Acquisition of cell polarity during cell cycle and oral replacement in *Tetrahymena*

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ABSTRACT The aim of this study was to search for a mechanism responsible for the acquisition of cell polarity in a ciliate *Tetrahymena*. Homologs of the mammalian genes coding for CDC42-GSK3β-MARK/PAR1-MAPs proteins were found in the *Tetrahymena* genome (Eisen et al., 2006, and this study). These proteins belong to a pathway which controls assembly and disassembly of microtubule bundles and cell polarity in neural cells. In *Tetrahymena*, there are two types of morphogenesis: divisional and oral replacement (OR). In divisional morphogenesis, an elongation of longitudinal microtubule bundles (LMs) takes place during cell division. In contrast, in OR type morphogenesis, which occurs in starved non-dividing cells, a polar retraction of LMs occurs. In *T. pyriformis*, the frequency of developmental switch to OR morphogenesis increases in the presence of wortmannin, an inhibitor of the CDC42-GSK3β-MARK pathway. In contrast, wortmannin when applied to dividing cells does not affect divisional morphogenesis. Using immunostaining with the antibody against mammalian mitotic phosphoproteins (MPM-2) we show that these proteins co-localize with the LMs and are distributed along the anterior-posterior gradient. In addition, we show that during OR type morphogenesis, the fate of LMs correlates with the anterior-posterior gradient of instability of the cortical structures. We used the conditional mouthless mutant of *T. thermophila* (Tiedtke et al., 1988) to test if the presence of the oral apparatus is required for the maintenance of cell polarity. We discuss our results in relation to the hypothesis of GSK3-β-MARK pathway involvement in the acquisition of cell polarity in *Tetrahymena*.

KEY WORDS: *Tetrahymena*, polarity, cytoskeleton, MARK, armadillo

The *Tetrahymena* (like other ciliates) is a permanently polarized cell. Longitudinal ciliary rows (CR) define its anterior-posterior axis and a sub-apically located oral apparatus (OA) marks its ventral side. The position of other cortical structures depends on the position of the oral apparatus (Frankel 1999) (Fig. 1A and Fig. 7A). In *Tetrahymena* the spatial deployment of the cytoskeletal proteins is under genetic control of a mechanism of global positioning (Kaczanowski 1975, Williams et al., 1987, Jerk-Dziadosz et al., 1995, Kaczanowska et al., 2003, Strzyzewska-Jowko et al., 2003). The aim of the present study was to gain an insight into the mechanism responsible for the acquisition and maintenance of cell polarity, during morphogenesis of *Tetrahymena*.

In *Tetrahymena* there are two types of morphogenesis: divisional and oral replacement (OR). The cortex of *Tetrahymena* contains a regular array of polarized longitudinal microtubular bands (LMs) running next to meridians of the rows of basal bodies. The assembly of LM-bands is unidirectional (Allen 1967; Ng 1978, 1979; Ng and Frankel, 1977) and they play an important role in divisional morphogenesis as a putative cytospindle (Jerk-Dziadosz et al., 1995). The anterior-posterior axis is preserved during cell division that results in anterior and posterior offspring (Frankel et al., 1981; Frankel 1989; Kaczanowska et al., 1993;

Abbreviations used in this paper: AF, anarchic field of oral primordium; CR, ciliary rows of basal body units; CVP, the contractile vacuole pore located at the posterior area of the cell to the right of the ventral side; EHT, end of heat treatment; LM, Longitudinal microtubule bands; nOA, newly formed oral apparatus that appears during oral replacement morphogenesis; OA,oral apparatus; OA1, parental oral apparatus; OA2, new oral apparatus for posterior daughter cell; OR, oral replacement; RM, remnant of engulfed and regressed anterior oral apparatus.

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Published online: 8th February 2008.

0214-6282/2008/$35.00
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Printed in Spain
process: the anterior-posterior (Nanney 1975; Kaczanowski, 1978; added into ciliary rows. There are two “gradients” of this addition divisional morphogenesis the new unciliated basal bodies are basal bodies and the middle region of the parental cell with the local intercalation of parental cell undergoes continuous unidirectional elongation in (Frankel, 1999)., 1995). The two types of morphogenesis in Tetrahymena. (A) Cortical patterns during segmental divisional morphogenesis. (B) Cortical patterns during oral replacement (OR) morphogenesis.

