and 3).

The antiserum against the Ca^{2+} pump peptide and cyclic AMP receptor fusion protein do not react with any proteins from PLC- or salt/ethanol-treated cells (Fig. 6B, lanes 1 and 2; Fig. 6C, lanes 1 and 3), indicating that these integral membrane proteins are probably not present in the PLC supernatant or salt/ethanol wash. Similarly, proteins in the supernatants from the sham- and PLC-treated pellicles do not react with anti-Ca^{2+}-pump (Fig. 6B, lanes 1 and 2) or anti-cyclic-AMP receptor (Fig. 6B, lanes 3 and 4) antiserum. Even though sufficient protein is present to produce a strong reaction with anti-CRD antibodies (Fig. 6B, lane 6).

Proteins from salt/ethanol washes also fail to react with anti-Ca^{2+}-pump and anti-cyclic-AMP receptor antisera (Fig. 6C, lanes 1 and 3) on western blots, even though sufficient protein is present to produce a reaction with anti-A and anti-B antisera (Fig. 6C, lane 2).

Positive and negative controls for the antiserum against the Ca^{2+} pump and cyclic AMP receptor are shown in Fig. 6A. The anti-A and anti-B antisera recognize proteins in the supernatant of PLC-treated cells (Fig. 6A, lane 1), but the anti-Ca^{2+}-pump and anti-cyclic-AMP-receptor antisera do not recognize proteins from the supernatant (Fig. 6A, lanes 2 and 3). In contrast, the anti-Ca^{2+}-pump and anti-cyclic-AMP-receptor antisera recognize their antigens because the anti-Ca^{2+}-pump polyclonal antiserum reacts with the Ca^{2+} pump C terminus fusion protein (filled arrow, Fig. 6A, lane 4), and the anti-cyclic-AMP-receptor polyclonal antiserum reacts with the receptor fusion protein (open arrow, Fig. 6A, lane 5). In addition, Fig. 6B (lane 6) demonstrates that there is sufficient protein in the PLC supernatant for recognition by anti-CRD antibody. Likewise, Fig. 6C (lane 2) shows that there is sufficient protein in the salt/ethanol wash to be recognized by the anti-A and anti-B antisera. Therefore, low antibody titers or insufficient protein loaded on the gels cannot account for the lack of recognition of the Ca^{2+} pump (133 kDa) or the

