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Topical Review

Genetically Controlled Expression of Surface Variant Antigens in Free-Living Protozoa

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Introduction

Besides parasitic protozoa some free-living ciliates have the ability to exhibit alternative types of proteins on their cell surface (Sonneborn, 1948; reviews: Schmidt, 1988; Bleyman, 1996; Schmidt, 1996). A range of these exchangeable structurally different surface proteins has been detected in species of the genera *Paramecium* (Ciliophora) and *Tetrahymena* (Ciliophora). The presence of variable surface proteins is detected by immunochemical techniques. Injections of *Paramecia* or cells of *Tetrahymena* species into mammals induce the production of antibodies against surface proteins of the injected cells. Therefore the proteins also are called surface antigens. Treatment of living *Paramecia* or of *Tetrahymena* cells with the homologous serum results in immobilization of the ciliates at low serum concentrations, or leads to cell death at higher concentrations. Because of the immobilizing effect of antibodies the corresponding surface antigens also are called "immobilization-antigens", or "i-antigens." Recent research involves the detection of mRNA specific for surface antigens by Reverse Transcriptase-PCR techniques (H.W. Breiner, H.J. Schmidt, J. Kusch, *unpublished results*), with the aim to investigate single cells from field samples and a possible ecological function of these molecules.

Cells that express the same type of surface antigen and therefore can be immobilized by the same antiserum belong to one serotype. Within genetically identical clones cells sometimes appear that are resistant against immobilization via antibodies. These cells induce the production of a different type of antibody after their injection into mammals. The cells represent a further serotype within the ciliate clone. In this way a range of serotypes, corresponding to different surface antigens, was observed in several species of *Paramecium* and of *Tetrahymena* (Nanney & Dubert, 1960; Koizumi, 1966; Hiwatashi, 1967; Juergensmeyer, 1969; Sonneborn, 1974; Steers & Barnett, 1982). Seven different serotypes are known for *Paramecium primaurelia* and twelve for *P. tetraurelia.* Most of the known strains of these species cannot express all of the surface antigen types, e.g., types S, G and D only can be observed in most strains of *P. primaurelia.*

Different surface antigens are generally mutually exclusive (Beale, 1957). Ciliates express only one type of surface antigen at constant environmental conditions, although genes for other surface antigens are present. A property of serotype expression is the ability to switch to another serotype. Among the stimuli for transformation are changes in temperature, pH, and the kind of growth medium, or UV radiation and proteolytic enzymes (Sonneborn, 1970). The inheritance of serotype genes follows Mendelian rules (Beale, 1957). The function of these surface proteins and the mechanism responsible for the variability of their expression are unknown. One suggestion is that they may be a buffer or defense against environmental biotic or abiotic factors (Preer, 1986). *Paramecia* lacking variable surface proteins have never been found.

Variant Antigens are High Molecular Weight Single Peptides

Surface variant antigens are distributed on the cell membrane (Fig. 1) including the cilia (Beale & Mott, 1962;

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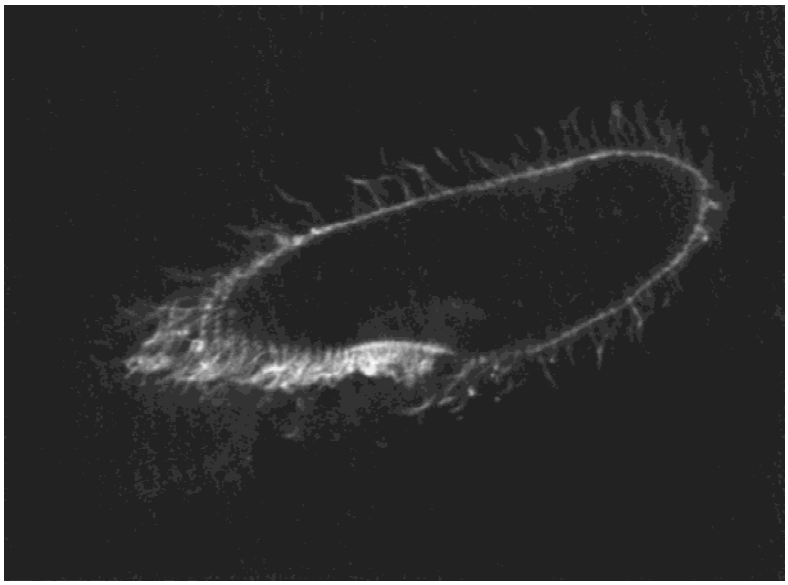