Nelsen et al., 1994) (Fig. 1A). Prior to cytokinesis, the LM- bands maintain their continuity until the fission line separates them. Eventually, after the cytokinesis, the apical band reforms (Jerka-Dziadosz et al., 2001; Thazhath et al., 2002; 2004). Thus the parental cell undergoes continuous unidirectional elongation in the middle region of the parental cell with the local intercalation of basal bodies and the in situ addition of microtubular subunits into the LM-bands; the process which is consistent with Frankel’s (1989) model of “the continuum growth cylinder”. During early divisional morphogenesis the new unciliated basal bodies are added into ciliary rows. There are two “gradients” of this addition process: the anterior-posterior (Nanney 1975; Kaczanowski, 1978; Frankel et al., 1981) and the circumferential gradient (Kaczanowski, 1978). In the nascent posterior daughter cell, the new oral primordium appears to the left of the postoral ciliary row #1 as an “anarchic field of basal bodies” (AF) (Fig. 1A). Its position corresponds to the site of the intersection of both gradients of the proliferation of the basal bodies (Kaczanowski, 1978). This anarchic field develops into the oral apparatus, which, at its anterior, contains fission line that runs perpendicularly across all ciliary rows (Frankel et al., 1981; Nelsen et al., 1994; Kaczanowska et al., 1999; Strzyzewska-Jowko et al., 2003) (Fig. 1A). This segmental pattern of cortex development is synchronized with a bipolar micronuclear mitosis in T. thermophila and with a bipolar macronuclear “amitosis” in both T. thermophila and T. pyriformis (reviewed by Frankel, 1999).

In Tetrahymena, the submembrane skeleton containing at least three epiplasmic proteins EpiA, EpiB and EpiC (Williams et al., 1995) and at least two Ca\(^{2+}\) binding proteins, (Hanyu et al., 1990, Williams and Nelsen, 1973). The two types of Tetrahymena morphogenesis are summarized in Fig. 1 A, B and Fig. 2 A-C.

In this study, we asked whether the re-formation of an anterior-posterior axis and the ventral positioning of the oral apparatus occurring during Tetrahymena morphogenesis are controlled by CDC42- GSK3β-MARK pathway regulated by a nutritional signal. It is known that this pathway is involved in the generation of cell polarity in metazoan neural cells (Doble and Woodgett, 2003) and non-neural cells (Etienne-Manneville and Hall, 2003). In differentiating neural cells, the effector mechanism for the establishment of cell polarity involves the cascade of activities of specific kinases. The GSK3β (glycogen synthase kinase 3), and MARK kinase (Microtubule Affinity Regulating Kinase) regulate the lengthening and linear assembly/ disassembly of the bundles of microtubular subunits during the neuritogenesis through specific phosphorylation of a set of microtubule-associated proteins (MAPs) (Drewes et al., 1997; Johnson and Stoothoff, 2004; Wiggins et al., 2005). The GSK3β kinase activates MARK/ PAR1 effector kinase by a structurally directed phosphorylation (Kosuga et al., 2005) and then MARK/PAR1 destabilizes MAPs. This pathway is controlled by CDC42 complex (Cau and Hall, 2005) involved in posterior localization of MARK/PAR1 kinase (Schonegg and Hyman, 2006). Hence, the primary task in Tetrahymena genome research is the search for the putative genes encoding the CDC42- GSK3β-MARK- MAP pathway. If these genes do really exist in Tetrahymena genome, the putative CDC42, GSK3β (ser9) and MARK/PAR1 proteins should be identifiable on Western blots.
of homogenized *Tetrahymena* cells with the available commercial antibodies against cognates metazoan proteins.

The next question concerns the character of the upstream components of this signaling pathway. During the neural differentiation of metazoan cells the anterior-posterior gradient of activity of GSK3\(\beta\) renders the stabilized posterior pole and a highly active and structurally unstable, growing anterior cone of the neurite (Biernat et al., 2002). This linear gradient of growth and structural stability of the neurite is induced by the upstream Wnt signaling transmitted to GSK3\(\beta\), and by the posterior location in cell of MARK/PAR1 protein (Doble and Woodgett, 2003). Therefore, we were also searching for the presence of possible orthologs of a Wnt signaling pathway in the genome of *Tetrahymena*.

The presence or absence of cell polarity during oral replacement (OR) morphogenesis in *Tetrahymena* can be tested by the use of the MPM-2 antibody against mitotic phosphoproteins. The MPM-2 monoclonal antibody binds to a plethora of phosphorylated proteins present in mammalian basal bodies and the mitotic spindle (Westendorf et al., 1993). Previously we described the cortical patterns and polarity of MPM-2 immunostaining during cell cycle of *Tetrahymena* (Kaczanowska et al., 1999). Our present studies were focused on the pattern of MPM-2 immunoreactivity in *Tetrahymena* cells during OR morphogenesis.

