



## Selective and programmed cleavage of GPI-anchored proteins from the surface membrane by phospholipase C

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### ABSTRACT

Many surface proteins of eukaryotic cells are tethered to the membrane by a GPI-anchor which is enzymatically cleavable. Here, we investigate cleavage and release of different GPI-proteins by phospholipase C from the outer membrane of the ciliate *Paramecium tetraurelia*. Our data indicate that different GPI-proteins are not equally cleaved as proteins of the surface antigen family are preferentially released *in vitro* compared to several smaller GPI-proteins. Likewise, the analysis of culture medium indicates exclusive *in vivo* release of surface antigens by two phospholipase C isoforms (PLC2 and PLC6). This suggests that phospholipase C shows affinity for select groups of GPI-anchored proteins. Our data also reveal an up-regulation of PLC isoforms in GPI-anchored protein cleavage during antigenic switching. As a consequence, silencing of these PLCs leads to a drastic decrease of antigen concentration in the medium. These results suggest a higher order of GPI-regulation by phospholipase C as cleavage occurs programmed and specific for single GPI-proteins instead of an unspecific shedding of the entire surface membrane GPI-content.

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### 1. Introduction

Across kingdoms surface membranes of eukaryotic cells show a great variety of surface proteins tethered by a glycosylphosphatidylinositol (GPI-) anchor [1]. As one would assume by their localization on the outer membrane, these GPI-proteins often serve signaling functions, representing receptors (e.g. mammalian folate receptor) and adhesive molecules (e.g. neural cell adhesion molecule) [2,3]. Moreover, alkaline phosphatase and renal dipeptidase are also known as catalytically active enzymes [4,5]. Due to their importance, loss of GPI-protein expression, either by defects in expression of single proteins (decay accelerating factor, DAF) or malfunctioning biosynthesis of GPI-anchors, lead to drastic patterns of disease or lethality in several systems [6,7].

Attachment of proteins to the membrane by GPI versus transmembrane domains may provide several advantages. First, the GPI-anchor of extracellular receptors has been postulated to allow transduction of the incoming signal into the cell [8]. This hypothesis seems surprising at first glance as the GPI-anchor does not completely cross the

membrane but data supporting this indicated that GPI-proteins associate with transmembrane proteins of the signal transduction pathway [9–11]. Second, GPI-anchors may be considered as pre-determined breaking points that allow release of the protein into the extracellular compartment upon enzymatic cleavage [8,12,13]. In this context, it was demonstrated that phospholipases, such as PI-PLC are able to remove the 1,2-diacylglycerol moiety of the anchor which separates the protein from the membrane. Such enzymatic release mechanisms were consequently suggested to be selective regulation mechanisms, for example, to disrupt an adhesive state between cells upon GPI cleavage [8]. However, such mechanisms need some kind of regulation. Uncontrolled, non-specific enzymatic release holds some risks for cells, as only rare events would require a complete shedding of the entire GPI content from the surface, and most likely, only one class of proteins needs to be specifically released [13]. If a cell, for instance, needs to disrupt adhesion by programmed release of the GPI-anchored adhesion molecules, non-specific cleavage of all the GPI-anchored receptors or surface enzymes should be avoided.

The ciliate *Paramecium* covers its surface membrane with several different classes of GPI-anchored proteins. Most prominent, in terms of expression and decades of scientific work, are the surface antigens which are a family of high molecular weight proteins exhibiting a very special mechanism of regulation: analogous to parasitic protists, the multigene family allows only for expression of one gene at a time. The ability to switch the expressed gene, and as a consequence the surface protein coat, is called antigenic variation. A serotype is defined by the presence of only one surface antigen protein-species at a time on the surface. It was recently shown that this is regulated

**Abbreviations:** GPI, glycosylphosphatidylinositol; PLC, phospholipase C; PI-PLC, phosphatidylinositol specific phospholipase C; GPI-PLC, glycosylphosphatidylinositol specific phospholipase C; CRD, cross-reacting determinant; ELISA, enzyme linked immunosorbent assay; HM, high molecular; SM, small molecular; DAF, decay accelerating factor; GAPDH, glyceraldehyde-3-dehydrogenase.

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by endogenous RNA interference mechanisms in *Giardia intestinalis* and *Paramecium tetraurelia* [14,15].

In *P. tetraurelia*, a series of previous studies demonstrated that besides the large surface antigens there is a series of smaller GPI-proteins on the cellular membrane [16] one of which has been identified as a folate receptor [17–19]. All these surface-proteins can be easily purified without cell-lysis by extraction with salt/ethanol solution [20]. This procedure was shown to specifically release GPI-proteins by activation of endogenous phospholipase C. This became apparent as the extracted proteins show the CRD-epitope (cross-reacting determinant) representing the 1,2-cyclic monophosphate of the remaining anchor [17,21].