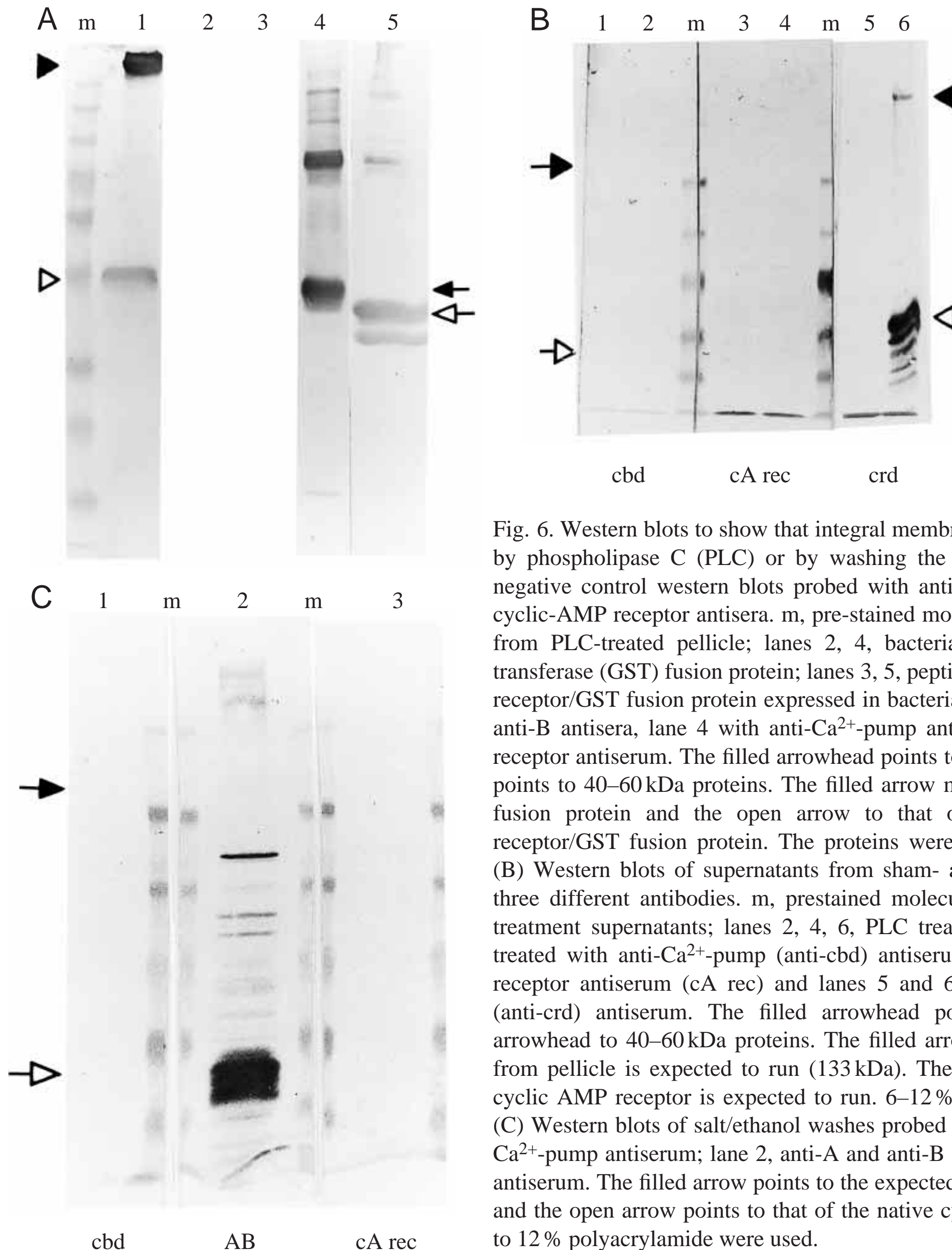

Fig. 6. Western blots to show that integral membrane proteins are not among those released by phospholipase C (PLC) or by washing the cells with salt/ethanol. (A) Positive and negative control western blots probed with anti-A and anti-B, anti-Ca^{2+}-pump and anti-cyclic-AMP receptor antisera. m, pre-stained molecular mass markers. Lane 1, supernatant from PLC-treated pellicle; lanes 2, 4, bacterially expressed Ca^{2+} pump/glutathione-*S*-transferase (GST) fusion protein; lanes 3, 5, peptide from the N terminus of the cyclic AMP receptor/GST fusion protein expressed in bacteria. Lanes 1–3 were treated with anti-A and anti-B antisera, lane 4 with anti-Ca^{2+}-pump antiserum and lane 5 with anti-cyclic-AMP receptor antiserum. The filled arrowhead points to surface antigen, and the open arrowhead points to 40–60 kDa proteins. The filled arrow marks the position of the Ca^{2+} pump/GST fusion protein and the open arrow to that of the N terminus of the cyclic AMP receptor/GST fusion protein. The proteins were separated on 12 % polyacrylamide gels. (B) Western blots of supernatants from sham- and PLC-treated pellicles developed with three different antibodies. m, prestained molecular mass markers. Lanes 1, 3, 5, sham-treatment supernatants; lanes 2, 4, 6, PLC treatment supernatants. Lanes 1 and 2 were treated with anti-Ca^{2+}-pump (anti-cbd) antiserum, lanes 3 and 4 with anti-cyclic-AMP-receptor antiserum (cA rec) and lanes 5 and 6 with the anti-cross-reactive-determinant (anti-crd) antiserum. The filled arrowhead points to surface antigen, and the open arrowhead to 40–60 kDa proteins. The filled arrow points to where the intact Ca^{2+} pump from pellicle is expected to run (133 kDa). The open arrow points to 48 kDa, where the cyclic AMP receptor is expected to run. 6–12 % gradient polyacrylamide gels were used. (C) Western blots of salt/ethanol washes probed with three different antisera. Lane 1, anti-Ca^{2+}-pump antiserum; lane 2, anti-A and anti-B antisera; lane 3, anti-cyclic-AMP-receptor antiserum. The filled arrow points to the expected molecular mass of the native Ca^{2+} pump, and the open arrow points to that of the native cyclic AMP receptor. Gradient gels from 6 to 12 % polyacrylamide were used.

cyclic AMP receptor (48 kDa) among the proteins of the PLC supernatant and salt/ethanol wash by the anti-Ca^{2+}-pump and anti-cyclic-AMP-receptor antisera in Fig. 6B (lanes 2 and 4) and Fig. 6C (lanes 1 and 3).