In non-neural metazoan cells, the signal upstream of PI-3-kinase drives the activation of Akt kinase regulating the polarity and asymmetry of the cytoskeleton (Ahringer 2003; Doble and Woodgett, 2003). In metazoan cells the specific inhibitor, wortmannin, inhibits activity of the PI-3K pathway and Akt kinase, hence induces abnormal activity of the GSK3\(\beta\) (Kozma and Thomas, 2002). Wortmannin acts on the PI-3K (class I) pathway in *T. thermophila* and *T. pyriformis* (Leonardiris et al., 2005) affecting, tentatively via insulin-like signaling (Christensen et al., 2003), the food-vacuole formation (Zackroff and Hufnagel, 2001) and various components of the cytoskeleton (Kovacs and Pallinger, 2003). Since wortmannin arrests degradation of proteins at the initial sequestration step (Petiot et al., 2000), we tested the effects of wortmannin on the divisional morphogenesis of *Tetrahymena*.

**Fig. 2. *Tetrahymena* in two types of morphogenesis. (A-C)** Immunostaining with the anti-B epiplasma antibody; “negative” staining is seen, i.e. the cortex remains unstained in the regions occupied by microtubular organelles. (D-F) Anti-MPM2 antibody immunoreactivity; positive staining of phosphoproteins associated with cortical structures of *Tetrahymena* in OR morphogenesis can be seen. (A) The early dividing cell with a “negative” pattern of cortical microtubules: in the OA1, AF region, basal body ciliary rows and the LM-bands. (B) The dorsal side of dividing cells with “negative” pattern of basal body ciliary rows and LM-bands, and with diminished immunostaining of apical area in parental cell, and in the area posterior to the fission line. (C) The cell in OR morphogenesis, Region of OA1 and the early anarchic field for new OA2 (AF) remain unstained; note the distinct “negative” pattern of the LM-bands. (D) Cell with extremely strong MPM-2 staining of the engulfed oral apparatus (RM) and with strong immunostaining of the newly formed oral apparatus (nOA) with the moderately immunostained dispersed anarchic field (AF). (E) Cell in OR at an advanced stage; the remnant (RM) of the engulfed oral apparatus is reduced to a strongly shining RM-pocket and some traces of the closed slit; the posterior shining field is subdivided into two entities (nOA and nOA?) along the ventral side. (F) Dorsal side of the cell in OR; note very intense and polarized immunostaining of LM-bands.

**Results**

**Search of *Tetrahymena* genome for the components of Cdc42-GSK3\(\beta\)-MARK pathway**

**GSK3\(\beta\)**

We searched *Tetrahymena* genome for the possible homologs of the mammalian gene encoding GSK3\(\beta\) protein. We used the query sequence of human GSK3\(\beta\) (GSK3\(\beta\)-human uniprot ID) and the tblastn computer program, provided by the www interface http://tigrblast.tigr.org/er-blast/index.cgi?project=tgt. We found DNA sequence Q248B3-TETH in the *Tetrahymena* genome encoding a protein homologous to a GSK3\(\beta\) kinase with an extremely low expected value: 3.8e-63. Another blast search showed that all other proteins most closely related to the candidate *Tetrahymena* gene belong to the family of GSK3\(\beta\) kinases present in various species of animals.

The results of blast analysis prompted us to test the antibody directed against metazoan inactivated GSK3\(\beta\) kinase i.e. the
GSK3β (ser9) (Santa Cruz BT) on *Tetrahymena* cells. This antibody recognized single protein band of about 47 kD in the homogenate (L) and microsomal (P) fraction of *Tetrahymena* cells (Fig. 3, arrow).

**MARK/PAR1**

According to Eisen *et al.* (2006) MARKs genes are present in *T. thermophila*. We carried out the tblastn search in a *Tetrahymena* genome using www interface http://tigrblast.tigr.org/er-blast/index.cgi? project=ttg and a human sequence of mark1 (uniprot ID mark1-human). We detected the homologous sequence with a very small 4.4.e-43 expected value. This sequence corresponds to the putative sequence of the SNF1 kinase of *Tetrahymena* (Q22XQ2). Alignment of this sequence with human MARK1 showed sites responsible for activation of phosphorylation 202 and 212 (Kosuga *et al.*, 2005) with corresponding sites in *Tetrahymena* Q22XQ2, and containing “T” motif typical for human MARK, but without a header sequence for centrosomal localization (Fig. 4) (Tassan and Le Goff 2004). The KIAA human domain of MARK1 is homologous to the KIAA Q22XQ2 domain with expected value 3e-29, different human aapk2 and marks kinases (e.g. to human protein p78/c-tak protein. This sequence is similar to the OSK1 protein of *Oriza sativa* (plant) and to human protein p78/c-TAK1, which in mammalian cells sequestrates MARK kinase in a cytoplasm (Peng *et al.*, 1998). The search for *Tetrahymena* homologs of mouse mark2 (ID MARK2_Mouse) PAR1B detected also many related sequences (Eisen *et al.*, 2006).