Fig. 1. Fluorescence laser-scanning light micrograph of a middle section of a *Paramecium primaurelia* cell, expressing surface variant antigen type G. The surface antigens were immunocytochemically stained with a monoclonal anti-G-antibody and Cy3-rabbit-anti-mouse-IgG. (Figure from Lars Czubatinski, Kaiserslautern, Diploma thesis, 1999, reproduced with permission of L.C.).

Mott, 1963, 1965; Wyroba, 1977; Doerder, 1981). They are large, single polypeptides of 251,000 to 308,000 MW in the genus *Paramecium* (Reisner, Rowe & Sleigh, 1969; Hansma, 1975; Preer, Preer & Rudman, 1981), but small, acidic polypeptides from 25,000 to 59,000 MW in *Tetrahymena* species (Bruns, 1971; Williams, Doerder & Ron, 1985; Doerder & Berkowitz, 1986; Smith et al., 1992; Ko & Thompson, 1992; Ron, Williams & Doerder, 1992). The layer of surface variant antigen on a cell is about 17–25 nm thick (Capdeville, Cardoso De Almeida & Deregnaucourt, 1987; Ramathan et al., 1981). Surface variant antigens contribute 3.5% of the cell's total protein (Preer, 1968). Similar to variant antigens of Trypanosomes (Kinetoplasta; Review: Ferguson, 1999) those of ciliates are anchored to the cell membrane by glycosylphosphatidylinositol, "GPI" (Capdeville et al., 1987). Sphingolipids stabilize GPI-anchored protein-rich membrane domains in ciliate and trypanosomatid cells, with ceramideaminoethylphosphonate (CAEP) being the only abundant sphingolipid (Zhang & Thompson, 1997). A high content of cysteines (11%) characterizes the variant surface proteins. A special periodicity of the cysteines and internal repeats has been observed for surface proteins of *Paramecium* (Prat et al., 1986), *Tetrahymena* (Deak & Doerder, 1995) as for *Giardia lamblia* (Diplomonadea; Gillin et al., 1990). The deduced amino-acid sequence of the α-51D gene of *Paramecium tetraurelia*, except for 103 N-terminal and 142 C-terminal amino acids, consists of 33 periods with eight cysteine residues. In addition four half periods containing four cysteines each are present (Breuer et al., 1996). In a small (31.5 kDa) surface protein of *Lembadion bullinum* (Ciliophora) cysteines form a more irregular periodicity (Fig. 2; Peters-Regehr, Kusch & Heckmann, 1997). A major 48 kDa surface antigen of the common fish parasite *Ichthyophthirius multifiliis* (Ciliophora) contains five repeats, each with six invariant cysteines. This spacing of periodic cysteine residues is entirely consistent with the structure of zinc-binding proteins (Clark et al., 1999). Deletion analyses identified a small segment within C-terminal repeating cysteine motifs to be required for expression of *Paramecium tetraurelia* A51 surface antigen (Thai & Forney, 2000). This region contains a number of conserved amino acid residues. All cysteine residues of variant surface antigens are apparently bound in cystine (Reisner et al., 1969), thus the three-dimensional structure of variant antigens is shaped by disulfide bridges (Jones, 1965). This conformation gives a high stability against degradation by proteases which may be important for the function of surface variant antigens.