In another set of positive controls (not shown), we demonstrated that the antisera can detect the Ca^{2+} pump and cyclic AMP receptor proteins in the whole pellicle at the dilutions used in the experiments shown in Fig. 6. We found that the antiserum against the Ca^{2+} pump reacts specifically with a protein with a molecular mass of approximately 133 kDa, while the antiserum against the cyclic AMP receptor reacts specifically with a protein of molecular mass 48 kDa in pellicle. We determined specificity by observing the disappearance of bands when the antiserum was pre-incubated with the antigens against which they had been made.

T-maze tests of cells treated with anti-A and anti-B antisera

The salt/ethanol wash of cells clearly contains many GPI-anchored proteins in addition to the surface antigen, because many proteins that react with the anti-A and anti-B antisera also react with the anti-CRD antiserum. It is unlikely that integral proteins are found among the salt/ethanol wash proteins, except as a result of proteolytic cleavage. Therefore, we hypothesized that the anti-A and anti-B antisera could contain blocking antibodies to the surface chemoreceptors that

Table 1. *T-maze assays after treatment with antisera to the A and B surface antigens*

	T-maze responses			
Treatment	Potassium-L-glutamate	Disodium folate	Sodium acetate	NH_4Cl
Buffer	0.76±0.13	0.81±0.05	0.75±0.05	0.89±0.07
Antisera	0.59±0.13	0.36±0.13*	0.67±0.11	0.84±0.09

Values are means ± S.D. (N=8–12).

*Statistically significantly different from the control (Mann–Whitney U-test).

A T-maze result of 0.5 indicates neutrality, a value greater than 0.5 indicates attraction, and a value less than 0.5 indicates repulsion.

All chemicals were tested at 5 mmol l^{-1} in chemokinesis buffer. Antiserum or pre-immune serum (4 or 8 μl ml^{-1} buffer) was used to pre-treat the cells.

govern attraction to stimuli such as acetate, folate, glutamate, cyclic AMP and biotin if these receptors were peripheral or GPI-anchored. All these stimuli appear to bind to specific sites, probably receptors, at the cell surface to mediate attraction, while ammonium does not (Van Houten, 1994). We therefore tested whether anti-A and anti-B antisera mixtures could block the chemoresponse in mutants lacking the A and B antigens (courtesy of J. Forney). These cells would not be immobilized by the antisera, but might show behavioral defects if their chemoreceptors to specific stimuli were blocked by the antisera. As shown in Table 1, the antisera produce significant inhibition of the response to folate, while the responses to acetate and ammonia were unaffected, showing that this is not a general effect on motility in the T-mazes. The A^-B^- cells show a definite attraction, but a more variable response, to glutamate than wild-type cells (index of chemokinesis, I_{che}, 0.70±0.07, mean ± S.D., N=8–12). This variability makes it difficult to determine whether the decrease in glutamate I_{che} with antibody treatment indicates that a GPI-anchored protein is involved in the glutamate chemoresponse. Responses to cyclic AMP and biotin (not shown) are affected, but only at higher concentrations of the antisera than are required to block the response to folate or glutamate, and the response to biotin is only partially reduced.

Antisera to a mammalian GPI-anchored folate-binding protein

Mammalian folate-binding proteins are GPI-anchored and mediate the uptake of folate into cells (Antony, 1996). An antiserum to bovine folate-binding protein recognized one protein band of approximately 37 kDa among salt/ethanol wash proteins (Fig. 7, lane 1). Probing of blots with anti-CRD antiserum shows a band of similar size among the GPI-anchored proteins on the blot (Fig. 7, lane 2). (Repeated use of anti-folate-binding-protein antiserum with salt/ethanol washes has confirmed that one protein of approximately 37 kDa is recognized.)

Subsequent use of this antiserum in a pre-incubation of the cells before T-maze tests blocks the chemoresponse to folate (Table 2). Chemoresponses to other stimuli do not appear to be affected by the antiserum treatment.

Fig. 7. Western blot of salt/ethanol wash proteins showing a band reactive with antiserum against a glycophosphatidylinositol (GPI)-anchored folate receptor and reactive with anti-cross-reactive-determinant antiserum. Lane 1, treated with anti-folate-binding-protein antiserum. The protein band at the arrow has an approximate molecular mass of 37 kDa. Lane 2, treated with anti-cross-reactive-determinant antiserum. Note that a band at the same molecular mass, 37 kDa, is recognized. Gels contained 12 % polyacrylamide.