Western blot analysis of the total homogenate and the microsomal fraction of *Tetrahymena* cells demonstrated that the antibody raised against C-TAK1 recognizes a single 36 kD protein band (Fig. 5, arrow). Peng *et al.* (1998) reported that c-tak1 protein is cleaved at its NH2 and COOH-terminal ends to generate the 36 kD catalytic domain. It is possible that similar process occurs in *Tetrahymena* and that 36 kD band represents a cleaved product. So far we were unable to visualize the presence of this kinase in the LM-bands in *Tetrahymena*.

**CDC42**

This GTPase is a common component of the GSK3β-MARK pathway (Cau and Hall, 2005) which is possibly involved in the localization of MARK/PAR1 kinase at the posterior pole of the polarized metazoan cells. A gene coding for putative small GTP-binding protein closely related to CDC42 proteins (uniprot ID Q23GR6-TETTH) was found in the *Tetrahymena* genome. When we carried out a blastp search against the uniprot data base we detected sequence with strong homology to the genes coding for animal CDC42 proteins. The candidate gene was also homologous to CDC42 of *Schizosaccharomyces pombe* with expected value 2e-52. Immunoblot analysis of *Tetrahymena* cell homogenate and microsomal material with monoclonal antibody against CDC42 showed a single 29 kD protein band (Fig. 6 arrow).

**MAPs: Search for MAP1B, MAP2, tau and MAP4**

These proteins contain PFAM domain tubulin binding repeat and are exclusively metazoan. We carried out a tblastn search in *Tetrahymena* genome using www interface http://tigrblast.tigr.org /er-blast/index.cgi? project=ttg and a human sequence of MAP1B (uniprot ID MAP1B_Human) and we detected a low complexity homologous sequence with a very high expected value (0.11). The same search with a sequence for human MAP2 protein (uniprot ID MAP2_Human) also detected a homologous sequence with a very high expected value (0.46). Search for the human tau protein (uniprot ID Q5XWF0) detected a low complexity sequence with expected value 0.99. Finally, a search for the human MAP protein 4 (uniprot ID MAP4_Human) detected a low complexity sequence with expected value 0.67. Western blot with anti-tau5 (anti-five isoforms of tau) antibody also yielded a negative result (not shown, Matui *et al.*, 2004).
Therefore in agreement with previous report (Eisen et al., 2006) it is unlikely that Tetrahymenagenome is encoding MAP1B, MAP2, tau and MAP4 proteins.

MAPs of Tetrahymena

So far there are two identified MAPs that are able to bundle subunits of microtubules in experimental assay (Matui et al., 2004). These are: the GAPDH protein and another protein probably corresponding to the fragment of the XMAP 215 microtubule-associated protein participating in the formation of the mitotic spindle in Xenopus cells. Eisen et al. (2006) reported in Tetrahymena two sequences of XMAP215: TThERM_00439010 and TThERM_00488190. Matui et al. (2004) also found another MAP in Tetrahymena, which has not been characterized yet.

In summary the in silico search of sequenced Tetrahymena thermophila genome (TIGR http://www.tigr.org/tdb/e2k1/ttg/) identified the orthologs of the GSK3β- MARK2/PAR1/kin1 pathway. The corresponding bands were also detected in fractionated Tetrahymenaby Western blotting with metazoan antibodies (Figs. 3, 5, 6).

Search of Tetrahymena genome for upstream components of Wnt signaling

Dishevelled and axin mediate upstream signaling cascades to regulate cell polarity in metazoan (Wallenga and Habas, 2005; Jiang et al., 2005). We used as a query, the sequences for human dishevelled (uniprot ID dvl1_human) and human axin (uniprot ID axn1_human). These proteins contain smart domain dax. This is exclusively animal domain according to smart server, and therefore, as expected, we were unable to detect any homologous sequences in Tetrahymena genome.