Flötenmeyer, Momayezi & Plattner (1999) isolated surface antigens and used them to produce antibodies for immunolocalization by confocal imaging and by quantitative immuno-gold EM-labeling. They derived a biosynthetic way that involves the endoplasmic reticulum, golgi apparatus, nonregulated/nondense core vesicle transport, and finally a diffusional spread over nonciliary and ciliary cell membrane (Fig. 3). Still intriguing is the site of surface protein integration into the cell membrane by unstimulated exocytosis. Clear vesicles probably export surface variant antigens via sites which most of the time are occupied by coated pits ("parasomal sacs", vesicles that are located in the ciliate's cortex in association with ciliary basal bodies).

Degradation of surface proteins follows after their concentration in the cytostome and the formation of nascent digestive vacuoles. These mature and release their digestion products at the cytoproct, with partial retrieval by "discoidal vesicles." A second internalization path-

	VA									
1.	CPTVASGAAAVP	CTT	CQTLTGTTTID	CET			CTNSLATYGNPSKVTN	CISATYTAQAQTIT		CTA
2.	CKPGFWVATATS	CTA	CTSP	CSA	CSTSATT	CTA	CITGQFLTGST	CAAYTAIAN	CKTQDTATT	CSA
3.	CNNKFAKTSTTV	CDA	CTDTN	CLL	CASAKGT	CTE	CSGLYFISGGA	CTGNAVTAAVAN	CTIYSNATT	CTN
4.	CSTGYVLASGTS	CVL					CSTELTNATS	CTVSGTTYTAST		
5.	CTTGFYVVTASATVKASA	CGA	CGTN	CST	CTSNTA	CTT				
6.	CATGYDVWNPSGSTVVTA	CSL	CPSASYL		CQTSK		CVTDAVTALLKSYEYLI	CAFAIFVLGIIG		

Fig. 2. Cysteine periodicity of the surface protein "L-factor" of *Lembadion bullinum.* The amino acid sequence is displayed starting with position 1 from the N-terminus to the last transcribed amino acid number 350 (data from Peters-Regehr et al., 1997). Adjacent sequences were aligned according to the occurrence of altogether 43 cysteine residues. Thereby 6 periods (numbered on left side) contain 4–10 cysteines. The spacing of cysteines is sixteen times C-X_2-C within a framework of larger motifs (C-$X_{3\text{-}17}$-C). A generally similar but more regular periodicity of cysteine residues has been observed for surface antigens of other ciliates.

