First publ. in: Journal of Membrane Biology 200 (2004), pp. 15-23

Temperature-Induced Change of Variant Surface Antigen Expression in *Paramecium* Involves Antigen Release into the Culture Medium with Considerable Delay between Transcription and Surface Expression

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Abstract. We analyzed temperature-induced changes of variant surface antigen (vsAg) expression in *Paramecium primaurelia*, using immuno-techniques and mRNA determinations. Upon a 23°C to 33°C shift, the old vsAg, type 156G, remains on the cell surface for a time, when already mRNA for the new form, 156D, is expressed. A considerable amount of 156D-specific mRNA is formed 45–48 h after the temperature shift, while 156D surface expression reaches maximal levels only after >72 h. A new aspect of these experiments is that, during this transition, the old vsAg is steadily released in high-molecular-weight form into the culture medium, as found by dot blot and Western blot analysis of concentrated culture medium. The new vsAg form is first inserted into the somatic cell membrane, before it spreads also into cilia. In the reverse transition, 33°C to 23°C, the adaptation on the level of transcription and surface expression is considerably faster. While we had previously shown, under steady-state conditions (constant temperature), the occurrence of a degradation pathway by endocytotic and phagocytotic ingestion of vsAg this may proceed in parallel to the steady release of old vsAg from the cell surface into the medium. Altogether these combined processes may facilitate the installation of the new vsAg type.

Key words: Cilia — Immobilization antigens — *Paramecium* — Protozoa — Variant surface antigens

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Introduction

A *Paramecium* cell is completely covered by densely packed ≥250 kDa large variant surface antigens (vsAg), which are inserted in the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. In the case of *Paramecium*, the GPI anchor is endowed with a ceramide basepiece (Capdeville, 2000). Antibodies (AB) against these proteins immobilize the cells and therefore vsAg are commonly called immobilization antigens. They are used to characterize different serotypes, since under steady-state conditions only one type of vsAg is expressed — a phenomenon called "mutually exclusive expression" (Antony & Capdeville, 1989). The different vsAg protein types are encoded by different genes (Preer et al., 1981; 1987) whose expression can be changed by a temperature shift (Antony & Capdeville, 1989; Benwakrim et al., 1998; Kusch & Schmidt, 2001).

For the present work we used *P. primaurelia*, for which the corollaries of selective vsAg expression are particularly well established (Beale, 1954). Cells cultivated at 23°C express the G-type vsAg (vsAg-G), while they change to vsAg-D upon transfer to 33°C (Antony & Capdeville, 1989). Under steady-state conditions, when a permanent turnover of vsAg takes place, we had traced in *P. tetraurelia* a degradation pathway by internalization via the endocytosis and phagocytosis route, ending up in digestive vacuoles (Flötenmeyer et al., 1999). In contrast to this, little is known about the mechanism how such exchange of vsAg on the cell surface takes place during experimental serotype change, e.g., caused by a temperature shift. In the present study, using AB binding and mRNA assays, we find evidence for a release into the culture medium, with considerable delay after transcription of