Discussion

We have attempted to build a progressive argument for the presence of GPI-anchored proteins on the surface of *Paramecium tetraurelia* by examining the effects of PLC and other treatments on the cells. Exogenous PLC releases proteins from the surface, including the large surface antigens, that are known to be GPI-anchored in *P. primaurelia* (Capdeville and Benwakrim, 1996). Inhibitors of PLC (Fig. 2, Fig. 3) and also low temperature (Fig. 2) reduce the release of these proteins by PLC, and the anti-CRD antiserum also reacts with many of these proteins (Fig. 3). These are solid indications that GPI-anchored proteins, which include the surface antigen, reside on the surface of *P. tetraurelia.*

As anticipated from work on *P. primaurelia* (Capdeville et al., 1987), the traditional method of harvesting the surface antigens for making antisera using a salt/ethanol wash of the cells also releases an array of proteins (particularly proteins of 40–60 kDa) that react with the anti-CRD antiserum (Fig. 3). These proteins can be slightly reduced in quantity using the same lipase inhibitors that inhibit the exogenous PLC used in

Table 2. *T-maze results for cells treated with antiserum against a GPI-anchored folate-binding protein*

Treatment	T-maze responses			
	Potassium-L-glutamate	Disodium folate	Sodium acetate	NH_4Cl
Sham treatment	0.80±0.08	0.79±0.08	0.85±0.08	0.89±0.05
Folate-binding protein antiserum	0.84±0.07	0.57±0.08*	0.83±0.11	0.89±0.06

Values are means ± S.D. (N=9–19).
*Statistically significantly different from the control (Mann–Whitney U-test).
All chemicals were tested at 5 mmol l^{-1} in chemokinesis buffer, except folate, which was at 1 mmol l^{-1} with control buffer at 2 mmol l^{-1}.
GPI, glycophosphatidyl inositol.
See Table 1 for further details.

these experiments (Fig. 3, Fig. 4), and many of these proteins show reactivity with the anti-CRD antiserum. However, another PLC inhibitor (PCMPSA), which does not greatly affect exogenous PLC, reduces the amount of protein in the wash and inhibits cleavage of the GPI anchor. These proteins of 40–60 kDa are not simply degradation products of the surface antigens; they cross-react with antisera from cells with surface antigens other than A and B (Eisenbach et al., 1983) and, in addition, salt/ethanol washes of A^-B^- cells show this same array of proteins when visualized with anti-A and anti-B antisera (C. A. Paquette, V. Rakochy, A. Bush, and J. L. Van Houten, unpublished observations).

We demonstrated by examining cells sham-treated in Dryl's solution and treated with salt/ethanol mixtures that the appearance of the surface antigen in the salt/ethanol washes of cells was not the result of accidentally harvesting cilia, which are enriched in membrane and the surface antigen proteins. We found that the cells from both treatments appeared intact and there was no measurable loss of cilia, as determined by visual inspection of fixed cells using phase microscopy (Fig. 4).

It was anticipated from the work of Capdeville et al. (Capdeville et al., 1993) on ciliary proteins of *P. primaurelia* that an endogenous lipase could be activated by salt/ethanol treatment. However, we have demonstrated here that, for *P. tetraurelia,* not only are more proteins with cleaved GPI anchors found in salt/ethanol washes than in buffer washes but that, upon Triton X-114 extraction and phase separation, the GPI-anchored proteins are found primarily in the aqueous phase with their anchors cleaved. It is interesting that constitutive endogenous lipases are found in other systems, such as in trypanosomes (McConville and Ferguson, 1993). It appears, therefore, either that both salt/ethanol and Triton X-114 activate a surface lipase or that *P. tetraurelia* also has a constitutive lipase. At present, we are unable to distinguish between these possibilities.

The anti-A and anti-B antisera produced against salt/ethanol washes of cells react with proteins that are liberated from the cell surface with exogenous PLC or with salt/ethanol washes (Fig. 3). Subsets of these proteins (particularly the surface antigens and 40–60 kDa proteins) react with the anti-CRD antiserum (Fig. 3). While this is far from proof of congruence between the two protein profiles, it is at least suggestive that the anti-A and anti-B antisera react with many GPI-anchored proteins. If there are other proteins present in the salt/ethanol wash of whole cells, and hence available as antigens for the antisera, they are likely to be peripheral proteins and not integral membrane proteins. We reasoned that the anti-A and anti-B antisera could contain blocking antibodies for chemoreceptors and found that these antisera block chemoresponse behavior in T-mazes, but not responses to all stimuli. The response to ammonium, which we believe crosses the membrane without the assistance of a receptor (Davis et al., 1998), is not affected by antiserum treatment, while the responses to folate and glutamate are always reduced to neutral or near neutral (Table 1). Other responses that we believe are membrane-receptor-mediated either are not affected (as in the case of acetate) or are affected only at high antiserum concentrations (cyclic AMP) and to a lesser extent (biotin) (data not shown). It is possible that these last cases are indicative of receptors that are not GPI-anchored and that their receptors are not among the proteins found in the salt/ethanol wash. Alternatively, the antibody titers may be too low to have a blocking effect on these specific receptors. These possibilities cannot be resolved until we purify the receptors. The cyclic AMP receptor, a Triton-X-100-soluble protein, has been purified to homogeneity, and antibodies against it do not react with any of the proteins in blots of salt/ethanol washes, supporting the view that this receptor is not a GPI-anchored receptor (Fig. 6).