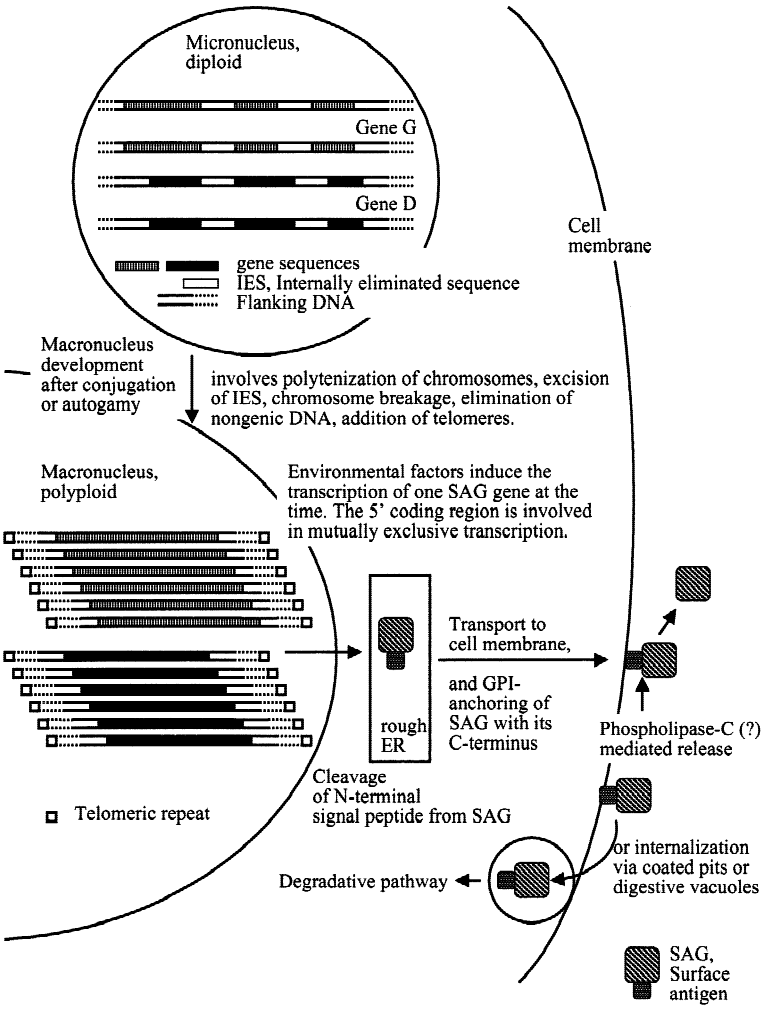

Fig. 3. Schematic summarization of the major events of surface antigen expression in ciliates. Two types of genes are shown, termed G and D, as examples for a set of genes that occur in the micronucleus and in the macronucleus. For references and further explanations, *see* text.

way proceeds via coated pits, early endosomes ("terminal cisternae") and digestive vacuoles. Dense packing of surface proteins in the glycocalyx may drive them into the endo-/phagocytic pathway (Flötenmeyer et al., 1999). Uptake of GPI-anchored surface proteins has been assumed to involve cleavage by a GPI-specific phospholipase C (Capdeville et al., 1986, 1987) or an equivalent enzyme (Assouz & Capdeville, 1992). GPI-anchoring

and enzymatic cleavage may enable high turnover rates of variable surface proteins (Freedman, Kern & Scheele, 1998). A surface antigen of *Lembadion bullinum* may be released, at least in part, into the surrounding medium (Peters-Regehr et al., 1997).

A Family of Genes Codes for Variant Antigens

Unlinked gene loci with several alleles code for surface variant antigens. Stocks of different geographical origin differ in the alleles for two loci in *P. primaurelia*, "D" and "G," expressed at 24 and 33°C respectively, in laboratory conditions. Allelic variant antigens can often be distinguished serologically (Beale, 1952, 1957). A stock expresses its specific G allele and represses the D locus, and *vice versa* (mutual exclusion). After genetic recombination each clone continues to express the serotype of the cytoplasmic parent. In heterozygotes both alleles are coexpressed, either the D variant antigen is specified by both D alleles, or the G antigen by both G alleles. In *Paramecium tetraurelia* at least 14 unlinked mutually exclusive loci could be identified. Each locus has multiple alleles, e.g., 9 alleles at the A locus. Also in *Tetrahymena thermophila* there are several mutually exclusive loci for variant antigens, some with multiple coexpressed alleles (Nanney & Dubert, 1960; Phillips, 1967; Grass, 1972; Juergensmeyer, 1969). Of 2600 isolates of *T. thermophila* from natural populations 43% appeared to have unknown surface antigens (Saad & Doerder, 1995).

Several structural genes for surface proteins have been isolated from *P. tetraurelia* (Forney et al., 1983; Godiska, 1987; Breuer et al., 1996), *P. primaurelia* (Prat et al., 1986) and from *Tetrahymena thermophila* (Tondravi et al., 1990; Deak & Doerder, 1995). The structure of these genes is remarkably similar, except that the antigen genes of *Tetrahymena* are much smaller. A typical feature, besides the periodicity of cysteine codons, is a codon usage that favors A/T in the third position of codons. All sequenced genes are free of introns, but a nucleic-acid splicing process occurs in the generation of macronuclear genes. The occurrence of two kinds of nuclei characterize ciliates, micronuclei with mainly generative functions, and transcriptionally active macronuclei with exclusively somatic functions. In the course of conjugation or autogamy the recombined micronucleus develops a new macronucleus. This macronucleus development involves polytenization of chromosomes, excision of internally eliminated sequences (IES), chromosome breakage, elimination of nongenic DNA, excision of gene-sized molecules, addition of telomeres, and further DNA replication (Reviews: Prescott, 1994; Schmidt, 1996; Fig. 3). In *Paramecium*, IESs are generally short (28-882 bp), AT-rich DNA elements. Data from a mutant cell line that cannot excise a 370-bp IES from the coding region of the 51A gene support a theory that *Paramecium* IESs evolved from transposable elements (Mayer, Mikami & Forney, 1998). The isolation of serotype genes has also shown that the genetic code is used with deviations from the universal code. The stop codon is different in serotype genes as it is in other genes of ciliates (Preer et al., 1985, 1987; Caron & Meyer, 1985; Kuchino et al., 1985; Prat et al., 1986; Martindale, 1989). There is probably only one copy of each variant antigen gene per haploid *Paramecium* micronucleus genome (Forney & Blackburn, 1983). The two genes 51A and 156G are located in the vicinity of the end of a macronuclear "chromosome" oriented with their 3′ ends towards the terminus (Preer, 1986). This parallels to surface antigens from trypanosomes where expression-linked copies of antigen genes were found at chromosomal ends (Myler et al., 1984).