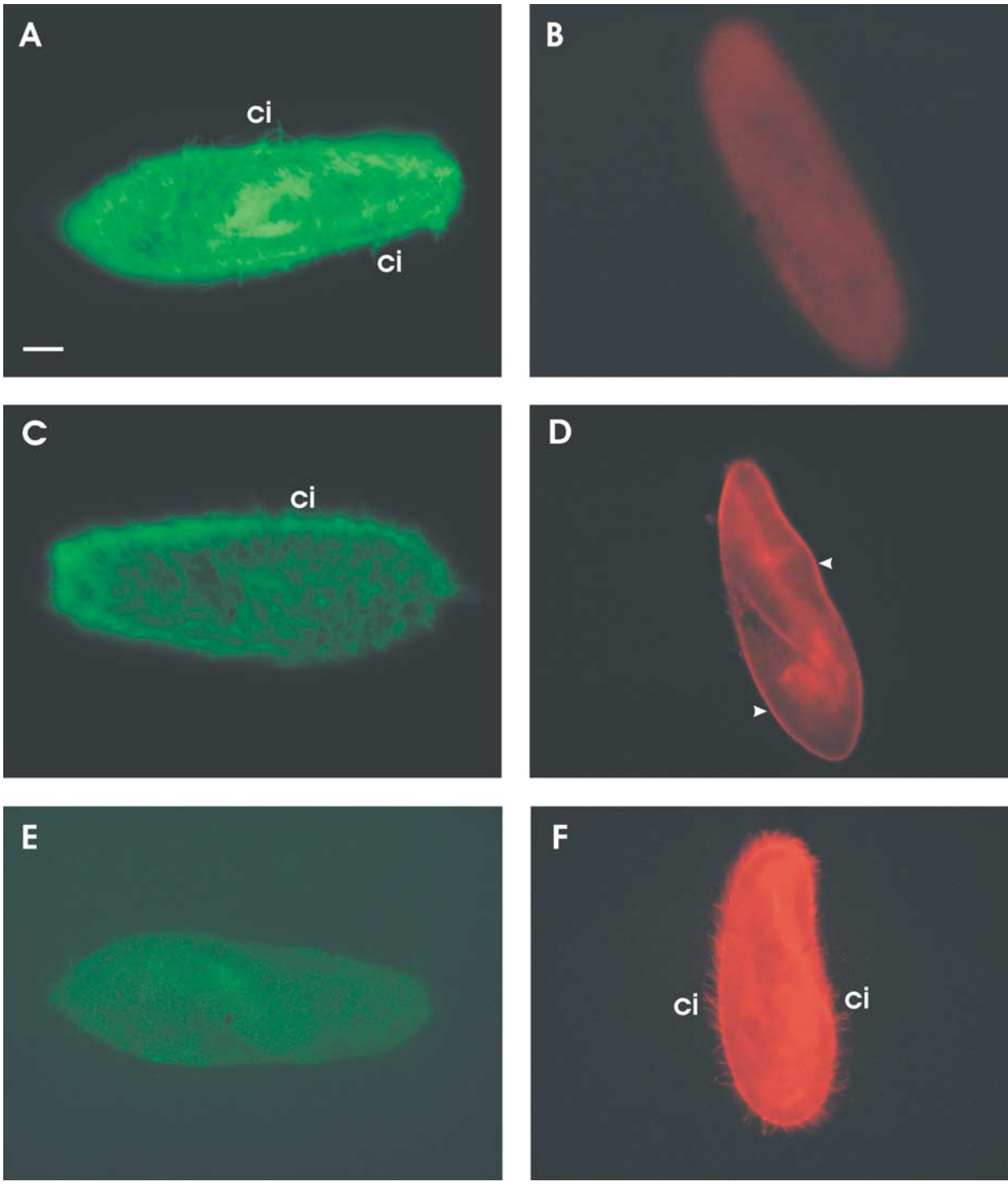

Fig. 1. Fluorescence analysis of the transition of vsAg-G to vsAg-D, as seen in *A–D*, 48, 72 and 96 h, respectively, after a temperature shift (23°C to 33°C), by labeling with AB against the respective antigens (*see* text). *Green*: AB against vsAg-G; *red*: AB against vsAg-D. (*A*) Persistence of vsAg-G on the cell surface, 48 h after transfer. Also note staining of cilia (*ci*). (*B*) Absence of vsAg-D staining 48 h after transfer. (*C*) Reduction of vsAg-G staining 72 h after the temperature shift (*compare* with [*A*]). (*D*) Emergence of vsAg-D staining 72 h after transfer on the somatic cell surface, but not in cilia (*compare* with [*A*, *C*]), e.g., at arrowheads. (*E*) Disappearance of vsAg-G staining after 96 h, while the surface staining, including cilia (*ci*), with AB against vsAg-D (*F*) is complete. Magnification 700 ×.

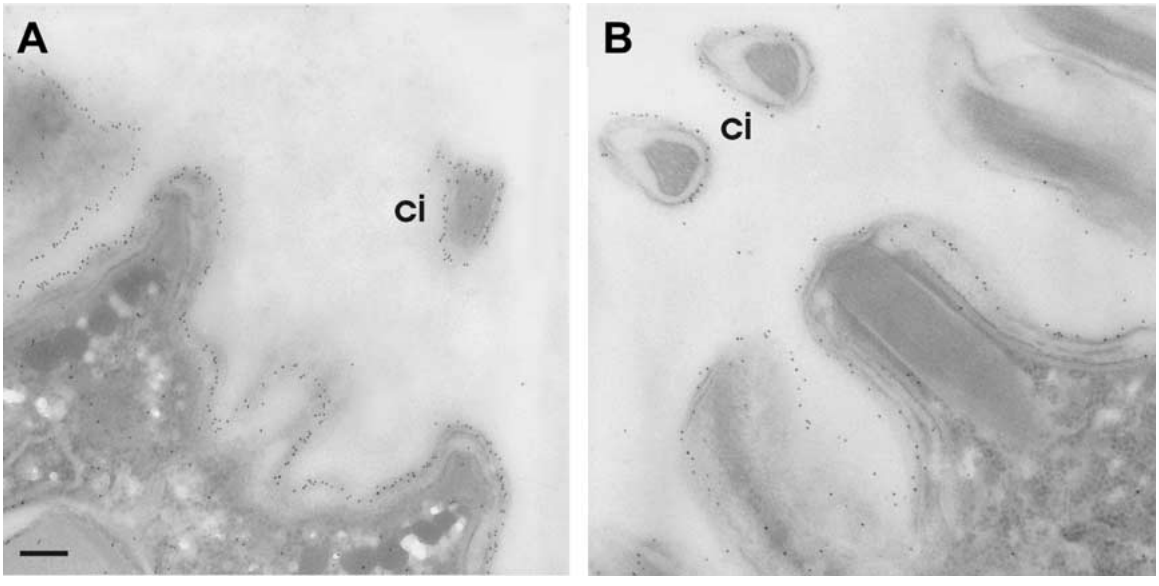

Fig. 2. Electron microscopy (EM) immuno-gold labeling of G- (*A*) and D-type (*B*) cells, analyzed under steady-state conditions, i.e., after latent cultivation at 23°C and 33°C, respectively. Note homogeneous labeling with protein A-$Au_{5\ nm}$ of the entire cell surface, including cilia, (*A*) with AB against vsAg-G in a G-type cell and with AB against vsAg-D in a D-type cell (*B*), respectively. Magnification 60,000 ×.

the mRNA for the induced new vsAg type and its surface expression.