## 2. Methods

### 2.1. Cultivation, RNAi, induction of serotype shifts

*Paramecia* were cultured in wheatgrass powder (WGP) bacterized with *Klebsiella pneumoniae*, supplemented with 0.8 mg/l  $\beta$ -sitosterol. RNAi by feeding was carried out as described before [21,22]. Positions of the individual feeding fragments were 658–1196 in *PLC2* (GSPATT00034681001) and 1267–1648 in *PLC6* (GSPATT00030070001). Silencing efficiency was checked by q-RT-PCR showing knock-down levels of  $20.6 \pm 0.1\%$  (*PLC2*),  $8 \pm 0.01\%$  (*PLC6*) relative to wt-expression. Serotype shifts from 51A to 51D were induced by RNAi against the expressed 51A gene resulting in expression of pure serotype 51D [15]. The position of the silencing fragment of surface antigen 51A was 380–874.

### 2.2. Surface protein extractions and culture medium concentration

200,000 cells were pelleted and resuspended in 400  $\mu$ l Volvic mineral water (Danone waters, Frankfurt, Germany). For salt/ethanol extractions of GPI-anchored proteins, 240  $\mu$ l 10 mM  $\text{Na}_2\text{HPO}_4$  pH 7, 150 mM NaCl, and 30% EtOH were supplemented according to [20]. For isolation of surface proteins by membrane solubilization [23], cells were treated with 1.3% Triton X-100 in 50 mM Tris-HCl, pH 7.5 with Complete™ Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Extraction was carried out for 1 h except as otherwise stated. Cells were then carefully pelleted (500 g) to avoid lysis. Proteins were subsequently precipitated by 1 Vol. Acetone and resuspended in 150  $\mu$ l 20 mM Tris-HCl, 1 mM EDTA, 25 mM KCl, and 50 mM sucrose, pH 7.4 [18].

For purification of proteins from culture medium, 4000 cells/ml were transferred to Volvic mineral water for 24 h and then gently removed to avoid lysis. Cellular supernatant was concentrated with two different Amicon ultra columns (Millipore, Billerica, MA): the high molecular (HM) fraction with 100 kDa cut-off and its flow-through using a 10 kDa cut-off, representing the small molecular fraction (SM). The concentrated proteins were then precipitated with a final concentration of 10% TCA (trichloroacetic-acid). Simultaneously, surface proteins of the re-isolated cells were isolated in a small-scale salt/ethanol extraction (20,000 cells).

### 2.3. Semi-Quantitative RT-PCR

Total RNA was extracted with Trizol™ (Invitrogen, Karlsruhe, Germany) according to manufacture instructions, DNaseI digested and extracted with phenol (pH 4.5). After an integrity check by denaturing agarose gel-electrophoresis, 500 ng total RNA were reverse transcribed using M-MuLV H-reverse transcriptase (Finnzymes, Espoo, Finland) and oligo dT-Primers. cDNA dilutions used for subsequent PCR were  $1/20$ ,  $1/100$ ,  $1/500$ , and  $1/1500$ . Positions of PCR-products were *PLC2*: 658–1196; *PLC6*: 40–807 and *GAPDH*: 503–908 (intron spanning primer). Control PCRs with noRT reactions were carried out with undiluted cDNA to show purity of the isolated RNA.

### 2.4. Immobilization, Western Blot and ELISA

For *in vivo* immobilization, a minimum of 50 cells were exposed to polyclonal sera (1/200 dilution) in a depression slide for 20 min. For Western blots, purified proteins were SDS-PAGE separated under (denaturing and reducing conditions) and blotted according to standard procedures described before [21]. The antibodies used were rabbit anti-51A polyclonal serum, anti-51D polyclonal serum, anti-51H polyclonal serum from the Sonneborn collection [24] and the anti-CRD antibody (Oxford GlycoSystem, Rosedale, NY). Before reprobing, blots were stripped with 0.2 N NaOH for 30 min and loss of antibodies was checked by probing with secondary antibodies. Densitometric analysis was carried out with the ImageJ software [25]. Indirect ELISA was performed according to a standard procedure. In brief, 100  $\mu$ l antigen solution was coated to polystyrene microtiter plates Nr. 655061 (Greiner-Bio One, Frickenhausen, Germany) in  $1/10$ ,  $1/50$  and  $1/100$  dilution. The procedure included the following steps: wash 3 $\times$  with phosphate buffered saline (PBS), 0.05% Tween-20 (= PBST), block with 5% BSA in PBS, wash 3 $\times$  in PBST, primary antibody  $1/1900$  in 1% BSA PBST for 1 h, wash 6 $\times$  in PBST, HRP-conjugated secondary antibody  $1/1900$  in 1% BSA PBST, wash 6 $\times$  in PBST, apply ready-to-use TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution (UPTIMA, Montluçon Cedex, France), stop reaction after 10 min with 1 M HCl and measure absorption. Relative optical density was calculated following ((OD450–OD690)-blank).