A subfamily of genes codes for the D type of variant antigens in *Paramecium* species. From *Paramecium tetraurelia* that expressed the D serotype Breuer et al. (1996) isolated high molecular weight mRNA, which corresponded to the molecular mass of the D surface protein. Using this D-specific mRNA as a probe for screenings in different genomic libraries a subfamily of five very similar genes was found, named α-51D, γ_1-51D, γ_2-51D, δ-51D and ε-51D. Each of them is about 8-kb long, they show regions of identity to each other, and there is no evidence that any are defective genes or pseudogenes. In *P. primaurelia* three very similar genes, Dα, Dβ and Dγ were cloned (Bourgain-Guglielmetti & Caron, 1996). Of these three genes, only the Dα mRNA was present in the cytoplasm of cells that expressed the D variant antigen. Up to now serotype D is the only known serotype showing this phenomenon. Two of the D isogenes in *P. tetraurelia* are closely linked. The sequences for the entire coding region of the α-51D gene in *P. tetraurelia* and of the Dα gene in *P. primaurelia* have been determined, as well as the upstream and downstream noncoding regions. The deduced amino acid sequence shows the same characteristic cysteine periodicity displayed by all other variant antigen genes from *Paramecium.* However, in contrast to most other such genes, tandem repeats are missing from the 7599-bp long coding region of the α-51D gene, and the 7632-nucleotide open reading frame of the Dα gene. The sequences of the type 51 D genes of *P. tetraurelia*, or the expressed and the nonexpressed D genes of *P. primaurelia*, are identical to a high percentage in the coding as well as in the noncoding regions. Similarity within noncoding regions is usually only observed for allelic antigen genes. Type D genes may constitute a family of isogenes that are nonallelic. They contain slightly different consensus sequences with possible functions as

regulatory regions. A region of variable DNA rearrangement was identified 1 kb upstream of the Dγ gene of *P. primaurelia*. This macronuclear region arises from the same micronuclear locus by alternative excision of internal eliminated sequences during macronuclear development.

Environmental Factors Regulate the Activity of Antigen Genes

Spontaneous changes of variant antigen types frequently happen in single cells or in total cultures of paramecia. Moreover a change of surface antigen type can be induced by changes of culture conditions. Serotype transformation after cultural changes is performed by the majority of cells in a culture, but is not an effect of selection of single cells that spontaneously transformed (Sonneborn, 1943). The changes of variant antigens are reversible, and are not caused by mutations of genes. Durations of transformations depend on the feeding state of the cells. Hungry cells cannot be induced to change their variant surface proteins.