Materials and Methods

Strains and Cell Culture

Paramecium primaurelia, strain 156 (New Haven, CT) was grown in a wheat grass powder medium bacterized with *Enterobacter aerogenes* the day before use. Cultivation of serotype 156G was carried out at 23°C according to Sonneborn (1947). Cells expressing serotype 156D were cultivated at 33°C.

Induction of Serotypes

To induce serotype 156D we utilized the method of daily isolating lines. Isolated cells were incubated overnight at 4°C and then the cultivation temperature was suddenly changed to 33°C. After division overnight, only one cell was isolated and transferred into fresh medium with *E. aerogenes*. After cultivation at 33°C overnight, this procedure was repeated for 10 days. By that time all cells had been transformed to serotype 156D. To induce serotype 156G, the procedure is simpler, i.e., cells expressing 156D at 33°C were cultivated at room temperature (23°C) for the time indicated in text and figure legends to transform them into serotype 156G. Henceforth these strains are designated as vsAg-G and vsAg-D, respectively.

This methodology implies the following. For inducing a change in expression of serotype, strong signals are most efficient. Changing the cultivation temperature from 4°C to 33°C is such a strong signal, resulting in cells clearly expressing serotype D. Although a rather high number of cells does not survive this harsh but most effective treatment, the number of surviving cells (50 to 70%) is sufficient when working with daily isolation lines. A similar experiment to induce serotype G by changing culture conditions from 33°C down to 4°C is lethal for all cells. Therefore, in this case, only a shift to 23°C has been applied, resulting in a reliable transformation from vsAg-D to vsAg-G.

Isolation of Surface Antigens

A defined number of vsAg-G or vsAg-D cells were washed in 10 mM PIPES-buffer pH 7.2 and divided into five portions, each with 1.5×10^5 cells, in 40 ml fresh bacterized culture medium. Cells were grown under the two conditions, (i) after a shift from 23°C to 33°C and (ii) after a shift from 33°C to 23°C.

For the isolation of vsAg samples the methods of Preer (1959) and Jones (1965) were applied. Briefly, 10 to 15 liters of mass culture were centrifuged in a liquid separator at 1,000 × *g* to obtain 400 ml of concentrated cells. This volume was washed twice in Dryl's phosphate buffer, pH 6.8 (in mM: 1 NaH_2PO_4, 1 Na_2HPO_4, 2 Na_3citrate, 1 $CaCl_2$). Cells were concentrated once more to a volume of 5 ml and then suspended for 1 h in extraction medium (12% ethanol; 7.7 mM NaCl) at 4°C. Then the suspension was briefly centrifuged (180 × *g*, 5 min) and the supernatant was again centrifuged (20,000 × *g*, 5 min, 4°C) to remove all cell fragments. Proteins were precipitated by adding solid $(NH_4)_2SO_4$ up to 75% saturation, followed by stirring on ice for 3 h. The isolated surface proteins were centrifuged at 10,000 × *g* (15 min, 4°C) and then resuspended in 10 ml bidistilled H_2O. This material was dialyzed over 48 h to remove ions and then lyophilized in a vacuum concentrator.

AB Production

Isolated vsAg were diluted in phosphate-buffered saline (PBS) pH 7.2. Protein was used in native form. AB against the respective type of vsAg, after expression by the respective strains and purification, were raised in rabbits. Pre-immune sera were taken and checked for non-reactivity on Western blots and in immunofluorescence.

Immobilization Reaction

The effect of the AB against the respective vsAg was tested under light-microscope control, as described previously (Flötenmeyer et al., 1999).