β-catenin

First, we searched for the gene encoding β catenin in Tetrahymena genome using the tblastn computer program provided by www.interface http://tigrblast.tigr.org/erblast/index.cgi?project=ttg using the sequence of Drosophila β catenin (ARM_DROME uniprot ID). We were unable to detect any homologous sequences in the Tetrahymena genome.

The catenin protein contains many armadillo repeats. Using a smart server http://smart.embl-heidelberg.de/ we identified a putative protein in Paramecium containing many armadillo repeats (Q6BGJ0_PARTE). When we used this sequence as a query and we carried out blast search against the uniprot database we detected homology to the different importines including human α.6, (IMA5-HUMAN uniprot ID), plant MOS6 (Palma et al., 2005) and 8 different importins in Tetrahymena.

Search for PAR6 with PAR6α as a query. PAR6 protein is a fusion of widely distributed smart domains (PB1 and PDZ). According to smart server http://smart.embl-heidelberg.de/ this type of fusion is present only in metazoan genes and is absent in Tetrahymena.

In summary, we concluded that the upstream components of a Wnt signaling pathway are not present in T. thermophila genome. In addition, it is likely that in Tetrahymena, the proteins with armadillo repeats may have totally different functions than β-catenin.

Morphological observations on re-polarization events in dividing cells and in cells in oral replacement

In mammalian cells the GSK3-β controls the mitotic spindle dynamics that is suppressed by such inhibitors like the lithium chloride (Wakefield et al., 2003). We tested the spatial distribution of mitotic phosphoproteins (detected by MPM-2 antibody) in cells in OR. Kiersnowska and Golinska (1996) found a very weak phosphorylation of the LM-bands in the morphostatic cells in contrast to the strong staining of LM-bands in cells in OR (Fig. 2D). The area of the regressing and engulfed anterior region (Fig. 2D, E; RM) and the area of newly produced parts of the oral apparatus (Fig. 2 D, E; nOA) immunostained very strongly with the MPM-2 antibody. The posterior anarchic field of basal bodies (Fig. 2D; AF) showed lower intensity of immunostaining but stained more intensely than the basal bodies in ciliary rows at the posterior part of the cells. At the more advanced stage of oral morphogenesis (Fig. 2E) a strong fluorescence visible at the apical area corresponded to the remnant (RM) of the engulfed OA1 and the new OA (Fig. 2E; nOA). In addition, an anterior-posterior gradient of fluorescence of the abnormally organizing additional oral apparatus and fluorescence of AF were also visible (Fig. 2E; nOA?+AF). In some cells in OR, their dorsal side showed an anterior-posterior gradient of the immunostaining of the LM-bands (Fig. 2F; LM).

All these results are consistent with the hypothesis that the LM-bands and the oral structures in Tetrahymena undergo certain spatial and cell cycle determined changes which correlate with the anterior-posterior intensity of fluorescence of the antibody against MPM-2 phosphoproteins.

Studies on the effects of wortmannin on synchronized Tetrahymena pyriformis

Frankel (1967) and Frankel and Williams (1973) found that in Tetrahymena, the progress of the cell cycle and the acquisition of cell polarity depend on the external signals of nutrition and that the starved cells are mitotically arrested. The effects of wortmannin, the inhibitor of PI-3 kinase pathway was tested in synchronized T. pyriformis in constant presence of 1% PPy, the chelated Fe chloride and salts. The data are presented in Table 1. These data indicate that wortmannin added 30 min or later after the end of synchronizing treatment (EHT) does not affect the morphology,
with the exception of the enlargement of the forming OA2 (Fig. 7A,B) or the timing of synchronous cytokineses (approximately 90 min after the EHT the 25% of cells were in cytokinesis). After the cytokinesis the post-divided and rounded cells still lacked of internal part of cytopharynx, i.e. the deep fiber structures, which usually are produced 20-30 min after cytokinesis. In two types of treated samples (30-90 minutes, and 50-90 minutes), fixed at the time of synchronous cytokinesis, about 50% of cells were in stages corresponding to the OR morphogenesis. Thus, it seems that the post-dividing cells and possibly the undivided cells (cells in synchronous rounding stage; Frankel, 1967) without internal part of cytopharynx start the oral replacement synchronously. Usually most of these cells form a single new oral replacement primordium (Fig. 7D), but some cells form a series of primordia in an anterior-posterior order with a tendency to curl the ciliary row #2 located posterior to OA1 that is reduced to a remnant engulfed pocket (Fig. 7C). Such behavior of ciliary rows #2 and rows n-1 and n-2 strongly suggest that the position of the newly formed apex become established at the posterior end of the disappearing OA1. Another type of cells was also present in wortmannin treated samples; these were mouth-less cells with an enlarged anarchic field (AF) of basal bodies marking the ventral side of the cell (Fig. 7E,F).