The antiserum against a bovine GPI-anchored, folate-binding protein is better defined than the anti-A and anti-B sera and provides better evidence for the participation of a GPI-anchored protein in chemoresponses. A single protein from salt/ethanol washes is the primary protein recognized by this antiserum. Moreover, a protein that shows similar migration on gels is reactive with the anti-CRD antibody, indicating that it is GPI-anchored. The same antiserum very specifically and thoroughly blocks the chemoresponse to folate in T-mazes. While these data are not conclusive, they nonetheless suggest that the *P. tetraurelia* chemoreceptor for folate is a GPI-anchored protein.

Previous observations have been made (Ramanathan et al., 1981; Ramanathan et al., 1983; Eisenbach et al., 1983) about

the effects of antisera against *P. tetraurelia* ciliary surface proteins, including the surface antigen and a cluster of proteins of lower molecular mass. There are some distinct differences between these earlier observations and those presented here. We have compared proteins from the pellicle (cell body surface membranes) and whole-cell salt/ethanol washes, while the previous studies focused on ciliary membrane proteins. Antisera in the previous studies were prepared against these ciliary proteins using membrane preparations as antigens rather than salt/ethanol washes (Ramanathan et al., 1983; Eisenbach et al., 1983). Nevertheless, there are distinct similarities between the pattern of proteins cleaved by PLC treatment of pellicle and salt/ethanol washes of whole cells and these earlier protein profiles. In short, we are probably observing many of the same proteins using different antisera. Insight into GPI-anchored proteins also helps to explain the effects of the antisera against ciliary surface proteins on a membrane Ca^{2+} conductance (see below).

The recent cloning by complementation of a subunit of the Ca^{2+} channel of *P. tetraurelia* introduced the possibility of further roles for GPI-anchored proteins (Haynes et al., 1998). This subunit appears to be GPI-anchored, on the basis of its deduced amino acid sequence. A trivial explanation for the absence of chemoresponse to chemical stimuli could be the inhibition of this channel rather than blockade of chemoreceptors by the anti-A and anti-B antisera, since an ability to reverse swimming direction in response to Ca^{2+}-based action potentials is required for chemoresponsiveness (Van Houten, 1978). Inhibition of the Ca^{2+} channel would cause a global effect on attraction chemoresponses. However, since the inhibition of chemoresponses by the antisera is selective, and not all responses to stimuli are affected, as would be expected if the Ca^{2+} channel were inhibited, our results suggest a more specific effect of the anti-A and anti-B antisera on receptors.

An alternative approach to demonstrating that the chemoreceptors are GPI-anchored would be to treat whole cells with PI-PLC. The volumes that would be needed for pre-treatment of the cells before T-maze assays make this prohibitively expensive, and this approach might fail if the PLC treatment removed many, but not all, of the GPI-anchored proteins. Considering the preponderance of the large surface antigens, especially on the cilia, we would predict that most of the proteins removed in partial digestions would be the surface antigens. Indeed, incubation of whole cells in PLC is not sufficient to remove enough surface antigen to protect the cells from immobilization by anti-A and anti-B antisera (J. L. Van Houten, unpublished observations).

The strongest inhibition of the chemoresponse by the antisera is of the folate chemoresponse. In the past, we have found that the folate-binding proteins on the surface of *P. tetraurelia* are difficult to solubilize in non-ionic detergents, which is a characteristic of GPI-anchored proteins (Sasner and Van Houten, 1989). Interestingly, the folate-uptake-binding proteins (folate receptors) of mammalian cells are GPI-anchored (Antony, 1996; Rothberg et al., 1990). If the *P. tetraurelia* folate chemoreceptor is GPI-anchored, its signal transduction pathway must include other membrane proteins that interact with the GPI-anchored protein and rapidly signal to the interior of the cell (uptake through potocytosis or other endocytotic pathways would be too slow). It will be interesting to look for these interacting proteins among the other proteins of the surface membranes.

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