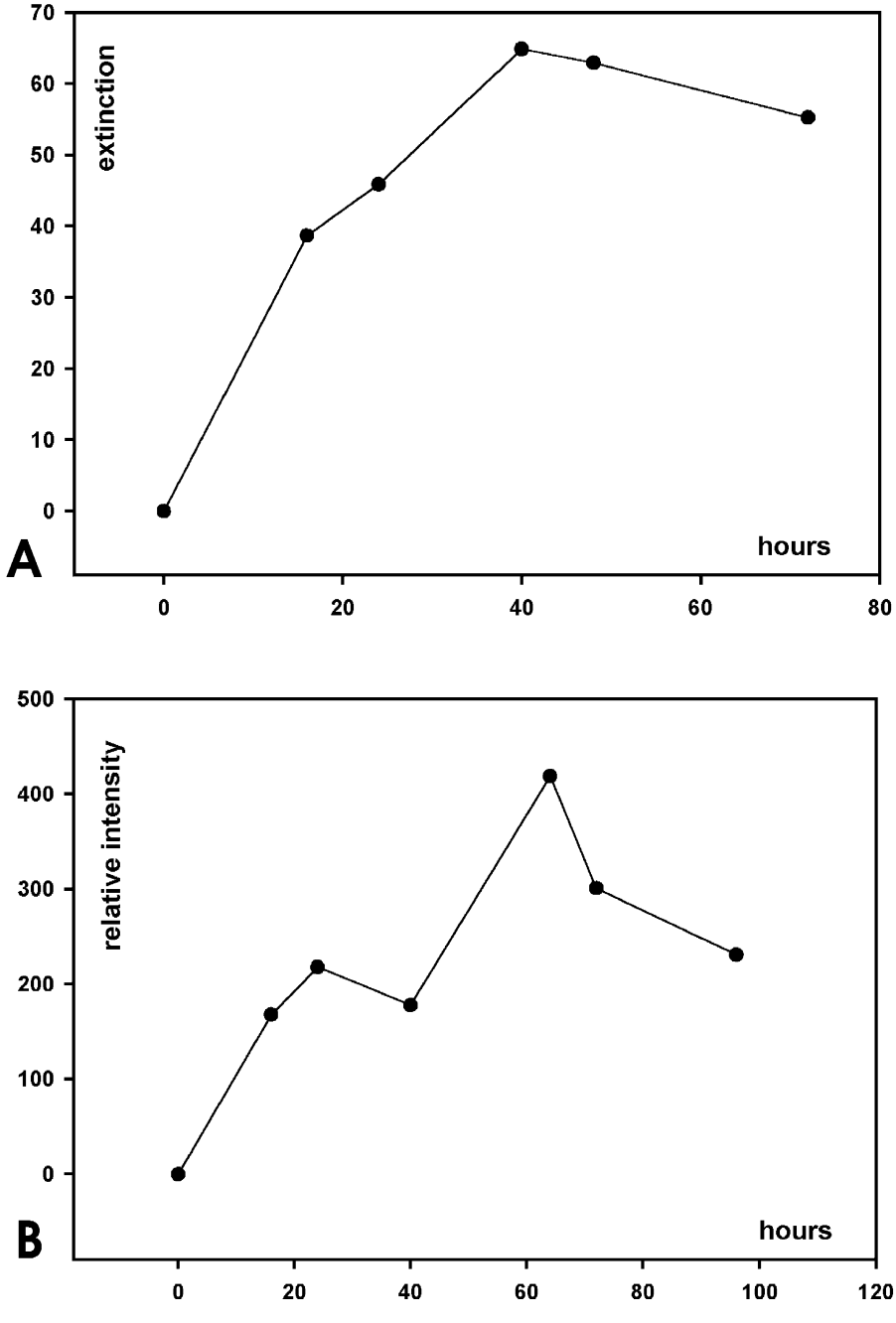

Fig. 3. Typical experiments showing the release of vsAg-G after transfer of cells from 23°C to 33°C, as derived from dot blot (*A*) and Western blot analysis (*B*). Concentrated supernatants from cell culture medium have been probed with AB against vsAg-G as specified in Materials and Methods.

ELISA, Dot Blots and Western Blots

For ELISA and dot blots, fractions to be analyzed were bound to the wells in different concentrations (to assure concentration-dependent linearity of the reaction) and further processing was by standard methods. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed with gels of 5 to 15%. Samples of concentrated culture medium were applied to gels after brief boiling. The sizes of markers (Pharmacia, Freiburg, Germany) varied between 10 and 250 kDa. Gels were stained with silver or alternatively processed for Western blots and treated with anti-vsAg AB followed by alkaline phosphatase-tagged anti-rabbit AB (Sigma, Taufkirchen, Germany) by standard techniques. Reaction intensities were quantified in a standard scanner.

Immunofluorescence

Cells were washed in PIPES buffer pH 7.2 and injected as a concentrated suspension into 4% formaldehyde in PBS pH 7.4 (20°C, 30 min). Then cells were pelleted, washed in 50 mM glycine-PBS (3 × 10 min) and incubated with antisera in PBS, supplemented with 1% BSA, followed by FITC- or Texas Red-conjugated goat anti-rabbit-IgG (ICN, Eschwege, Germany). For further details, *see* Flötenmeyer et al. (1999).

Immuno-Gold Electron Microscopy

Cells were washed in Pipes buffer pH 7.2 and fixed in 8% formaldehyde supplemented with 0.25% glutaraldehyde in PBS pH 7.4. After

Fig. 4. Western blot analysis of vsAg-G released after transfer of cells from 23°C to 33°C. The vsAg-G released 16 h and 40 h after temperature change is of the high molecular weight form (≥250 kDa, between arrowheads) and its intensity increases with time, in agreement with Fig. 3*A*. The higher molecular weight band represents material retained in the stacking gel, while low-size bands at the bottom are of unidentified type and invariably found, independently of the vsAg released.

blocking the reactive aldehyde groups by 50 mM glycine (3 × 10 min) the samples were dehydrated in a graded ethanol series, impregnated with Unicryl (British BioCell Int., London) at 4°C (2 × 3 h) and UV-polymerized at −20°C according to the manufacturer's advice.

Ultrathin sections were floated on 0.02 M glycine in PBS (10 min), washed in 0.3% BSA-C (BioTrend, Köln, Germany) in PBS (2 × 10 min), incubated for 1 h with one of the anti-vsAg AB, washed in 0.3% BSA-C/PBS (3 × 10 min) and incubated with protein A-gold of 6 nm diameter ($Au_{6\ nm}$) obtained from University of Utrecht (Dept. Cell Biol., School of Medicine, The Netherlands). After washing with distilled water, sections were routinely stained with 2% aqueous uranyl acetate for electron microscope (EM) analysis.

