A new class of *Paramecium* surface proteins anchored in the plasma membrane by a glycosylinositol phospholipid

Membrane anchor of *Paramecium* cross-reacting glycoproteins

Christiane DEREGNAUCOURT, Anne-Marie KELLER and Yvonne CAPDEVILLE

Centre de Génétique Moléculaire, Département 1, Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France

Treatment of paramecia with ethanol or Triton X-100 solubilizes a major membrane protein, namely the surface antigen (SAG), and a set of glycopeptides in the range 40–60 kDa, which cross-react with the SAG. We demonstrate that these glycopeptides, called 'cross-reacting glycoproteins' (CRGs), are distinct molecules from the SAG. First, after purification of CRGs from ethanolic extracts of *Paramecium primaurelia* expressing the 156G SAG, the amino acid composition of a given CRG was found to be different from, and incompatible with, that of the 156G SAG. Secondly, we showed that the CRGs, although not immunologically detectable, are present in fractions containing the myristoylated form of the 156G SAG. The treatment of these fractions by phosphatidylinositol-specific phospholipases C enables us to reveal the CRGs through the unmasking of two distinct epitopes. One is the 'cross-reacting determinant' (CRD), initially described for the variant surface glycoproteins (VSGs) of *Trypanosoma*; the other determinant, called 'det-2355', is specific to the SAG and to the CRGs. Our results suggest that (1) phosphatidylinositol is covalently linked to the CRGs and (2) the CRD and the det-2355 are localized in the same region of the CRGs. We propose that the CRGs are a new set of surface proteins anchored in the cell membrane of *Paramecium* via a glycosylinositol phospholipid, in the same way as the SAGs.

INTRODUCTION

The cell coat of *Paramecium* is mainly composed of a 250–300 kDa glycoprotein called 'surface antigen' (SAG) (Preer, 1959b; Steers & Davis, 1977) that undergoes antigenic variation. Biochemical and immunological studies (Capdeville *et al.*, 1985) have demonstrated the existence of two forms for a given antigen: a soluble (sSAG) and a membrane-bound form (mSAG). The mSAG is myristoylated and anchored in the plasma membrane via a phosphatidylinositol (Capdeville *et al.*, 1987). The appearance of the sSAG is mediated by an endogenous phospholipase C-like hydrolase (Capdeville *et al.*, 1987), which is activated when cells are incubated in ethanolic solution or in Triton X-100. The conversion from the mSAG into the sSAG form can be inhibited by *p*-chloromercuriphenylsulphonic acid (pCMPSA), a compound also known to be an inhibitor of the phosphatidylinositol-specific phospholipases C (PI-PLCs) from *Trypanosoma brucei* (Bülow & Overath, 1985) and *Bacillus cereus* (Ikezawa & Taguchi, 1981).

After lipid cleavage by the endogenous phospholipase C, or by the PI-PLCs from *B. cereus* and *T. brucei*, a particular antigenic determinant, partly glycosidic and called the 'cross-reacting determinant' (CRD), becomes accessible to specific antibodies in the soluble form of the SAG (Capdeville *et al.*, 1986). The CRD was first characterized (Barbet & MacGuire, 1978) in the soluble form of the variant surface glycoproteins (VSGs) of *Trypanosoma* (Cardoso de Almeida & Turner, 1983).

In the course of the present study we observed that, when ethanolic or Triton extracts containing the soluble form of SAG are reduced by β -mercaptoethanol, a serum

raised against a purified SAG recognizes on immunoblots not only the SAG, but also two bands of lower molecular mass (in the range 45–50 kDa). The bands were first (Capdeville *et al.*, 1985) called 'cross-reacting light chains' (CRLCs). The cross-reaction observed between sSAG and CRLCs was shown to involve at least two determinants accessible only on the reduced molecules: the CRD, and one or several determinant(s) called 'det-2355', recognized by the serum 2355 (AS 2355) raised against a purified soluble G antigen and free of anti-CRD antibodies. Since it has been demonstrated (Capdeville *et al.*, 1985) that the CRLCs are not S–S-bond-linked subunits of the SAG, the name 'cross-reacting light chains' was no longer suitable, and we rebaptized them 'cross-reacting glycoproteins' (CRGs).