Antony & Capdeville (1989) showed that the temperature during transformation significantly affects the duration of changes. Serotype changes depend on supply of energy and metabolites for RNA- and protein synthesis (Austin, Pasternak & Rudman, 1967*a*). If the feeding state allows changes of variant antigens, e.g., strain 156 of *Paramecium primaurelia* changes from antigen type D at 33°C to type G at 23°C. One hour after decrease of the temperature new G type antigens appear on the cell cortex and the cilia. For approximately 24 hr antigen G accumulates on larger areas at the cell surface, without considerable loss of the old antigen type D. Immobilization tests with D antisera still detect this antigen type. During this intermediate phase both antigen types are present simultaneously. Thereafter the old type of antigens is lost quickly. Thirty-six hours after change of temperature the cell surface is covered by a "coat" of new G type antigens, that is without the old antigen type. Transformations are usually accompanied by four-to-six mitotic cell divisions (Antony & Capdeville, 1989). Yet, serotype transformations don't need cell divisions, they also can take place within one cell generation (Austin, Widmayer & Walker, 1956).

Until now numerous factors were observed which induce serotype transformation. Most effective in laboratory cultures has been the change of cultivation temperatures (Beale, 1957; Austin, 1963*a*). Other environmental factors that induce antigenic changes are the type, pH and salinity of media, proteolytic enzymes, UV and X ray, the alcaloid patulin, actinomycin, puromycin, chloramphenicol, fluorophenylalanine, colchizine and acetamide (Kimball, 1947; Sonneborn, 1947; Van Wagtendonk, 1951; Sonneborn & Schneller, 1950; Sonneborn, 1951; Beale, 1954; Austin et al., 1956; Austin, 1963*b*; Austin et al., 1967*a,b*; Finger, 1967; Finger et al., 1968; Finger, 1974).

Serotype Expression Does Not Seem to Involve DNA Rearrangements

Rearrangements of DNA as they were observed to occur concomitantly with shifts in gene expression in trypanosomes (Myler et al., 1984) are doubtful due to distinct peculiarities of the serotype system. The speed of the serotype transformation, the high level of ploidy in the macronucleus and the fact that nearly all cells in a culture participate in the transformation process are difficult to reconcile with a regulation of gene expression by DNA rearrangements. Indeed, rearrangements of the sizes typical for the other organisms have never been found for *Paramecium*, despite numerous experiments to detect them (Forney et al., 1983; Meyer, Caron & Baroin, 1985; Schmidt, 1987).

The differential expression of type A and B surface antigen genes in *Paramecium tetraurelia* stock 51 is regulated at the level of transcription (Leeck & Forney, 1996). The 5′ coding region of the antigen genes controls the mutually exclusive transcription. A region downstream of the transcriptional start site between nucleotide positions +1 and +885 (relative to translational start) is necessary to control differential transcriptional activity.

Serotype Expression is Generally Stable

Serotype expression is generally stable, that is the cytoplasmic state is perpetuated after genetical recombination. This inheritance of a cytoplasmic state also applies to other features in some ciliates, e.g., mating types in the *Paramecium aurelia* species group. A significant role of cytoplasmic factors in this clonal inheritance of the variant antigen phenotype is indicated by the observation that cytoplasmic exchange can interrupt the clonal lineage (Bleyman, 1996). A "no A" mutant in *P. tetraurelia* (Epstein & Forney, 1984) showed an inability to incorporate properly the micronuclear gene into the macronucleus. During the processes of genome fragmentation and telomere addition that occur during macronuclear differentiation (Blackburn & Karrer, 1986), the A gene in the mutant is truncated, and the 5′ end of the gene gets the telomere addition (Forney & Blackburn, 1988). This defect could be cured by macronuclear differentiation in the presence of wild-type cytoplasm or by the injection of wild-type macronucleoplasm into either macronuclei or cytoplasm of mutant cells (Harumoto, 1986). The DNA segment coding for the information that allows for the correct differentiation processes

which then results in a functioning copy of the micronuclear A gene in the macronucleus has been identified (You et al., 1991). It may itself be the processing factor (Jessop-Murray et al., 1991). This gene segment is apparently present in the cytoplasm only between the 1st and 2nd post-zygotic divisions, when fragments of the old macronucleus are present. It remains within the macronucleoplasm at other times. It is quite likely that it is the fragmentation that releases the product and causes the cytoplasmic effects (Koizumi & Kobayashi, 1989). Essentially, then, the DNA organization of the post-zygotic macronucleus is controlled by the preconjugant macronucleus, resulting in an apparent self-perpetuating pattern of cytoplasmic inheritance, or, a stable nuclear differentiation (Sonneborn, 1977).