RT-PCR Test

Isolation of mRNA

The cells were washed twice in Dryl's phosphate buffer. Cell lysis and isolation were based on the mRNA Capture Kit from Roche (Mannheim, Germany). The cells were lysed in 50 µl buffer, followed by the addition of a biotin-labeled oligo-dT-probe to the lysate. This mixture was incubated at 37°C for 10 min for hybridization of probe and mRNA. The solution was then transferred into a streptavidin-coated micro-testtube (Roche). After further incubation (10 min, 37°C) the mRNA/probe hybrids bound tightly to the micro-testtube. The cells were washed twice with washing solution (Roche).

Single-Step RT-PCR

The Titan-One-Tube RT-PCR-System® (Roche) was used to carry out the RT-PCR, with the advantage that all reactions took place in the streptavidin-coated micro-testtube used for mRNA isolation.

Two Master Mixes were prepared for reaction.

Master-Mix 1: 4 µl dNTP (each 10 mM), 1 µl primer forward (100 mM), 1 µl primer reverse (100 mM), 2.5 µl dithiothreitol (100 mM), 0.5 µl RNAse inhibitor from human placenta, 16 µl bidistilled water, 25 µl total volume.

Master-Mix 2: 10 µl 5 × RT-PCR buffer, 1 µl enzyme mix, 14 µl bidistilled water, 25 µl total volume.

Program parameters of the single-step RT-PCR were as follows. Reverse transcription: 30 min, 50°C. Denaturation: 2 min, 94°C. 10 cycles (denaturation 10 s, 94°C; primer annealing 30 s, 45°C; primer elongation 45 s, 68°C). 25 cycles (denaturation 10 s, 94°C; primer annealing 30 s, 45°C; primer extension 45 s + 5 s/cycle, 68°C; 1 cycle: primer extension 7 min, 68°C).

After the reaction, the product was visualized by Agarose gel-electrophoresis.

Results

Transformation of serotype 156G to 156D was induced by a rapid temperature change as described in "Materials and Methods". This includes incubation of the cells overnight at 4°C, before transfer to 33°C. The temperature shock thus produced causes a rather rapid and efficient change of vsAG expression. This change has been analyzed by immuno-fluorescence labeling and in parallel the transcription of the respective genes has been monitored by RT-PCR. Immobilization tests, combined with Western and dot blot analyses, as well as immunolabeling studies showed specifity of the AB used.

After transfer from 23°C to 33°C, *P. primaurelia* cells expressing the vsAg type G change to type vsAg-D only after a rather long time, as visualized by immunofluorescence using AB against the respective vsAg type (Fig. 1). After 72 h, both vsAg can be seen on cells in transition. One would expect that this change occurs over the entire cell surface, since its localization under steady-state conditions includes the somatic and the ciliary cell membrane. In fact, under such conditions, as we show on micrographs obtained by immuno-gold EM analysis (Fig. 2), the overall localization is rather homogeneous. However, during the temperature-induced vsAg change, we observe after 72 h a transitional stage where only the somatic cell membrane is labeled by AB against the new vsAg type, whereas cilia are not (Fig. 1*D*).

During the temperature-induced change we have measured by immunological methods the time-dependent occurrence of vsAg-G in the cell culture medium. Assays were done by ELISA or dot blots (Fig. 3*A*) and by Western blot analysis of the ≥250 kDa large immuno-reactive bands (Fig. 3*B*). For these tests the cell culture medium was concentrated as described in "Materials and Methods". In pilot assays, the amount of protein applied per dot or per lane was varied over two orders of magnitude to ascertain linearity of the reaction with AB. Evaluation was by scanning the intensity of the reaction per dot and by scanning the high molecular weight peak areas in Western blots, respectively, also as described. We thus have determined the relative intensity of the

Table 1. Time-dependency of the change from vsAg-G to vsAg-D following a temperature shift 23°C → 33°C, analyzed by immunofluorescence and mRNA determination by RT-PCR[§]. Evaluation derived from Figs. 1 and 4.

Time after 23°C to 33°C	Immunofluorescence/mRNA	
Transfer (h)	Anti-vsAg-G AB/mRNA-G	Anti-vsAg-D AB/mRNA-D
0	+++/+++	−/−
42	+++/+++	−/−
45/48	+++/−	−/++
72	++/−	++/+++
96	−/−	+++/+++

[§]Rating: (−) no reactivity; (+) to (+++) low to maximal intensity.

immuno-staining achieved with either one of these AB under identical conditions, e.g., with equal amounts of protein applied and equal AB dilution. Figures 3*A* and 3*B* each present data from typical experiments with the two methods described. Clearly, during the 23°C to 33°C transition, vsAg-G is released into the medium in intact form, i.e., without change in molecular weight (Fig. 4). This may allow new vsAg molecules to be inserted into the cell membrane, as they substitute for old ones. In all runs, the intensity of the vsAg-G-reactive material contained in the cell culture medium declined roughly after 2 to 3 days following the 23°C to 33°C transition.

How does that compare with immunofluorescence experiments? According to Fig. 1 and the summary of data contained in Table 1, the release of vsAg-G decreases during the time when the new type, vsAg-D, is increasingly incorporated into the cell surface. Therefore, the change in the surface composition is not due to an acute release of the old vsAg, but this change is mainly due to a gradual replacement of old vsAg molecules by those of the new type.