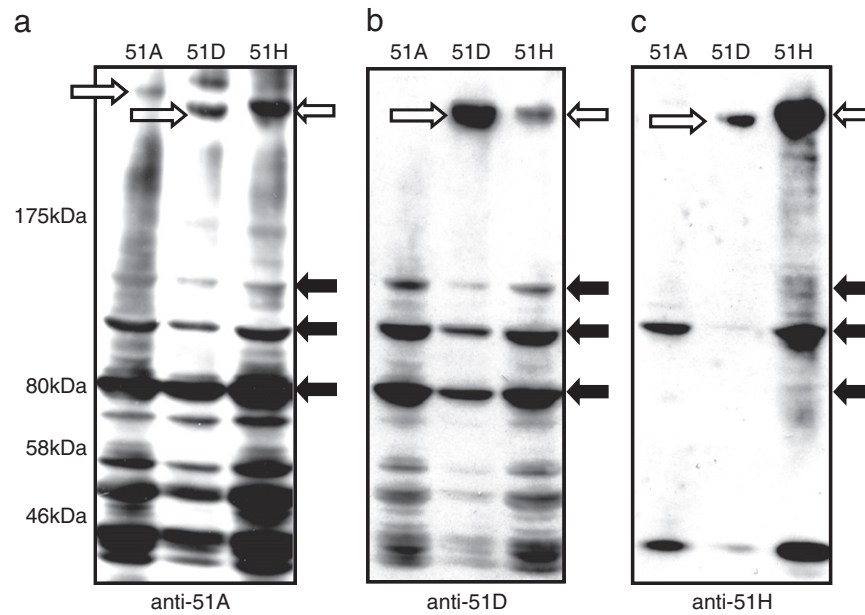
## 3. Results

### 3.1. Small invariant GPI-proteins are expressed independently from the serotype

We first analyzed the expression of the small molecular mass GPI-proteins in context of serotype expression. As the cultivation temperature highly affects surface antigen expression in *Paramecium*, serotype pure cells were isolated from different cultivation temperatures. Fig. 1 shows a Western blot of salt/ethanol extracted surface proteins from different serotypes (51A, 51D, 51H) cultured at 31 °C, 26 °C and 16 °C respectively.

As indicated in previous studies, polyclonal sera which specifically detect the serotype proteins by *in vivo* immobilization also detect several smaller surface proteins in Western blots of denatured proteins [17,21,26]. Fig. 1a shows the GPI-anchored proteins from cells expressing three different serotypes recognized by antisera against serotype 51A cell antigens; the high molecular surface antigen is indicated by the open arrow.

Note that in Fig. 1a, the small mass proteins from cells of serotype 51A, 51D and 51H all show the same pattern with anti-51A antiserum. Fig. 1b and c shows that the anti-51D and anti-51H antisera detect patterns of small molecular mass proteins different from that detected by anti-51A. However, for any given antiserum, the small molecular mass protein patterns are the same across the three serotypes. Therefore, we consider the small molecular mass proteins to be invariant. Furthermore, the blot indicates a strong cross-reactivity of the surface antigen-specific sera with both, other high molecular mass serotype proteins and also the smaller invariant GPI-proteins. As the antisera do not show sufficient cross-reaction to native proteins *in vivo* to immobilize the cells, signals in the Western blots of denatured and reduced proteins are likely due to the conservative C- and N-terminal regions of the serotype-proteins. These areas appear to be masked in the native tertiary structure *in vivo* but accessible when a protein is released from the surface [27–29]. However, single sera do not detect all the smaller GPI-proteins, as for instance anti-51H does not detect the prominent ~80 kDa protein nor the ~130 kDa band, which show strong signals with anti-51A and -51D sera. We suggest that sera show different affinities (i) to other serotype proteins and (ii) to different small GPI-proteins as seen by the



**Fig. 1.** Expression of invariant surface GPI-proteins in different serotypes. Western blot of (denatured) salt/ethanol extracted surface proteins (8–16% gel) from serotypes 51A, 51D and 51H developed with antisera specific for each of the antigen-proteins. The high molecular variant surface antigens are marked by an open arrow and the relative migration of these different proteins fits with previous experimental results: 51A – 300 kDa; 51D – 280 kDa; 51H – 288 kDa [20]. Three of the discussed invariant proteins are indicated by black arrows (~80, ~100, ~130 kDa).

stronger signals of the ~100 kDa and ~130 kDa proteins in Fig. 1b. By the cross-reactivity between the different serotype proteins and by the lack of a general cross-reactivity among all the smaller proteins, we conclude that this behavior appears not due to a protein contamination during immunization, but more likely is the result of a high degree of similarity of the small GPI-proteins to the serotype proteins. This assumption is supported by the genome data that show a large variety of candidates for the small GPI-proteins showing homology to the classical serotypes and intact GPI-anchoring signals [Simon M. & Meyer E., unpublished; 19]. However, we do not know the function of the smaller GPI-proteins, although the constitutive expression may be an argument for a receptor function, similar to the GPI-anchored folate receptor [17–19].