The CRGs are not recognized on immunoblots of reduced ethanolic or Triton extracts prepared in the presence of pCMPSA. The question arose as to whether they were fragments of SAG arising from proteolytic events taking place after the conversion process (mSAG $\rightarrow$ sSAG) or membrane proteins distinct from the SAG. The results of our biochemical and immunological studies performed on 156G CRGs (CRGs present in the strain 156 of *P. primaurelia* expressing the G antigen) allow us to answer this question and provide new information on the structure of the CRGs.

MATERIALS AND METHODS

Growth of *Paramecium primaurelia*

Cells from the geographical strain 156 of *P. primaurelia* (Sonneborn, 1975) expressing the surface antigen G were

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; (m or s)SAG, (membrane-bound or soluble) surface antigen; pCMPSA, *p*-chloromercuriphenylsulphonic acid; PI-PLCs, phosphatidylinositol-specific phospholipases C; CRD, cross-reacting determinant; VSGs, variant surface glycoproteins; CRLCs, cross-reacting light chains; CRGs, cross-reacting glycoproteins; IDC, incompletely demembrated cilia.

grown at 24 °C in hay-grass medium supplemented with β -sistosterol (0.8 mg/l). Cultures and identification of the expressed SAg were performed as previously described (Capdeville, 1971).

Cellular fractions

Cellular ethanolic extractions leading to the solubilization of the soluble form of the SAg were performed as described by Preer (1959a). When indicated, the following cocktail of proteinase inhibitors was added: 1 mM-EDTA (Merck), 1 mM-EGTA (Merck), 1 mM-*o*-phenanthroline (Sigma), 1 mM-iodoacetamide (Sigma), 1 mM-*N* α -*p*-tosyl-L-lysylchloromethane (Sigma), 0.05 mM-leupeptin (Sigma), 0.28 mM-phenylmethanesulphonyl fluoride (Sigma) and 0.26 mM-*N* α -*p*-tosyl-L-arginine methyl ester (Sigma). Ethanolic extractions leading to the solubilization of the membrane form of the SAg were performed in the presence of 1 mM-pCMPSA (Sigma).

The preparation of incompletely demembrated cilia was by the method of Hansma & Kung (1975) modified by Adoutte *et al.* (1980).

Purification of 156G CRGs

The purification of the CRGs was based on an extension of the technique described by Preer (1959a) for the purification of sSAg. The pH of an ethanolic extract from strain-156 cells expressing the G antigen was adjusted to 2.0 with 0.2 M-HCl, and the precipitate was removed by centrifugation at 25000 *g* for 5 min at 4 °C. The pH was raised to 7.0 with 0.2 M-NaOH and the precipitate again removed. Fractional precipitation with solid (NH₄)₂SO₄ was then carried out such as to achieve the following desired levels of saturation: 30, 40, 50, 60, 80 and 100%. The 60–80%-(NH₄)₂SO₄ pellet was redissolved in 50 mM-Tris/5 mM-EDTA, pH 7.5, and extensively dialysed against the same buffer. It was then subjected to h.p.l.c. at 30 ml/h on a 4.15 ml DEAE IEX 540 column (Beckman) equilibrated with 100 mM-Tris, pH 7.3, at room temperature. The CRGs were eluted from the anion-exchange column with a linear gradient of NaCl (0–0.5 M). Absorbance was measured at 280 nm.

Amino acid composition of 156G CRG2

Protein hydrolysis was carried out at 110 °C in 5.7 M-HCl/0.1% phenol in evacuated sealed tubes. Amino acid composition was determined as described by Lederer *et al.* (1983). The amounts analysed were of the order of 1.6 nmol.

Protein electrophoresis

One-dimensional SDS/PAGE using 5–15%-(w/v)-polyacrylamide gradient slab gels was performed as described by Laemmli (1970). For electrophoresis of non-reduced proteins, β -mercaptoethanol was omitted from the denaturation buffer. Gels were silver-stained (Gelcode color silver stain kit; Pierce Chemical Co., Rockford, IL, U.S.A.). Molecular-mass standards (Bio-Rad) were used. Protein concentrations were determined by using the BCA Protein Assay Reagent (Pierce). Lanes were loaded as follows: 40–60 μ g of protein for ethanolic cellular extracts; 20–40 μ g for 60–80%-satd.-(NH₄)₂SO₄ pellets; about 1 μ g for purified CRG2 and CRG3.