In *Tetrahymena thermophila* SerH genes are variably and reproducibly rearranged during macronuclear development (Kile et al., 1988). As in *Paramecium, T. thermophila* wild-type cytoplasm apparently contains gene products necessary for the normal macronuclear differentiation leading to expression of the *serH* locus (Doerder & Berkowitz, 1987). However, unlike in *Paramecium* mutants, these gene(s) are not part of the variant antigen locus. Regulatory elements map at or close to the structural locus (Doerder, Berkowitz & Skalican-Crowe, 1985), and also exist as unlinked genes, which do not specify any variant antigen (Doerder, 1979). Some of these regulatory genes affect the transcription of the mRNA for the variant antigen, while others allow transcription, but block the synthesis of the variant antigen by affecting the stability of the mRNA (McMillan et al., cited in Bleyman, 1996).

Surface Antigens in Protozoa may Function as Defenses Against Environmental Chemical or Biotic Factors

Selection obviously favors the expression of variable surface antigens in paramecia and other ciliates genera, despite physiological costs, since strains without surface proteins have never been found. One suggestion on possible functions of variable surface antigens in protozoa is that they may affect the function of the calcium channel and thus play a role in membrane excitability (Ramathan et al., 1983). Variable antigens could be part of the cell's signal reception cascade. Depending on the prevailing environmental conditions, like temperature, the type of antigen that is best functioning in signal reception or transmission at these conditions is expressed (Capdeville, 1979*a,b*). Other authors favor defensive functions against environmental biotic or abiotic factors (Preer, 1986).

Biochemical characteristics of variable surface antigens, like the number of cysteines, indicate a defensive function. Surface antigens of *Giardia lamblia* are also very rich in cysteine residues. They were postulated to be responsible for an increased resistance of this gut parasite to proteases of the host (Gillin et al., 1990). Possibly *Paramecium* is defended by its cysteine-rich surface antigens against proteases, that are secreted from bacteria and fungi living in the natural habitat of *Paramecia* (Capdeville et al., 1993).

A hypothesis of Harumoto and Miyake (1993) gives variable surface antigens of paramecia a defensive function against predators. *Didinium* species (Ciliophora) are protozoa specialized on paramecia as food organisms. Specialization on single food species requires a recognition system for this food, that may be a receptor for surface proteins in the *Didinium/Paramecium* system. Variation of surface proteins could interfere with this prey recognition system.

Information on functions of surface proteins in free-living protozoa came from research on regulation of prey-predator relationships via released chemical signals. In several ciliates, e.g., of the genus *Euplotes*, morphological or behavioral defenses are induced by signal substances ("kairomones") from predators (Kusch, 1993*a,b*). Released kairomones are recognized by the potential prey ciliates and favor their survival via defense development. Purified kairomones of different predators were identified to be small proteins of 4.5–31.5 kDa. The gene sequence, as well as immunocytochemical staining, showed the kairomone of the ciliate predator *Lembadion bullinum* (Ciliophora) to be a surface protein with typical cysteine periodicity and having high general homology to variant antigens of *Paramecium* and *Giardia* species (Peters-Regehr et al., 1997). Biological tests with purified kairomone of the predator *Amoeba proteus* (Amoebozoa, Gymnamoebia) showed that surface-bound kairomones in protozoa presumably function as self-recognition signals. Coating of agarose beads with kairomone inhibited phagocytosis of the beads by amoebae. The beads were taken up if they were covered with albumin, or were uncoated (Kusch, 1999). Self-recognition inhibits phagocytosis of clonemates in these asexually reproducing organisms. Turnover of surface proteins by cleavage from the GPI-anchor releases small amounts of the surface protein into the environment where it is recognized by potential prey ciliates. This led to the evolution of predator-induced defense in ciliates. Whether variant types of kairomones from predacious protozoa exist, is not known, but kairomones give us one example of possible functions of surface proteins in free-living protozoa.

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