### 3.2. Rapid release of surface antigens *in vitro*

To analyze whether all GPI-proteins are released in the same manner, and to gain insight in the kinetics of the individual release, salt/ethanol extractions were carried over five increasing time periods. Fig. 2a indicates release of four different surface proteins from cells expressing serotype 51A. The blots, as well as the densitometric quantification of the bands show that the ~80, ~100 and ~130 kDa are constantly released indicated by continuous increasing of band intensity. In contrast, the surface antigen band shows a strong signal after 5 min. Fig. 2c indicates that approx. 60% of the maximally released surface antigen is already present in the supernatant after 5 min. Therefore, the kinetics suggests that different GPI-proteins are not released in the same manner and release of surface antigens appears to be much faster compared to other GPI-proteins in the *in vitro* experiment.

### 3.3. PLCs predominantly release serotype proteins *in vitro*

Next, we analyzed phospholipase C activity involved in release of both the invariant GPI-proteins and the high molecular surface antigens. Fig. 3a shows salt/ethanol extractions of a 51A culture undergoing silencing of an uninvolved control gene (*ND169*) and a 51A culture with silenced *PLC2* and *PLC6*. These isoforms of phospholipase C have previously been identified to affect release of different surface

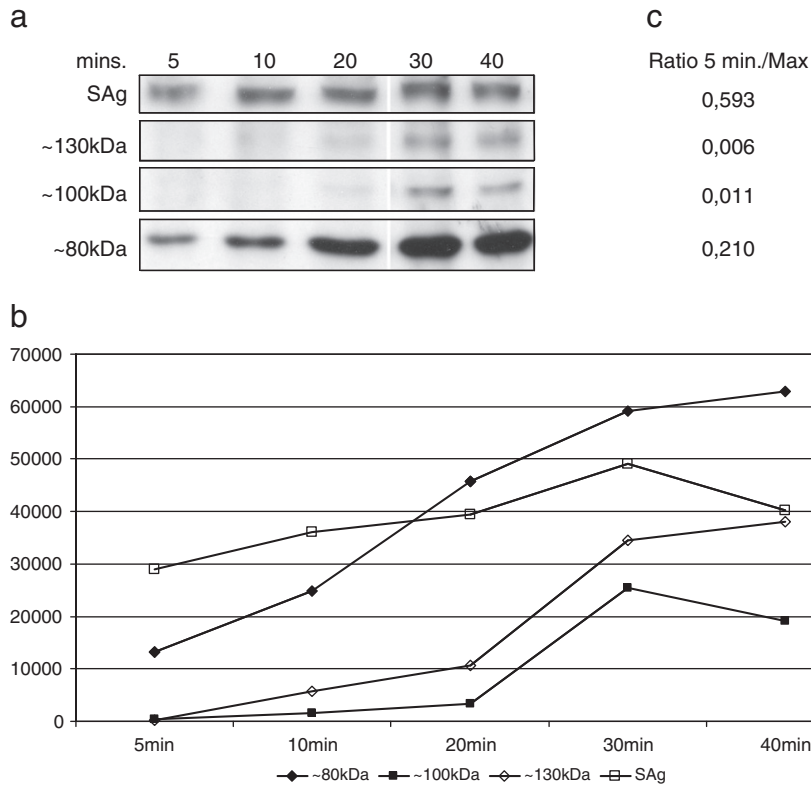
proteins during salt/ethanol extraction [21]. Here, the Coomassie stained gel indicates that silencing of *PLC2* and *PLC6* predominantly affects release of the large surface antigen in salt/ethanol extractions, whereas release of the majority of smaller proteins seems only slightly reduced (Fig. 3a).

To compare the PLC released GPI-surface proteins with the total amount of detergent solubilized surface membrane proteins, surface proteins extracted by salt/ethanol treatment were compared to membrane proteins solubilized by Triton X-100 treatment.