Protein immunoblots

Immunoblotting was performed as described by Towbin *et al.* (1979) with slight modifications (Capdeville *et al.*, 1985). The serum 2355 (raised against the purified 156G sSAg in the denatured state) was diluted 100-fold, and the purified anti-CRD antibodies (3 mg/ml) were diluted about 500-fold. These purified antibodies to the CRD of *Trypanosoma brucei* were a gift from Dr. L. Cardoso de Almeida (Escola Paulista de Medicina, São Paulo, Brazil). Peroxidase-conjugated sheep anti-rabbit IgGs (Institute Pasteur, Paris, France) were used at the dilution 1:200, with 4-chloro-1-naphthol (Sigma) as a chromogenic agent.

PI-PLC treatment of blots

After saturation with bovine serum albumin, the nitrocellulose sheets were incubated for 2 h at 37 °C in 10 mM-Tris/150 mM-NaCl, pH 7.3, in the presence of 2% (w/v) Triton X-100 and proteinase inhibitors (0.25 mM phenylmethanesulphonyl fluoride, 0.01 mM-leupeptin, 1 mM-EDTA). When indicated, PI-PLC (type III) from *Bacillus cereus* (Sigma) was added to a final concentration of 2 units/ml. The blots were then washed three times for 10 min with phosphate-buffered saline (10 mM-KH₂PO₄/0.15 M-NaCl), pH 7.5, before incubation with the given antibodies.

Fig. 1. Effect of a 'cocktail' of proteinase inhibitors on the appearance of the CRGs

Whole cells expressing the 156G SAg were submitted to ethanolic extraction in the presence (+) or in the absence (-) of proteinase inhibitors and the extract was electrophoresed in a 5–15% (w/v) gradient polyacrylamide gel with (+) or without (-) proteinase inhibitors in the sample buffer. After transfer, the nitrocellulose filter was allowed to react with the antiserum 2355 (anti-156G sSAg). sSAg, 156G sSAg migrating at 300 kDa; CRGs, 156G cross-reacting glycoproteins, with two major bands migrating in the range of 45–50 kDa.

Fig. 2. Purification of the CRGs

Elution profile of a 156G 60–80 %-satd.-(NH₄)₂SO₄ pellet by high performance ion-exchange chromatography (DEAE IEX 540 column). The numbers above the abscissa indicate the collected elution fractions.

RESULTS

Proteinase inhibitors do not prevent the appearance of the CRGs during ethanolic extraction

In order to check whether the CRGs are proteolytic fragments of the SAg, ethanolic extracts of whole cells from strain 156 expressing the G antigen (156G strain) were performed in the presence of a cocktail of metallo-, thiol- and serine-proteinase inhibitors. Proteinase inhibitors were added during the extraction and/or during SDS solubilization of the samples. The corresponding reduced samples were analysed by SDS/PAGE, blotted on to nitrocellulose and the filters allowed to react with an antiserum (AS 2355) raised against the purified 156G sSAg. Fig. 1 shows that the CRGs, in addition to the SAg, are recognized by the anti-sSAg antibodies in every sample, whether inhibitors were added or not.

Purification of the CRGs

An ethanolic cellular extract from 156G strain, prepared in the presence of proteinase inhibitors, was submitted to sequential precipitations (see the Materials and methods section). The CRGs were shown to precipitate between 60% and 80% saturation of (NH₄)₂SO₄. Immunoblotting analysis of a reduced 60–80 %-satd.-(NH₄)₂SO₄ pellet with AS 2355 showed several bands (at least four) in the 40–60 kDa range (results not shown). The high protein concentration in the pellet is responsible for the observation of several bands.

The dialysed 60–80 %-satd.-(NH₄)₂SO₄ pellet was then subjected to h.p.l.c. on a DEAE column. Each of the CRGs was excluded from the DEAE column at a different concentration of NaCl (Fig. 2). SDS/PAGE analysis of the elution fractions 13 and 17, respectively containing CRG2 and CRG3, the two

Fig. 3. SDS/PAGE of purified CRG2 and CRG3

SDS/PAGE was performed by using a 5–15% gradient polyacrylamide gel and silver staining was by the Gelcode procedure. The samples are non-reduced (NR). A, h.p.l.c. elution fraction no. 17; B, h.p.l.c. elution fraction no. 13; C, 156G 60–80 %-satd.-(NH₄)₂SO₄ pellet. The values on the right correspond to molecular-mass standards. The 'continuous' band at 70 kDa is probably due to a contaminant of the sample buffer or of the electrophoresis buffer.