How does that compare with the change in the amounts of the mRNA for the respective vsAg types, vsAg-G and vsAg-D, during the 23°C to 33°C shift? Figure 5 presents a typical example of a RT-PCR evaluation. The readings for the mRNA encoding the G-type vsAg, mRNA-G, disappear between 45 and 48 h after the temperature shift, while mRNA-D is transcribed to a significant extent after 45 h and then still increases to a maximal level after 72 h. As summarized in Table 1, the change in mRNA remarkably precedes the expression of the new vsAg on the cell surface. About 3 days are required to achieve the new steady state on the transcriptional level, while about 4 days are required for steady state on the translational level and on the level of transcellular transport to the cell surface. At this time also the original vsAg-G is fully removed from the surface (*see* Fig. 1).

How does the vsAg expression change during a temperature change in the opposite direction? Changes in vsAg after a temperature shift, 33°C to 23°C, were analyzed in similar experiments, again by

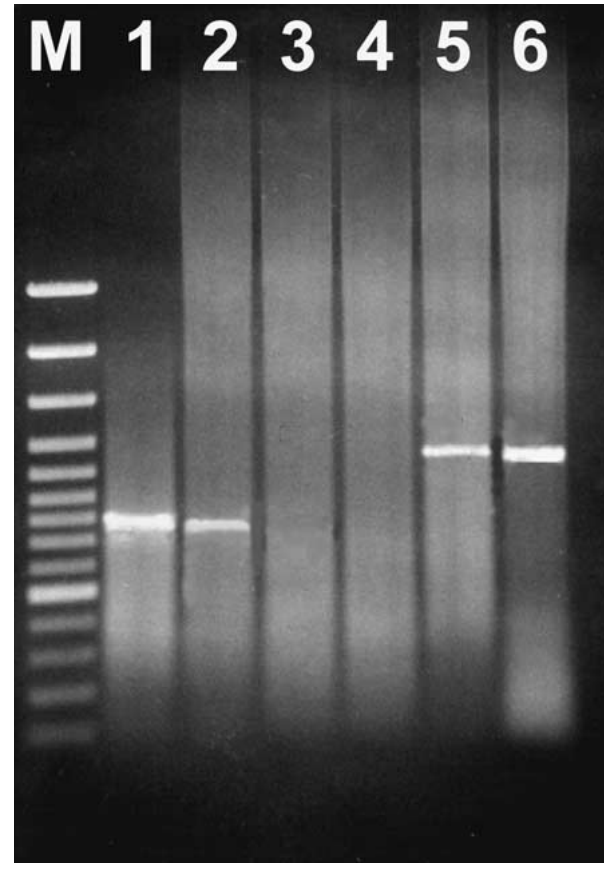

Fig. 5. RT-PCR analysis of the vsAg-G to vsAg-D transition induced by a temperature shift, 23°C to 33°C. *M* = Marker Gene Ruler 100 bp Ladder Plus MBI; (*1–3*) vsAg-G specific probe, (*4–6*) vsAg-D-specific probe. Times after temperature change: 42 h (*1*, *4*), 45 h (*2*, *5*), 48 h (*3*, *6*). Note that the vsAg-G-specific signal disappears only after 48 h, while the vsAg-D specific signal appears already 3 h earlier.

combining immunofluorescence and mRNA determinations (Figs. 6, 7). Again a change of vsAg, this time vsAg-D to vsAg-G, can be recognized. In this case, both methods indicate that the change occurs much more rapidly than in the opposite direction. Examples of immuno-fluorescence analysis are shown in Fig. 6, examples of mRNA change are found in Fig. 7. In brief, after a temperature shift from 33°C to