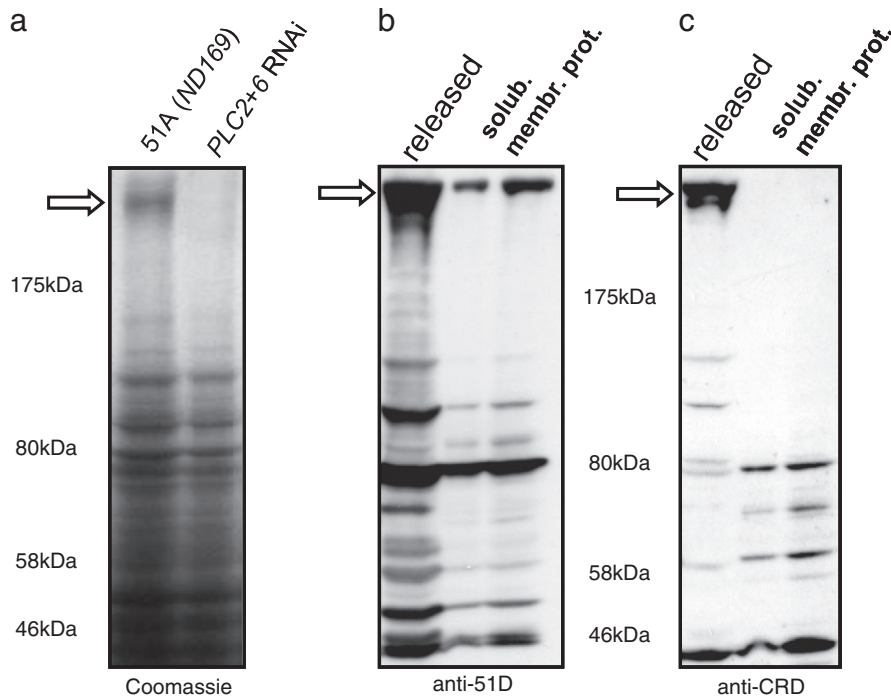
Extractions were carried out only for 5 min to avoid saturation of the reaction. The corresponding Western blot in Fig. 3b indicates that salt/ethanol extracts have a much higher amount of surface antigen compared to solubilization (last two lanes). The same blot developed with anti-CRD-antibodies (Fig. 3c) reveals that PLC cleaved the salt/ethanol extracted surface antigens but not the solubilized antigens (open arrows). In agreement with results from PLC silencing, the strongest signal in Fig. 3c can be seen on the surface antigens. The small GPI-proteins in the same lane show significantly weaker signals, suggesting that PLC acts predominantly on the large surface antigens and only to a lesser extent on the smaller GPI-proteins. However, this kind of artificial PLC activation does not necessarily reflect the PLC activity *in vivo*, as the conditions during extraction might influence substrate affinity and/or access of the cleaving enzymes.

### 3.4. PLCs release serotype proteins but not the smaller GPI-proteins *in vivo*

To clarify whether PLC activity also releases surface proteins *in vivo*, we developed a procedure to concentrate the medium from cultures to analyze the released proteins in Western blots and ELISAs. We used a non-nutrient medium (Volvic, mineral water) in order to hold cell numbers constant by stopping cell division. The use of water also eliminated potential effects from the bacteria in the culture medium. To characterize *in vivo* release of proteins we utilized a concentrated medium separated by filtration into high (HM) and small molecular (SM) mass molecules and compared them to the proteins that could be removed by salt/ethanol extraction of the surface after incubation of the cells in the water medium.



**Fig. 2.** Kinetic analysis of surface protein release *in vitro*. a: Western blot of salt/ethanol extractions of serotype 51A expressing cells stopped after 5, 10, 20, 30, and 40 min. Pictures indicate release of four different proteins detected with anti-51A serum including the surface antigen (SAg). Data are representative of three individual experiments. b: Densitometric analysis of the band intensity was performed using the ImageJ software [25]. c: The ratio was calculated for densitometric measurements (5 min relative to the maximal value).



**Fig. 3.** Involvement of phospholipase C in GPI-release and specificity of the anti-CRD antibody a: Coomassie stained SDS-PAGE (6–18%) with salt/ethanol extracted proteins from serotype 51A expressing cultures: a wild type 51A culture (control silencing, ND169) and a culture undergoing simultaneous silencing of PLC2 and PLC6. b and c: Comparison of PLC released surface proteins of serotype 51D (first lane) and such isolated by membrane solubilization with Triton-X 100 (lane two and three). In contrast to the general isolation protocol, extraction was carried out for only 5 min to avoid saturation of the reaction. Panel b shows the blot developed with anti-51D serum, panel c with anti-CRD antibodies indicating PLC cleaved GPI-anchors.



The blot in Fig. 4a shows surface antigen 51A in the high molecular fraction of the medium and also a slight smear. However, the media show weak or no signals of the small GPI-proteins, as well as none in the small molecular mass fraction. In this context, disappearance of the small molecular GPI-proteins from the cell-surface by other mechanisms during the procedure can be ruled out as they are still present on cells re-isolated from the medium after the experiment (first lane). Similar results can be seen in Fig. 4b showing the same experiment with a 51D expressing culture. Comparing the 51A and the 51D surface antigens, less smearing and a stronger signal for the serotype protein can be identified in the HM fraction of the 51D medium supernatant (Fig. 4a vs. b). The same is obvious in Fig. 1 that shows the salt/ethanol extractions of these two serotypes suggesting that the 51A protein is somehow degraded faster than the 51D protein. Similar observations were reported earlier [21].

We subsequently checked for an involvement of PLC activity in release of the surface antigens and analyzed surface antigens isolated from culture medium by anti-CRD antibodies. Using concentrated water medium (HM-fractions) from a serotype pure culture and a culture undergoing a serotype shift from 51A to 51D, the blot indicates the presence of the CRD-epitope in proteins from the medium, suggesting that they were cleaved from the surface by PLC (Fig. 4c). For the shifting culture, the blot reveals the “new” 51D protein on the cell surface which fits with the immobilization data as more than 90% of the cells already completed the shift (data not shown). In agreement with previous studies [30], the blot also indicates an increased release of antigens in the shifting culture, which may be due to increased PLC activity.