CRGs usually detected in a 156G ethanolic extract, was performed to test their degree of purification. Results are presented in Fig. 3. Only the band corresponding to

Table 1. Amino acid composition of 156G CRG2

The purified 156G CRG2 was hydrolysed in 5.7 M-HCl at 110 °C *in vacuo* and the amino acid composition was analysed. Data are compared with those for the 156G SAg.

Amino acid	Amino acid composition (residues/100 residues)	
	156G CRG2	156G SAg
Half-cystine	2.59	11.4
Aspartic acid	12.7	11.6
Threonine	8.96	16.5
Serine	9.47	8.87
Glutamic acid	13.2	2.99
Proline	3.36	1.75
Glycine	6.96	7.34
Alanine	8.5	13.4
Valine	5.29	4.92
Methionine	0.37	0.45
Isoleucine	4.18	2.8
Leucine	6.18	4.39
Tyrosine	3.0	3.2
Phenylalanine	2.9	1.69
Histidine	1.0	0.72
Lysine	8.0	6.8
Arginine	2.45	1.4

CRG2 appears in elution fraction 13, the band corresponding to CRG3 appearing in fraction 17.

Amino acid composition of 156G CRG2

The purified 156G CRG2 was analysed for amino acid composition (Table 1) and compared with the amino acid composition of 156G SAg [composition deduced (Prat *et al.*, 1986) from the 156G gene sequence]. 156G CRG2 was found to have lower contents of cysteine and threonine and a higher content of glutamic acid/glutamine. Since the 156G SAg is characterized by a highly periodic structure, with a large number of cysteine residues distributed at regular intervals along the amino acid sequence (on average every sixth or eighth amino acid), the amino acid composition of CRG2 cannot reflect any part of the amino acid sequence of 156G SAg.

CRGs are a set of proteins all presenting the CRD

The question arose as to whether the several bands recognized by AS 2355 in a 156G 60–80 % -satd.-(NH₄)₂SO₄ pellet are also recognized by anti-CRD-purified antibodies. We investigated the presence of the CRD on the molecules corresponding to these bands by immunoblotting of a reduced 156G 60–80 % -satd.-(NH₄)₂SO₄ pellet with anti-CRD antibodies (Fig. 4). The pellet used in the experiment displays four bands recognized by the AS 2355, and on the sister blot the same bands are revealed with the anti-CRD-purified antibodies.

After the action of a PI-PLC, the CRGs become immunologically detectable in membrane fractions

A 156G ethanolic extract prepared in the presence of pCMPSA was submitted to SDS/PAGE. The polypeptides were then transferred on to nitrocellulose sheets,

Fig. 4. Presence of the CRD on all the CRGs

Samples were submitted to SDS/PAGE on a 5–15 % gradient polyacrylamide gel and blotted on to nitrocellulose. The sister blots were incubated with: A, AS 2355; B, anti-CRD-purified antibodies. 1, reduced purified CRG3; 2, reduced purified CRG2; 3, reduced 156G 60–80 % -satd.-(NH₄)₂SO₄ pellet. The middle part of the blots is presented.

and the sister blots respectively incubated with or without PI-PLC from *B. cereus* before immunodetection with anti-CRD-purified antibodies or with the serum 2355. Results are presented in Fig. 5. The CRGs and the SAg present in such an extract are recognized by anti-CRD antibodies only on the filter treated with PI-PLC, and in the same way the CRGs are recognized by AS 2355 only after PI-PLC treatment. It will be noticed that the apparent molecular mass of the CRGs present in ethanolic extract prepared in the presence of pCMPSA is slightly lower than that of the CRGs present in ethanolic extract prepared without pCMPSA.

A parallel experiment was performed with 156G incompletely demembrated cilia fractions containing the membrane form of the SAg, and the blots were incubated with anti-CRD antibodies. Analogous results (not shown) were obtained: the CRGs and the SAg present in such fractions were recognized only after PI-PLC treatment.

Identical results (not shown) were obtained by action of the PI-PLC from *T. brucei* (partially purified). Throughout these experiments, it was checked that unreduced CRGs were not recognized by the different antibodies used.