**Analysis of the phenotypes of I18G mutant of Tetrahymena thermophila**

We used anti-Epi B and anti-fenestrin antibodies to test polarity of I18G conditional mutant cells in different stages of morphogenesis. Overall, the I18G mutant cells kept in conditions yielding mutated phenotype were much smaller than control cells, but a subpopulation of mutant cells had normal size (Fig. 8 A,B). The mouth-less cells were descendants of dividing cells with underdeveloped OA2 (not shown) or cells with more or less advanced engulfment of the anterior oral apparatus. In I18G cells the process of regression of apex and accompanying LMs and oral structures results in the formation of temporary pocket-like structure. We found that the border (slit) of this pocket is positive for the presence of EpiB (Figs. 8 C,D). After the completion of the resorption of old apex and the disappearance of the pocket-like structure, EpiB staining disappear and a new apex becomes temporarily positive for the presence of fenestrin (see below, Fig. 8H).

### TABLE 1

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Effects of wortmannin treatment (2 µg/ml) on the progress of divisional morphogenesis and on the appearance of the oral replacement phenotypes in synchronized *Tetrahymena pyriformis*. Times of addition of wortmannin and sample fixation indicate time in minutes that elapsed after EHT. Percentages of morphological stages counted in sample of about 200 well stained specimens.
The results of fenestrin immunostaining of II8G cells (Fig. 8 E-H) were also instructive. Some of the II8G cells showing the apical fenestrin staining (Fig. 8E; APEX) are either in the particular stage of the resorption of anterior oral apparatus or in the early stages of the development of the posterior oral structures (Fig. 8E; OA?, nOA?, and AF?). One of the cells in cytokinesis shows the anterior cell with the slightly engulfed oral apparatus with apical fluorescence signal indicating presence of fenestrin, and the posterior mouth-less cell also with a very strong apical signal for fenestrin (Fig. 8F; APEX). Fig. 8 (G, H) shows two small cells without oral apparatuses. These cells have strong signal of apical fenestrin (Fig. 8 G, H; APEX), and a very weak immunostaining of some ciliary rows at more distant positions.

All these observations indicate that the presence of the anterior oral apparatus is not required for the appearance of the fenestrin at the anterior pole, and that the engulfed slit is a marker of the ventral side of mouth-less cells.

Discussion

The first part of this study described the identification of the components of CDC 42-GSK3β-MARK pathway in a ciliate *T. thermophila* and tested their relationship to the upstream Wnt signaling pathway (as observed in neural metazoan cells, reviewed by Doble and Woodgett, 2003). From the search in silico of the sequenced genome of *Tetrahymena thermophila* (TIGR [http://www.tigr.org/tdb/e2k1/ttg/]) we identified the orthologs of the GSK3β-MARK2/PAR1 pathway. Some of the components of this pathway were also identified by Western blotting of fractionated *Tetrahymena*, using commercial antibodies against cdc42, mammalian GSK3β (ser9), and mammalian MARK78 kD. The upstream components of a Wnt signaling pathway were not found in *T. thermophila* genome. On the other hand we found in *Tetrahymena* a subset of proteins with armadillo repeats, which may play totally different role than β-catenins.

In non-neural metazoan cells, upstream of GSK3β, the PI-3-kinase-related signaling drives the activation of Akt kinase that controls polarity through the stabilization of structural bundling between MAPs and microtubules (Ahrringer 2003; Doble and Woodgett, 2003). Accordingly, in synchronized *T. pyriformis* the addition of the inhibitor (wortmannin) to this pathway yielded the dividing cells with enlarged new oral apparatus, which were prone to enter into an oral replacement morphogenesis (Fig. 7 A-F).

We also wanted to determine whether the appearance of cell polarity during morphogenesis of *Tetrahymena* correlates with the gradient of structural stability of cortical microtubules, which might be controlled by the GSK3β-MARK pathway. There are some indirect evidences that support the hypothesis of the involvement of GSK3β-MARK pathway in cortex stability. Jerk–Dziadosz and Frankel (1995) showed that in *Tetrahymena*, during divisional morphogenesis, the lithium chloride, the inhibitor of GSK3β, affects the organization of opistor oral structures.

Williams and Nelsen (1973) correlated turnover of microtubule proteins with oral replacement. It is possible that deficiency of MAPs brings about decreased stability of cytoskeletal structures and increased propensity of starved cells for OR morphogenesis.