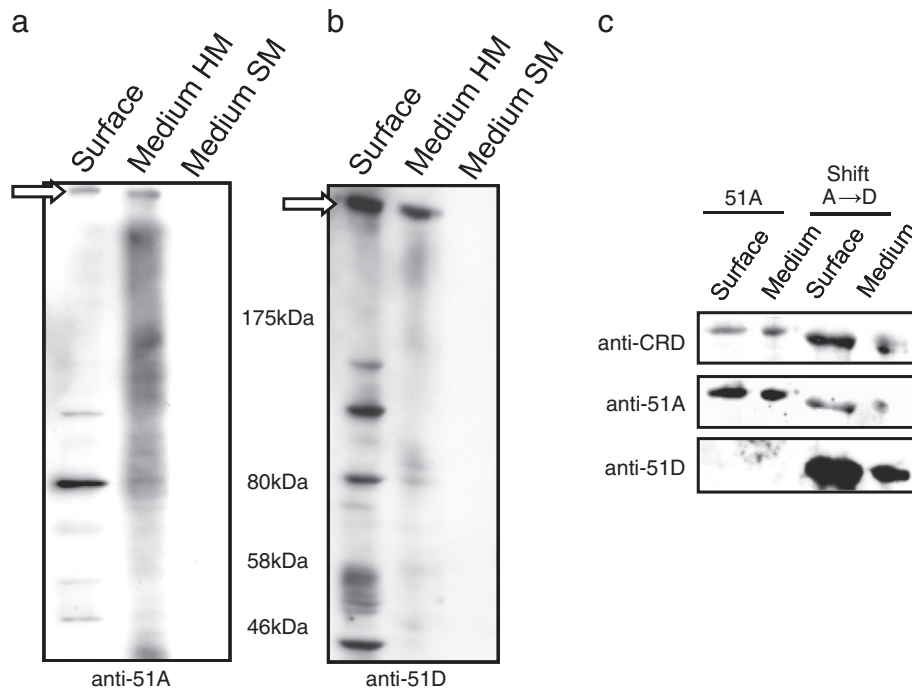
The data indicate that in our experimental setup, PLC activity specifically releases the large surface antigens (51A and 51D). This was observed for serotype stable cultures and to a higher degree in shifting cultures. In our experimental setup, *in vivo* release of the small invariant GPI-proteins cannot be detected even to a limited degree.

### 3.5. Programmed PLC activity during serotype switching

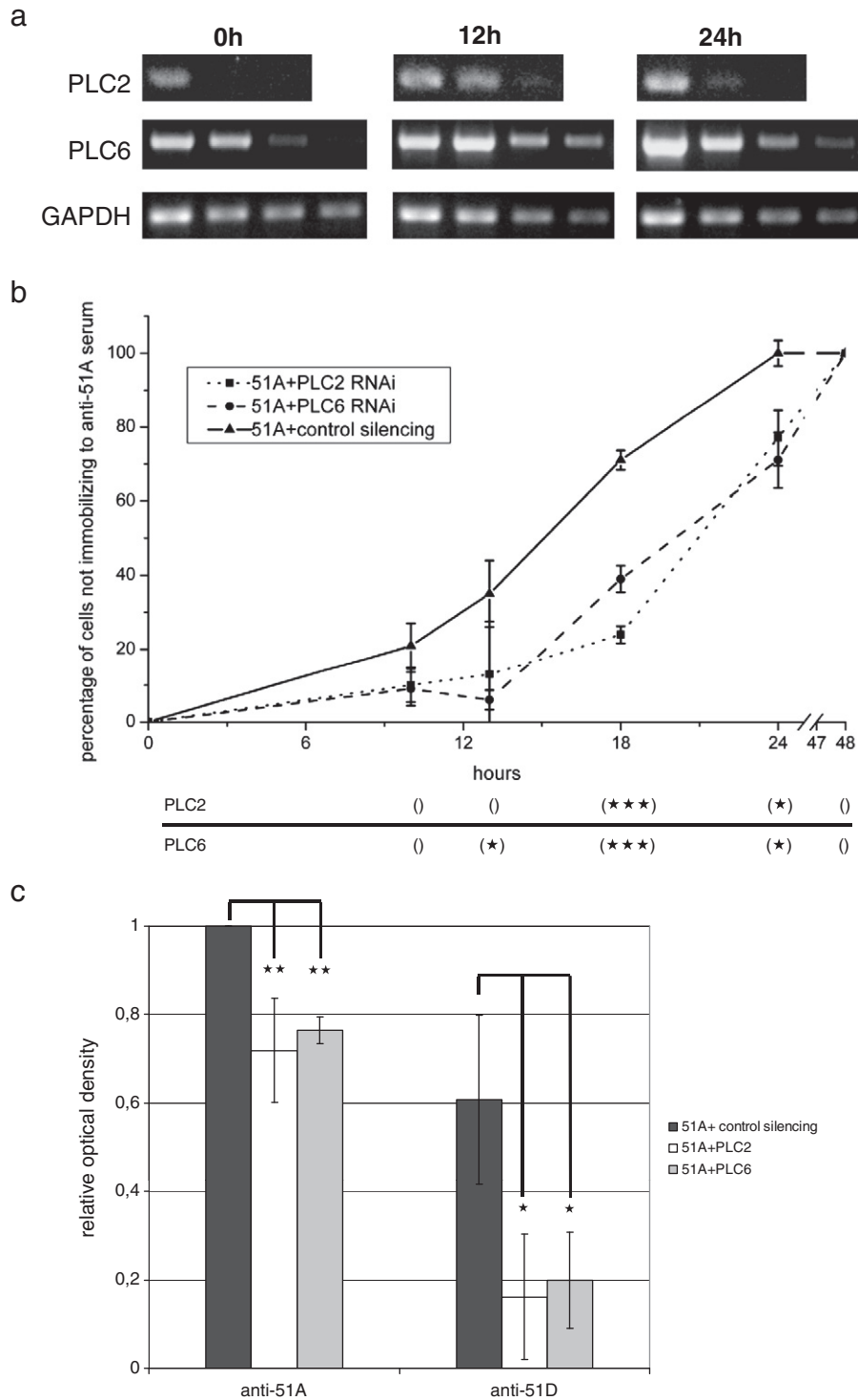
Speculating that release of GPI-proteins is also controlled by regulation of gene expression of the individual PLCs, we examined *PLC2* and *PLC6* during antigenic switching. Triggering a serotype shift (51A to 51D) and analyzing the transcript level of *PLC2* and *PLC6* during the shift, Fig. 5a indicates an increase of mRNA for both PLCs in samples taken 12 and 24 h after triggering the surface antigen shift. This suggests that the cleaving enzymes are activated during the antigen switch to guarantee a fast shedding of old antigens from the surface.

