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Inefficient serotype knock down leads to stable coexistence of different surface antigens on the outer membrane in *Paramecium tetraurelia*

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Abstract

Expression of surface antigens is usually mutually exclusive, meaning that only one protein is present on the cell surface. With the RNAi feeding technology we induce serotype shifts in *Paramecium tetraurelia* which are demonstrated to be incomplete, meaning that the cells remain in a shifting state. The coexpression of "old" and "new" protein on the surface can be detected to be stable for more than 15 divisions over a 5-day feeding procedure, a time period different from that reported for temperature-induced shifts. A characteristic heterogenic distribution of the different surface antigens is demonstrated by double indirect-immunofluorescent-staining and we show antigen transport mechanisms related to the tips of cilia. Therefore, we discuss release mechanisms, potential sorting mechanisms for glycosylphosphatidylinositol-anchored proteins and the localizations of surface antigens, which are important for the reported classical immobilization reaction.

Keywords: Surface antigen; *Paramecium*; Sorting mechanism; RNAi by feeding; Immobilization reaction

Introduction

The expression of variant surface antigens is a phenomenon which is to be observed in many protozoa. These proteins are located on the outer membrane and are inserted by a glycosylphosphatidylinositol (GPI) anchor (Capdeville et al. 1987a). The number of different surface antigen proteins known to be expressed by *Paramecium tetraurelia* is 11, but recently the genome project revealed that the multigene family of surface antigens contains many more members than expected (Sperling et al. 2002). A serotype is defined by the mutually exclusive expression of surface antigens, which means that only one antigen is expressed at a time. *Paramecium* shows a temperature-dependent expression of serotypes (for reviews see Beale 1954; Kusch and Schmidt 2001), and a coexpressive state, where two proteins are detectable on the surface, was reported to exist for an extremely short time period during the temperature-induced serotype shift (Antony and Capdeville 1989). Release of the "old" protein into the culture medium during such a temperature-induced serotype shift was described by Momayezi et al. (2004). The name antigen is due to the discovery by Rössle (1905) that, after Paramecia were injected into a mammal, there was a specific reaction of live Paramecia to isolated antiserum. This immobilization reaction resulting from fusions of the outer membrane by the binding of

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homologue antibodies in the added antiserum is still the easiest test to check a culture for the type of expressed surface antigen.

We report here that we can induce the stable coexistence of two antigens on the cell surface. In contrast to temperature-induced shifts, the described RNAi-induced serotype shifts can result in such a coexpression. This is documented in this study by parallel indirect immunofluorescence staining of Paramecia with two antibodies against the ''new'' and the ''old'' protein. For the target serotype we chose serotype 51A of *P. tetraurelia,* which is described as stably expressing 51A protein at 27 °C, but at 14 °C this protein is replaced by serotype 51H under standard cultivation conditions (Schmidt 1983).

Serotype function and expression was analyzed in the past by ''knock out'' mutants produced by X-radiation (Epstein and Forney 1984) but we have now the possibility to produce ''knock down'' cultures in which the old antigens are only partially ''silenced'' by the RNAi technology. RNAi phenotypes or so-called ''silencing'' phenotypes do not show a complete knock out of the expression of a target gene and are made using the feeding technology, where dsRNA-producing bacteria are fed to the Paramecia, resulting in post-transcriptional degradation of homologue transcripts in Paramecia. The level of knock down depends on several parameters, which cannot at present be defined because these mechanisms are not yet completely understood.

The silencing phenotypes were determined at different temperatures, revealing a heterologous distribution of the remaining 51A protein and the up-coming alternatively expressed protein (51D or 51I). Our results indicate that cortex membrane and ciliary membrane play different roles in serotype distribution and we propose a sorting and transport mechanism for GPI-anchored proteins in connection with intraciliary transport systems. We show that only cilia-surface antigens are involved in the reaction of the cells in the immobilization reaction. The possible role of an active release mechanism is discussed.

Materials and methods

Cultivation of Paramecia

Wild type cells of stock 51 (*P. tetraurelia*) were grown in wheat-grass-powder-medium (WGP) (Pines International Co., Lawrence, KS), supplemented with 0.8 mg/l β-sitosterol (Merck, Darmstadt, Germany), inoculated with *Klebsiella minuta* the day before. Expression of serotype 51A was induced at a cultivation temperature of 27 °C.

Immobilization reaction

Serotype expression was analyzed by standard immobilization test by adding homologous polyclonal antisera (1:100) to washed cells, resulting in a specific reaction of the cells. Results were assessed after 30 min.

RNAi by feeding

PCR-products of target-sequences of gene 51A were cloned into the LITMUS 38i-Vector (NEB, Beverly, MA) by restriction with EcoRV (Promega, Mannheim, Germany) and adding T-overhangs according to Marchuk et al. (1990) and were then transformed into *Escherichia coli* HT115DE3. The location in the open reading frame of the 51A gene of the fragment used in RNAi experiments was 7458–8154. 25 ml Luria Bertani (LB)-Medium (+100 µg/ml Ampicillin) were inoculated with 250 µl pre-culture of the *E. coli* and were incubated with shaking at 37 °C. 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were added when the culture reached OD_{595}0.4 and three hours later bacteria were separated from the medium by centrifugation for 15 min at 3000g. They were then transferred into 250 ml of WGP complemented with 100 µg/ml Ampicillin, 0.5 mM IPTG and 0.8 mg/l β-sitosterol. Paramecia were washed twice in Volvic™ and introduced into this bacterial suspension in WGP; this procedure was repeated each day for 5 days, and every day the same number of cells was transferred into fresh medium to provide a stable division rate of the cells. The division rate was also measured during the experiments. As a negative control, bacteria transformed with the LITMUS 38i-Vector lacking the cloned gene fragment were fed to Paramecia in the same way. Silencing experiments were performed during 5-day culturing of Paramecia at two different temperatures, 27 and 14 °C.

Immunofluorescence microscopy

Paramecia were washed twice in Volvic™ and fixed in paraformaldehyde (4% in phosphate-buffered saline, PBS: 0.137 M NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) for 1 h. The methodology was as follows: washing briefly in PBS, washing in 50 mM glycine-PBS, blocking 45 min in 3% BSA, 30 min incubation with primary antibodies (1:700 in PBS), 30 min washing with PBS, 30 min incubation with secondary antibodies (1:100 in PBS) and finally washing twice in PBS. Stained cells were set into Vectashield® (Vector, Burlingame, CA) to image them by microscopy. The primary antibodies used were the mouse-Y4 monoclonal antibody (kindly provided by Y. Capdeville, description in Capdeville et al. 1987b), rabbit-anti-51D polyclonal serum and rabbit-anti-51I polyclonal