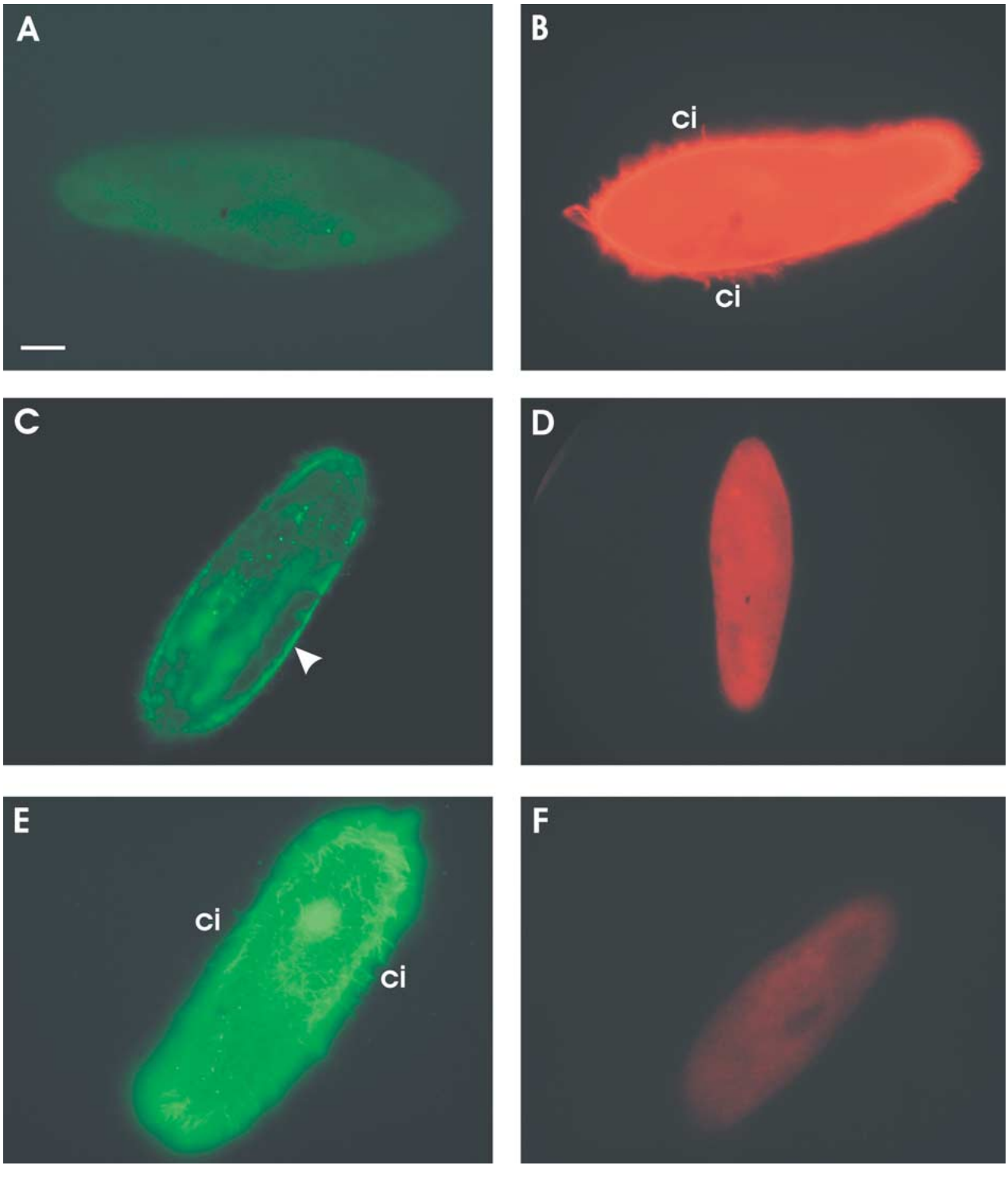

Fig. 6. Fluorescence analysis of the vsAg-D to vsAg-G transition during a 33°C to 23°C transfer, using AB against the respective antigens (*see* text). *Green*: AB against vsAg-G (*A*, *C*, *E*); *red*: AB against vsAg-D (*B*, *D*, *F*). After 6 h, staining with AB against vsAg-G is absent (*A*), while vsAg-D staining occurs all over the surface, including cilia, *ci* (*B*). After 24 h, vsAg-D reactivity is diminished (*D*), while vsAg-G reactivity occurs on the cell surface, largely with the exception of cilia, as pointed out by the arrowhead (*C*). After 48 h, staining with AB against vsAg-D has disappeared (*F*), while that with AB against vsAg-G is fully developed, including on cilia, *ci* (*E*). Magnification 700 ×.

23°C, the new vsAg type can be observed already after 24 h on the somatic surface (but not on cilia), when the old one is already considerably reduced. After 48 h the old vsAg type is no longer detectable on the cell surface by immunofluorescence. The change of mRNA species also occurs more rapidly

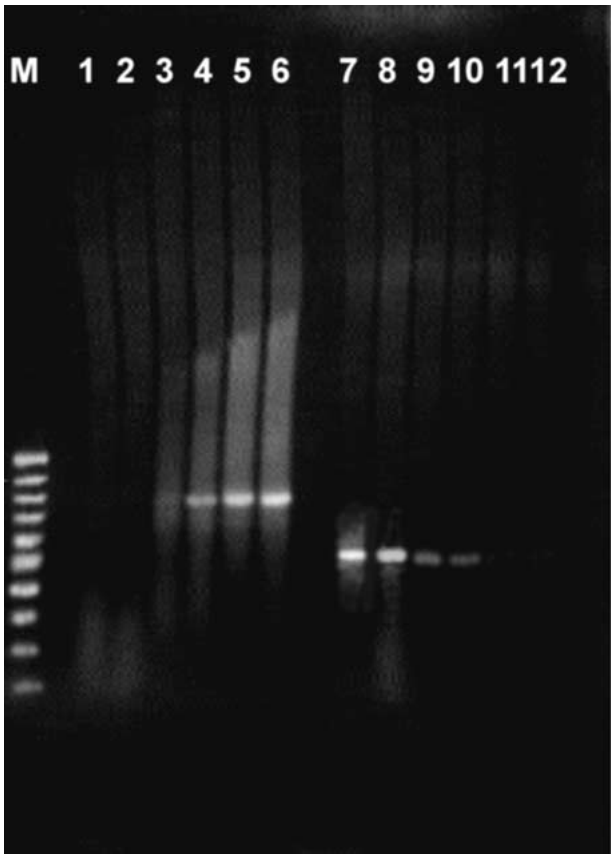

Fig. 7. RT-PCR analysis (*see* Materials and Methods) of the vsAg-D to vsAg-G transition after a temperature shift, 33°C to 23°C. *M* = 100 bp DNA Ladder Promega, (1–6) vsAg-G specific probe, (7–12) vsAg-D specific probe. Times after temperature change: 0 h (*1*, *7*), 4 h (*2*, *8*), 6 h (*3*, *9*), 8 h (*4*, *10*), 12 h (*5*, *11*) and 24 h (*6*, *12*), respectively. Note that the vsAg-D-specific signal disappears after 8 h, when the vsAg-G- and -D-specific signals are equally pronounced.

during a 33°C to 23°C shift than during one in the opposite direction (Fig. 7). As during the 23°C to 33°C shift, transcription of the respective mRNA significantly precedes translation, or—more precisely—positioning at the cell surface.