To test this hypothesis, we analyzed the duration of a serotype switch with and without silenced PLCs combining the data of temporal duration and quantification of released antigens in the cellular medium by ELISA. Again, serotype switching was induced by RNAi and the duration of the shift was followed by immobilizing samples at different time points. As expected from a previous study [21], PLC silencing showed a delayed shift, as cells showed stronger immobilization to anti-51A serum over the period of time compared to the wild-type shift (Fig. 5b). Although decelerated, PLC silencing cultures completed the serotype shift after 48 h. To demonstrate whether this corresponds to lower antigen-concentrations in the cellular medium, ELISA quantification of supernatants of these cultures was carried out. We found significantly lower signals for both PLC RNAi cultures compared to the culture undergoing control silencing (Fig. 5c). This was true for both the new (51D) as well as for the old antigen (51A). We therefore conclude that the longer persistence of 51A antigen on the cells surface (Fig. 5b) is due to a decreased release of the proteins into the medium and that *PLC2* and *PLC6* are in large part responsible for enhanced GPI-cleavage during antigenic switching.

PLC mediated GPI-release therefore strongly accelerates cellular serotype transformation. However, the data show that PLC silencing not only affects release of the old antigen (51A) but also cleavage of the new antigen 51D indicating that PLCs obviously cannot discriminate between different surface antigens.



**Fig. 4.** PLC mediated release of surface antigens. Western blots of concentrated medium supernatants (High molecular fraction (HM) and small molecular fraction (SM)) in comparison to the surface-coupled protein content. a: Isolates from serotype 51A expressing cells developed with anti-51A serum. b: Isolates from serotype 51D expressing cells developed with anti-51D serum. c: Comparison of surface antigen release in a serotype pure culture (control silencing of an uninvolved gene, *ND169*) and a culture undergoing a serotype shift from 51A to 51D, triggered by RNAi against the 51A antigen. The blot was first developed with anti-CRD antibodies and subsequently with anti-51A and anti-51D serum. Cutouts show the high molecular surface antigens.



**Fig. 5.** Programmed release of surface antigens into the medium. **a:** Semi-quantitative RT-PCRs of *PLC2* and *PLC6* of cultures undergoing antigenic switching from serotype 51A to 51D triggered by RNAi. Samples for RNA extraction and subsequent cDNA synthesis were taken at 0, 12 and 24 h after triggering. GAPDH serves as a housekeeping gene and all noRT controls did not show any signals. Gels show increasing cDNA dilutions from the left to the right. **b:** Effects of PLC silencing on serotype switching (RNAi triggered serotype shift 51A to 51D), determined by immobilization with anti-51A serum; Median  $\pm$  standard deviation; p-values, \*  $\leq 0.05$ ; \*\*\*  $\leq 0.001$  versus the control culture, students *t*-test. **c:** Indirect ELISA of concentrated cellular medium using anti-51A and 51D sera. Absorption (median  $\pm$  standard deviation) values were referred to values of anti-51A serum of the wild type shifting culture (value of 1). Data represent three independent experiments; samples were taken from cultures in panel b, isolated after 24 h incubation. p-values, \*  $\leq 0.05$ ; \*\*  $\leq 0.01$  versus the respective value of control cultures, factorial ANOVA followed by Fisher's LSD *post hoc* analysis.