In this study we showed that the anterior-posterior difference in intensity of MPM-2 immunostaining correlates with the changes in the structural stability of microtubular organelles. At the anterior pole, both LM-bands and parts of the "old" oral apparatus were extremely unstable and underwent regression, the neighboring posterior area was also unstable, undergoing futile attempts to organize oral structures, whereas the posterior pole seemed to be normal (Fig. 2D-F). This was reminiscent of *T. thermophila* phenotypes observed in cells with the EPC1 gene (epiplasm C.
protein) gene knockout (Williams, 2004). This suggests (but does not prove) that during starvation of a wild type cells, or in mutants (mouth-less II8G and EPC1 knockout cells) the gradient of structural stability is present along the anterior-posterior axis of cells. At the anterior apical cortex, the regression and instability of microtubules and microtubule-associated structures and epiplasm are very high. The moderate structural stability is sufficient for attempts to form the new oral apparatus posterior to the apical zone. The posterior pole appears to be the most stable cortical zone. Studies with apical markers (Kaczanowska et al., 2003) were consistent with the hypothesis that in the mouth-less cells (both after wortmannin treatment and in II8G phenotypes) the apical fenestrin immunostaining was independent of the presence of an oral apparatus. Nevertheless, some II8G phenotypes showed a spiraling of the LM-bands and associated ciliary rows. This indicates that the structural stability of the oral apparatus in Tetrahymena enforces a cylindrical pattern of cell growth.

The question arises whether this anterior-posterior spatial arrangement of regions of increasing instability of cortical structures represents the polar gradient of activation of some proteolytic activity in Tetrahymena? In this context MARK kinases with UBA domain (Fig. 4A) and c-TAK1 kinase which function depends on the ubiquitinating factor (Peng et al., 1998; Wang et al., 2001) are the first indications of a putative involvement of these kinases, or their upstream regulators in the mechanism of setting up cell polarity in Tetrahymena during OR. In polarized metazoan cells the MARK/ PAR1 kinase is active at early step of embryonic axis formation (La Carbona et al., 2004) and is inactive at the posterior pole that is more resistant to proteolysis (Benton and St.Johnston, 2002). The GSK3β-MARK-MAPs pathway also controls changes during mitotic cycle (Drewes and Nurse, 2003). It seems that the OR morphogenesis in starved Tetrahymenawith anterior retraction of LMs and associated apical structure may parallel the events occurring during polar retraction of neurites in axon pruning events in neural cells (Hooper et al., 2006).

In conclusion our data support hypothesis that GSK3β-MARK-MAPs pathway may be involved in setting up a global cell polarity in a ciliate Tetrahymena.

Materials and Methods

Strain culture and synchronization

Tetrahymena thermophila CU428.1 (MPR1, ms, mt VII) were kindly provided by Dr J.Gaertig (originally derived from Cornell University, U.S.A.), and Tetrahymena pyriformis (GL-C) was kindly provided by Dr J.Nilsson (University of Copenhagen, Denmark). These strains were routinely cultured in 1% proteose-peptone (Difco) and 0.1% yeast extract (1% PPY) in salt solution with FeCl3 at 28°C (Nelsen et al., 1981). Heat shock was applied to T. pyriformis, according to the routine procedure of Zeuthen (Williams and Scherbaum, 1959, Nelsen et al., 1981). The end of the seventh shock was designated EHT (end of heat treatment) and all shock was applied to (1% PPY) in salt solution with FeCl3 at 28°C (Nelsen et al., 1981). The end of heat treatment is given in minutes (Table 1).

The mutant II8G homozygous for single mutation in Tetrahymena thermophila (Tiedtke et al., 1988) was a gift from Dr Leif Rasmussen (University of Odense, Denmark). Stock cultures were grown in proteose peptone-yeast extracts supplemented with salts at the permissive temperature of 28°C. They exhibited wild type phenotype. Experimental cultures were grown at 37°C (non permissive temperature) in the same medium supplemented with 1 µg/ml folinic acid and salts of Fe³⁺ and Cu²⁺. In this condition cells are able to proliferate yielding small cells with very variable phenotypes including some cells without oral apparatuses and some cells in OR morphogenesis.

Study of the effects of wortmannin on the morphogenesis of T. pyriformis

These were performed according to the protocol of “synchronous rounding” (Frankel 1967, 1970; Williams and Frankel, 1973) except that T. pyriformis were permanently kept in 1% PPY, FeCl₃ and salt culture medium, and were given a standard seven shock heat synchronizing treatment. Control samples served to test the occurrence of the OR morphogenesis without the drug, whereas the experimental samples contained wortmannin (dissolved in DMSO) in final concentration of 2µg/ml. Wortmannin was added at 30min, 50min and 70 min after EHT i.e. when cells entered into divisional morphogenesis. The results of two different experiments containing cells with a high propensity for oral replacement after wortmannin treatment are shown in Table 1.