Discussion

General Aspects

The vsAg-type proteins are widely distributed among protists (Ferguson, 1999), from pathogenic to harmless forms, including the ciliate *Paramecium* (Capdeville, 2000). Many details on vsAg expression are known, like the "mutually exclusive expression" in *Paramecium* when exposed to a temperature shift (Antony & Capdeville, 1989). The unsettled question, how this change from one type of vsAg to another may occur, was addressed in the present study.

We had previously analyzed the pathways of vsAg turnover under steady-state conditions (Flötenmeyer et al., 1999). Under these conditions, using AB labeling on the fluorescence and EM level, we could ascertain uptake by endocytic and phagocytic vacuoles and delivery to digestive vacuoles, which would result in degradation. We now find a permanent release into the culture medium. This is a new pathway, which has not been followed up experimentally so far in previous work. We also observed during the studies presented here that AB against vsAg molecules stain food bacteria, which then undergo phagocytotic uptake (*data not shown*).

For protozoan life under laboratory conditions it may appear irrelevant that the functional significance of vsAg molecules and of their experimentally inducible change is still enigmatic (Bleyman, 1996). Since their discovery in *Paramecium* (Preer, 1959) widely different hypotheses have been proposed for the function of protozoan GPI-anchored proteins in general, and for those of *Paramecium* in particular. For *Paramecium*, hypotheses range from adaptive responses to environmental conditions (Capdeville & Benwakrim, 1996) to a role in folate receptor signaling (Paquette et al., 2001). In parasites, hypotheses range from host-cell invasion in the cases of *Plasmodium* (Blackman, 2000), *Toxoplasma* (Dzierszinski et al., 2000) and *Trypanosoma* (Lillico et al., 2003) to transmembrane signaling in the fish pathogenic protozoan, *Ichthyophthirius* (Clark et al., 2001), and induction of tumor necrosis factor in macrophage host cells by *Toxoplasma* (Debierre-Grockiego et al., 2003). Considering this ever expanding list of potential functions of GPI-anchored surface proteins in these enormously important parasites it is not surprising that the state of knowledge in *Paramecium* is even less advanced.

An interesting observation we now present is that newly formed vsAg first appears in the somatic cell membrane, and only later on in cilia. This would be compatible with a delivery in the form of "rafts" into the ciliary membrane, just as proposed for other ciliary membrane proteins (Rosenbaum & Witman, 2002).

How and where Could vsAg Shedding Occur?

Normally vsAg molecules cover the entire surface of a *Paramecium* cell (Adoutte et al., 1983; Barnett & Steers, 1984; Capdeville et al., 1987). To explain release into the medium one would have to consider transport to internalization sites and/or release directly from the surface, i.e., by surface-bound enzymes. Endocytic and phagocytic uptake for intracellular degradation has been analyzed in detail by Flötenmeyer et al. (1999). In addition, in the present study we find a permanent release into the culture medium, which may be prerequisite to phagocytotic uptake (including bacteria with vsAg adsorbed). This new pathway requires a tentative explanation.

One possibility to release vsAg molecules would be detachment from the GPI anchor by the GPI-specific phospholipase C (PL-C). This enzyme is known to occur in *Paramecium* where it has been shown that it can cleave vsAg in vitro (Assouz & Capdeville, 1992). However, its precise localization in the cell is not known. Partial release of an acylated form of ^{35}S-labeled vsAg has been analyzed in *Paramecium* under steady-state conditions (Deregnaucourt, 1992). The half-life so determined is longer than that of the time course we find during temperature-induced change. Any role of PL-C is enigmatic also in trypanosomatids since their GPI-specific PL-C is not essential for host-cell invasion, though it influences parasitemia (Webb et al., 1997). In *Plasmodium*, some GPI-anchored surface proteins were shown very recently to be shed by a protease (Howell et al., 2003). Considering the occurrence in the culture medium of vsAg with its original molecular weight, the endo-/lysosomal pathway previously described (Flötenmeyer et al., 1999) may be just one route for vsAg turnover, which may be relevant under steady-state conditions. The situation may be different when a new type of vsAg is induced. This change may then take place—additionally or exclusively—by the mechanism shown here.

In sum, although potential mechanisms of release are known, it remains to be settled how and where in the cell this may take place—possibly on the cell surface itself.

We gratefully acknowledge the valuable help of Dr. J. Hentschel in image processing, the skillful technical assistance of Ms. Sylvia Kolassa and Lauretta Schade in the EM work and of Ms. I. Traub in evaluating the blots. This work was supported by grants from the Deutsche Forschungsgemeinschaft to H. P and to H. J. S.

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