#### 4. Discussion

GPI-PLC activity of eukaryotic phospholipase C is found in several systems; however, there is little data addressing the responsible enzymes [31]. Indeed, most studies on phospholipase C investigate intracellular

PI-PLC activity involved in signal transduction rather than surface phenomena.

Unlike other unicellular eukaryotes, ciliates contain not only a single PLC gene but obviously distinct classes [21,32]. We previously identified two isoforms of phospholipase C involved in antigenic variation in the

ciliate *P. tetraurelia* [21], and in the present study we focus on the PLC mediated release of different GPI-proteins. *In vitro* salt/ethanol extractions of surface proteins indicate that PLCs predominantly release the large surface antigens and the analysis of cell media supports this finding as PLC mediated shedding of GPI-anchored proteins *in vivo* occurs specifically with the large surface antigen family and not other GPI-proteins. We therefore conclude that PLCs somehow are able to discriminate between different GPI-proteins and, moreover, our data indicate specific activation during serotype switching. As a result, specific release of surface GPI-proteins by phospholipase C exhibits an important step of regulation as cells and organisms are able to trigger the specific release of proteins in contrast to non-specific shedding of the entire surface. Furthermore, our data also indicate that PLC mediated release is a very rapid process allowing fast adaption to environmental changes. As surface antigens show a high turn-over accompanied with release into the medium, their potential impact on extracellular signaling remains speculative.

Two possible mechanisms may contribute to discrimination among proteins by PLC. First, the localization of either the PLC or its substrate may play a role. In contrast to the equally distributed large antigens, membrane proteins and other GPI-anchored proteins have been observed to segregate into different membrane domains, e.g. the cortex membrane [10,33]. However, we do not know exactly where cleavage and release takes place. Data from PLC2:GFP fusion proteins in *Paramecium* suggest secretion of the phospholipase into the medium [21]. Similar to our findings, a *Trypanosoma cruzi* PI-PLC shows developmentally regulated expression and localization. This enzyme was shown to localize on the outer membrane of intracellular amastigotes but is surprisingly secreted during extracellular differentiation [34,35]. Further investigation is necessary to clarify where on the surface cleavage takes place. Hints exist that in *Paramecium* the tips of the cilia are somehow involved as antigenic switching is accompanied by transport of the “old” antigens towards the tips of the cilia [21,36,37]. Since this indicates an active site of surface antigen release on the cilia, the cleavage of cortex bound proteins may not be affected by such a mechanism.

Second, modifications on the GPI-anchors may affect substrate discrimination by the PLC. Some GPIs for instance have an additional fatty acid (e.g. palmitic-acid) on the 2-hydroxyl of the inositol which makes the anchor resistant to PLC cleavage [38]. In this context, the biochemical analysis of *Paramecium* GPI-anchors demonstrated a rare core glycan modification identified for a mannosyl phosphate and a lipid-moiety composed of a ceramide [39,40]. Interestingly, the amide-linked fatty acid composition was shown to alter with different cultivation temperatures [40]. Such modifications may affect PLC affinity to its substrate and as a consequence, further biochemical analyses are necessary to identify modulated GPIs on different surface proteins. Comparing the pre-proteins, the C-terminal GPI-signal sequence is quite different from the large surface antigens and the candidates of the smaller GPI-proteins leading us to speculate that these trigger different modifications during GPI-addition [Simon, unpublished]. It seems also very likely that both hypotheses come together as different GPI-modifications target segregation of the proteins into different microdomains of the outer membranes and next to this separation effect influence substrate affinity to the endogenous PLC.

Finally, it is possible that other release mechanisms may yet play a role in shedding of antigens, as for instance surface antigens isolated from a log-phase culture medium with feeding-bacteria did not show the CRD-epitope [41]. Dual modes of release were also reported for GPI-anchored variant surface proteins in *Trypanosoma* as shedding involves PLC cleavage and proteolysis [42]. Similarly, PLC- or proteolytic-cleavage presumably by a zinc-metalloprotease causes the release of neurodegenerative disease causing prion proteins [43]. The comparison of surface half-life of DAF and prions on the same cell type suggests preferential shedding of the prion [44].

We have just begun to understand the important mechanisms of selective and programmed GPI-protein shedding from eukaryotic cell surfaces, which appear to play important roles in so many systems. The mechanisms that we propose here for GPI-modification and localization need to be explored in order to gain a better understanding of programmed release.

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