Transactions of the Illinois State Academy of Science
(2004), Volume 97, #1, pp. 33-44

received 7/22/03
accepted 12/15/03

The Effect of *p*-Fluoro-Phenylalanine on Differentiation of the Macrostomal Form of the Polymorphic Ciliate, *Tetrahymena vorax*

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ABSTRACT

Tetrahymena vorax is a polymorphic ciliate that exhibits two dominate cell types: a microstomal cell that filter-feeds on small particles and bacteria, and the macrostomal cell which feeds carnivorously on smaller eukaryotic cells including other species of *Tetrahymena*. When a population of microstomal cells is suspended in stomatin (a transforming principle released by a potential prey, *Tetrahymena pyriformis*), a high percentage differentiate synchronously into the macrostomal form within 360-420 min. By applying para-fluoro-L-phenylalanine, a protein synthesis inhibitor at 30 min intervals throughout the process of differentiation, we identified a critical time period between 150-180 min following stomatin addition, when most of the inhibitor-treated cells were no longer competent to form the macrostomal cell type. Inhibitor treatment before or after this period had little effect on levels of differentiation. One interpretation consistent with this result, is that proteins required for differentiation of the macrostomal form are synthesized during this time period.

Key Words: active principle, buccal apparatus, protein synthesis inhibitor, stomatin, transformation, transforming principle.

INTRODUCTION

Tetrahymena vorax is a polymorphic ciliate that exhibits two dominate cell types: the microstomal form is designed as a filter feeder and can be grown axenically in the laboratory. The small buccal apparatus of this cell type is designed to capture small particles including bacteria through a cytostome of approximately 2 um in diameter. The macrostomal cell type is a voracious carnivore capable of engulfing and feeding on smaller cells including other species of *Tetrahymena*. In nature, the macrostomal cell engulfs prey through a large cytostome (15-20 um in diameter). The prey then passes into a large receptacle called the cytopharyngeal pouch where digestion takes place (Fig. 1).

Division or macrostomal differentiation of *T. vorax* can be controlled under laboratory conditions (Buhse, 1967a). If a population of stationary growth phase microstomal cells is washed and suspended in a non-nutrient inorganic medium, the microstomal cells divide doubling the population within 360 min. If the population of microstomal cells is washed and suspended in stomatin[1], a transforming principle released by the potential prey, *Tetrahymena pyriformis*, they differentiate synchronously into the potentially carnivorous macrostomal cells within 360-420 min following suspension in stomatin (Buhse, 1967a). In this system, cell division and macrostomal differentiation are mutually exclusive processes. During cell division, a new oral apparatus for the potential posterior daughter cell begins as an anlagen of kinetosomes in the mid-region of the cell and organizes into the oral structures of the posterior daughter (Holsen, 1969). Then the cell divides transversely forming two microstomes. During differentiation into the macrostomal cell-type, the oral apparatus of the microstomal cell is resorbed and replaced from an oral anlagen that forms directly behind the resorbing oral apparatus (Buhse, 1966). Macrostomal differentiation is dependent on new RNA and protein synthesis (Buhse et al., 1974, Nicolette et al., 1971). Initiation of macrostomal differentiation is correlated with increased levels of transcription and translation (Buhse and Cameron, 1968). Inhibitors of transcription and translation prevent stomatin-induced macrostomal formation (Buhse, 1967b). For a current review of the biology of *T. vorax*, please see Ryals et al., 2002.

In order to obtain a better understanding of the events of macrostomal differentiation, stomatin-stimulated differentiating microstomal cells were subjected to a series of sublethal heat-shocks at defined times following stomatin addition (Holsen, 1969, Holsen and Buhse, 1969). This approach of using selectively applied heat-shocks has been successful for biochemical dissection of the cell cycle in *T. pyriformis* (Rasmussen & Zeuthen, 1962, Frankel, 1962, 1967). Experiments performed by Holsen, (1969) and Holsen and Buhse, (1969) using this technique showed that microstomal cells heat-shocked before 120 or after 180 min following stomatin addition differentiated at levels that were comparable to non-heat-shocked controls. However, when cells were subjected to 30 min sub-lethal heat shocks between 150 and 180 min, there was a significant decrease in the levels of macrostomal differentiation. Based upon these findings, they hypothesized that this period of heat sensitivity depended upon the synthesis of differentiation-specific proteins because heat shocks had been previously shown to interfere with the synthesis of "division protein" in *T. pyriformis* (Rassmusen and Zeuthen, 1962). Furthermore, Holsen and Buhse observed that the majority of cells that were heat shocked during this period of time did not form macrostomal cells, but rather divided into two microstomal cells.