Permanent preparations

Protargol staining was performed according to routine protocol (Kaczanowska et al., 1993). The preparations were used for analysis of the fractional distributions in different morphogenetic stages in accordance with accepted morphological criteria for divisional morphogenesis and for the oral replacement (Frankel, 1967; Williams and Frankel, 1973).

Immunostaining

The following antibodies were used: the MPM-2 antibody against a set of the mammalian mitotic phosphoproteins (1:200 dilution;gift from Dr P.N.Rao, Boulder, Colorado, U.S.A.), the anti B epilipsem serum (1:100 dilution; gift from Dr. N.E. Williams, Iowa University, U.S.A.) and the antifenestrin antibodies (9a7 against T. thermophila and 3A7 against T. pyriformis, both in dilution 1:40; (gifts from Drs J Frankel and N. Williams, Iowa University, U.S.A.). Immunostainings was performed exactly as described earlier (Kaczanowska et al., 1993; 1999; 2003).

Four commercial antibodies were also used: anti-tau5 (1:1000 dilution; Chemicon, Temecula,California, USA), anti-GSK3β (ser9) (1:500 dilution; Santa Cruz BT, Santa Cruz California, USA), anti-C-TAK1 (1:500 dilution; Upstate, Lake Placid, NY, USA) and anti-cdc42 (1:1000 dilution; Santa Cruz BT, Santa Cruz, California, USA). Storage and handling of antibodies were done according to donators’ or manufacturers’ protocols.

Cell fractionation

Cells were fractionated according to the method of Bollag et al. (1996). In short, cells were harvested by centrifugation at 1 000 rpm for 2 min at room temperature. Pellets were washed twice in a Tris medium containing 10 mM Tris (pH 7.3), 150 mM NaCl and 250 mM sucrose supromixed with the mixture of protease inhibitors (10 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM PMSF). Final pellets were resuspended in two volumes of cold Tris medium and transferred to chilled Poter homogenizer. Cells were broken by 40 hand strokes. The extent of cell breakage was monitored regularly with microscope inspection. The cell homogenate was centrifuged at 500g for 5 min to remove nuclei and debris. The supernatant was next centrifuged at 100 000g for 1h at 4°C to obtain cytosolic and particulate fractions. Protein concentration was determined using Bradford methods (Bradford, 1976). After boiling for 5 min in Laemmli sample buffer (Laemmli, 1970) the cell fractions were analyzed by SDS-PAGE and Western blotting.

Electrophoresis and Western blot analysis

Equal amounts of proteins (30 µg) from each fraction were separated by 10% SDS-PAGE with a Hoefer System (Amersham, USA) at pH 8.8 in the presence of SDS (Laemmli 1970). The resolved proteins were transferred to nitrocellulose membranes (Bio-Rad) at 100 V for 1h in transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine and 20% methanol (Towbin et al., 1979) by the Hoefer transfer system (Amersham, USA). Filters were blocked in blocking buffer of 2% BSA in TBS-Tween for 2h and subsequently were incubated with primary antibody overnight.
at 4°C, and with the HRP conjugated secondary antibody for 1h at room temperature. Finally, the immunoreactive protein bands were visualized using the Enhanced Chemiluminescence (ECL) detection system (Amersham, USA).

Research in silico

Search for putative components of CDC42- GSK3β-MARK/PAR1- MAPs effector pathway. Macronuclear genome of T. thermophila had been sequenced and is available at http://www.tigr.org/db/e2k1/tg/. We performed BLASTp search (Altschul et al., 1997) for proteins that may control the patterned growth and oral replacement in Tetrahymena with reference to the polar differentiation of neural cells. The details of another searches are described in the Result section.

Acknowledgements
The authors thank Drs Joseph Frankel, Jacek Gaertig, Jytte Nilsson, Potu Rao and Cheryl Ashorn, Leif Rasmussen, and Norman Williams for cell lines used in this study and for gift of antibodies. Many thanks are due to Dr Dorota Dymny-Miezcz for help in workmnn–linked experiments, and to Mrs Marzena Kiatowska-Dabrowska for the technical and computer assistance. We are very thankful to Dr M. Kloc for valuable discussion and comments. This work was supported by the grant from Polish Science Committee # 2P04C 074 28 to Dr Andrzej Kaczanowski.

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