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The Membrane Skeleton in *Paramecium*: Molecular Characterization of a Novel Epiplasmin Family and Preliminary GFP Expression Results

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Previous attempts to identify the membrane skeleton of *Paramecium* cells have revealed a protein pattern that is both complex and specific. The most prominent structural elements, epiplasmic scales, are centered around ciliary units and are closely apposed to the cytoplasmic side of the inner alveolar membrane. We sought to characterize epiplasmic scale proteins (epiplasmins) at the molecular level. PCR approaches enabled the cloning and sequencing of two closely related genes by amplifications of sequences from a macronuclear genomic library. Using these two genes (*EPI-1* and *EPI-2*), we have contributed to the annotation of the *Paramecium tetraurelia* macronuclear genome and identified 39 additional (paralogous) sequences. Two orthologous sequences were found in the *Tetrahymena thermophila* genome. Structural analysis of the 43 sequences indicates that the hallmark of this new multigenic family is a 79 aa domain flanked by two Q-, P- and V-rich stretches of sequence that are much more variable in amino-acid composition. Such features clearly distinguish members of the multigenic family from epiplasmic proteins previously sequenced in other ciliates. The expression of Green Fluorescent Protein (GFP)-tagged epiplasmin showed significant labeling of epiplasmic scales as well as oral structures. We expect that the GFP construct described herein will prove to be a useful tool for comparative subcellular localization of different putative epiplasmins in *Paramecium*.

Key words: *Paramecium*; membrane skeleton; epiplasmins; multigene family; GFP; morphogenesis.

Introduction

A stabilizing network of plasma membrane-associated proteins has been described in the cortex of many eukaryotic cells. This network, termed the membrane skeleton, was initially characterized in erythrocytes. After these pioneering studies, proteins of the erythrocyte membrane skeleton have been found in a variety of non-erythrocytic cell types. Spectrins, a family of actin-binding proteins, were identified as the major protein components of the membrane skeleton, which comprises several additional proteins linking the spectrin/

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actin complex to the inner face of the plasma membrane. The growth in sequence information has resulted in the identification of key motifs in protein components of the spectrin-based network which indicate specific interactions and functions which are of particular interest in the comprehension of cortical dynamics and cell morphogenesis (Bennett and Baines 2001).

However, a spectrin-based membrane skeletal system does not appear to be a general feature of protozoan cells. An alternative to this system is the epiplasmic membrane skeleton sustaining the membranous complex (cell membrane and underlying alveolar sacs) in ciliated protozoans. Available molecular data indicate that major epiplasmic proteins are structurally unrelated to spectrins and lack key motifs required for interaction with actin-based structures. Ciliate epiplasmic proteins include articulins, plateins, and Epc1p characterized respectively in Pseudomicrothoracidae, hypotrichs and Tetrahymenidae (Bouchard et al. 2001; Huttenlauch et al. 1995, 1998a; Kloetzel et al. 2003; Williams 2004). They also include a family of poorly defined proteins, termed the epiplasmins, which are prominent epiplasmic components of *Paramecium*. Epiplasmins were previously identified by cell fractionation, protein biochemistry, and immunological techniques (Coffe et al. 1996; Nahon et al. 1993). Strikingly, the data indicate an extremely complex family of antigenically related proteins, comprising up to 40 polypeptides with apparent molecular masses ranging from 30 to 50 kDa and showing remarkable in vitro reassembly properties consistent with the membrane skeletal function ascribed to the epiplasm in *Paramecium* cells. Attempts at epiplasmin molecular characterization were initiated by direct internal peptide microsequencing and use of deduced DNA primers in PCR amplification experiments (Coffe et al. 1996). However, the data were inconclusive due to the shortness of available sequences.

In this study, we have pursued the previous work and obtained, for the first time, the complete sequence of two *Paramecium* epiplasmins. To elucidate the heterogeneity of epiplasmins indicated by immunological approaches, we also sought homologous genes within DNA supercontigs obtained from sequencing of the *P. tetraurelia* macronuclear genome. Forty-one paralogous genes were identified, establishing that *Paramecium* epiplasmins represent a new multigenic protein family. The results of preliminary attempts to transform *Paramecium* cells using Green Fluorescent Protein (GFP)-tagged epiplasmins are also reported.

Results

Molecular Characterization of Epiplasmins 1 and 2 in *Paramecium tetraurelia*

Before this study, preliminary data on *Paramecium* epiplasmin sequences were published by Coffe et al. (1996). These authors described experimental conditions for solubilization and in vitro reassembly of epiplasmins. In the same study, the authors also reported partial characterization of *Paramecium* epiplasmins using internal peptide microsequencing. DNA primers were deduced from these peptide sequences and used for PCR on crude genomic DNA. The sequences obtained (maximal length: 162 bp) were clustered in three distinct groups, which yielded an initial insight into the genetic complexity of *Paramecium* epiplasmins. We used these data to synthesize new primers to obtain more extended amplification products. *Paramecium* macronuclear genomic DNA cloned in an EMBL3 λ phage bank was used as a template for a new series of PCR experiments. Primers Para-992 and Para-QPVsens allowed us to amplify Epipara 7-6, a 401 bp fragment which was then cloned and sequenced. Analysis of the Epipara 7-6 sequence confirmed the presence of some heptad repeats (QPVQ-h-, in which "h" is a hydrophobic residue) also revealed by Coffe et al. (1996) and previously considered as a possible hallmark of *Paramecium* epiplasmins (see Discussion). The fragment size was unexpected: 401 versus 54 bp, due to the fact that the Para-QPVsens primer annealed to a matching sequence localized 347 bp upstream of the expected target sequence. Screening of the EMBL3 λ bank using the Epipara 7-6 probe revealed several positive plates. However, for an unknown reason, selected clones displayed unusual instability preventing subcloning or sequencing of DNA of interest. This prompted us to screen another macronuclear genomic DNA library, the *P. tetraurelia* macronuclear genomic DNA indexed library constructed in the plasmid pBluescript II KS- (Keller and Cohen 2000). This library is composed of 61 440 clones containing $\approx$10 kb inserts from the macronuclear genome. Initial screening consisted of probing a replica of 160 polyclonal "mother wells", each corresponding to a mix of 384 clones. For each positive response, a second screening was performed to identify the clone(s) of interest among the 384. The Epipara 7-6 probe was used to screen this indexed genomic DNA library. Strong signals were observed for 40p22 and 103h9 clones. Plasmid