DISCUSSION

Some of the results presented here allow us to infer that the CRGs are distinct molecules from the SAg. A 'cocktail' of proteinase inhibitors used during ethanolic extraction (i.e. when the conversion takes place) does not prevent the appearance of the CRGs. The CRGs, although not directly immunodetected in cell membrane preparations, are present along with the mSAg. Furthermore, analysis of the purified 156G CRG2 amino acid

Fig. 5. Unmasking of CRD and det-2355 in CRGs after treatment by PI-PLC

Ethanol extracts from cells expressing the 156G SAg were prepared either in the absence (B) or in the presence (B') of pCMPSA (inhibitor of *Paramecium* PLC-like hydrolase). After electrophoresis of reduced samples, sister blots were incubated in the presence (+PL) or in the absence (-PL) of PI-PLC from *B. cereus*. Pairs of blots were reacted with either anti-CRD purified antibodies (a) or with the antiserum 2355 (b). Additional bands recognized by polyclonal antibodies 2355 correspond to degradative fragments of the SAg. In (b), track B, the analysed ethanol extract displays clearly CRG1 along with CRG2 and CRG3. Other experiments (results not shown) have demonstrated that CRG1 is not regularly expressed. This extract comes from a culture expressing CRG1.

content, when compared with the amino acid composition of the 156G SAg, rules out the possibility of CRG2 being a fragment of the SAg.

Since they can be found in incompletely demembrated cilia (IDC) and in purified membrane vesicles, the CRGs appear to be membrane proteins. Experiments carried out with the PI-PLCs from *B. cereus* and *T. brucei* indicate that they are, most likely, anchored in the cell membrane by a phosphatidylinositol lipid. Furthermore, CRG2 and CRG3 present in IDC fractions and in ethanol extracts prepared in the presence of pCMPSA, an inhibitor of PI-PLC, migrate faster in SDS/polyacrylamide gels than do CRG2 and CRG3 present in classical ethanol extracts, and this might be attributed to the presence of the lipid, affecting their migration in SDS/polyacrylamide gels as has been observed for the membrane form of the *Trypanosoma* VSG (Cardoso de Almeida & Turner, 1983) and *Leishmania* P63 (Bouvier *et al.*, 1985).

Lipid cleavage from the CRGs by PI-PLCs allows the access of specific antibodies to two determinants: the CRD and the det-2355; thus these determinants are masked by a lipid moiety. As has been shown for CRG2 and CRG3, the recognition of the two determinants by antibodies requires the reduction of S-S bonds after the activity of a PLC, indicating that these determinants are buried in the molecules. This probably explains why they are not detected in unreduced ethanol extracts. Taken

together, these results suggest that the two determinants are buried and localized in the same region of the molecule, close to the position where the lipid would be linked. Furthermore, amongst our battery of antisera and monoclonal antibodies raised against different SAg, the CRGs are only recognized in their reduced soluble form, which also suggests a limited area for the cross-reaction.

Preliminary experiments performed on the purified 156G CRG2 indicated the presence of mannose, galactose and (*N*-acetyl) glucosamine (G. Strecker, personal communication). The same sugars are found in the phosphoethanolamine glycopeptide located at the C-terminus of the VSG and are involved in the CRD structure (Holder & Cross, 1981; Strang *et al.*, 1986). This recognition of CRGs by antibodies against the CRD of sVSGs leads us to postulate that the structure of the *Paramecium* CRD is close to the structure of the *Trypanosoma* CRD.

From these results we infer that the CRGs (at least CRG2 and CRG3) are surface proteins anchored in the plasma membrane by a glycopospholipid in the same way as several other surface proteins [reviewed by Low *et al.* (1986) and Cross (1987)]. However, we cannot rule out the possibility that the different CRGs may be the same polypeptide presenting various secondary modifications, rather than different polypeptides. Like the SAg, the CRGs display two forms: a membrane-bound one and a soluble one, and the conversion of the

membrane-bound form into the soluble one can be induced by the action of PI-PLC. The phospholipase C-like hydrolase of *Paramecium*, activated during ethanolic or Triton extractions may be a single enzyme, able to act indifferently on several membrane proteins when they are anchored by the same structure. The physiological function of the endogenous phospholipase C remains an open question, but it is attractive to think that it might play a key role in antigenic variation when the cells have to renew their cell coat.

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