The purpose of this current study is to examine macrostomal differentiation by subjecting stomatin-stimulated microstomal cells to the protein synthesis inhibitor *para*-fluoro-L-phenylalanine (*p*-F-L-Phe). This inhibitor has been shown to block translation by interfering with the formation of L-phenylalanine-tRNA (Maclean and Herbert, 1971). There are several reasons for selecting *p*-F-L-Phe for our inhibitor experiments[2]. First, *p*-F-L-Phe has been used previously to analyze the molecular and morphogenetic events of cell division in *T. pyriformis* (Rasmussen and Zeuthen, 1962, Frankel, 1962, 1967). Second, its effects should be reversed by addition of phenylalanine which will allow us to apply "pulses"of the inhibitor at different times during differentiation, imitating the same pro-

cedure used for the heat shock experiments described earlier. Third, the proposed inhibition-reversal pulse treatment should cause minimal non-specific trauma to the fragile differentiating cells.

MATERIALS AND METHODS

Cell maintenance

Populations of *T. vorax* and *T. pyriformis* were maintained in 500-ml Erlenmeyer flasks containing 50 ml of Loefer's medium, 1.5 % proteose peptone (Difco laboratories-Detroit MI), 0.5 % bacto-casitone (Difco), 27 mM dextrose, 34 mM NaCl, 7.3 mM K_2HPO_4, 7.0 mM NaH_2PO_4, 0.05 % yeast extract, 1.2 mM $MgSO_4$ (Loefer et al., 1958). For experiments, 10 ml of cell/medium suspension were transferred every 48 hours into fresh medium. Cells were grown in the dark at 20° C.

Production of stomatin

Fifty ml of Loefer's medium containing 48 hr *T. pyriformis* cells were inoculated into a 2.8 L low form flask containing 1 L of Loefer's media. The cells were incubated at 20° C for 48 hr and then washed into glass distilled water by centrifugation (three times) at 360 g using a Sorvall GSA rotor. They were then suspended overnight in a final volume of 200 ml of glass distilled water contained in a 2.8 L low form flask. The next day, the cells were removed by three sequential centrifugations at 460 g for 10 min in an IEC model HN-SII-Centrifuge (International Equipment Company, Needham Heights, MA), followed by a 4000 g for 20 min, and the final spin at 16,000 g for 20 min in a Sorvall model RC5B centrifuge using a SS34 rotor head. The liquid was evaporated to dryness by roto-evaporation. The dried stomatin was rehydrated to give a final concentration of 6 mg/ml. This volume was passed through a 0.45 um filter (Nalgene syringe filter) and aliquated into 1-ml samples, and then stored at minus 70° C for later use.

Macrostomal cell induction

Forty-eight hr *T. vorax* microstomal cultures were washed three times (1500 rpm using an IEC model centrifuge) in inorganic medium containing 7.3 mM K_2HPO_4, 7.0 mM NaH_2PO_4, 1.2 mM $MgSO_4$ (Sherman et. al, 1983) and the cell density was adjusted to 8 x 10^4 cells/ml. Five-tenths ml of the cell suspension was mixed with 0.5 ml of stomatin solution and placed in 120 x 20 mm screw cap culture tubes. This yielded a stomatin concentration of 3 mg/ml and a final cell density of 4 X 10^4 cells/ml. The stomatin-cell suspension was incubated at 20° C for 480 min. The cells were then fixed by addition of 5 drops of Bouin's fluid, and the number of cells that differentiated into the macrostomal form was determined by direct observation using a Leitz Ortholux microscope. At least 500 cells were examined for each determination. All experiments were repeated at least twice and only experiments where differentiation in the positive control were between 50-70 % are reported.

General design of inhibitor experiments

p-fluoro-L-phenylalanine (*p*-F-L-Phe), *p*-fluoro-D-phenylalanine (*p*-F-D-Phe), L-phenylalanine, (L-Phe) and D-phenylalanine (D-Phe) were purchased from Sigma Corporation (St. Louis, MO). All compounds were suspended in inorganic medium on the day of the experiment. At the end of 480 min all cells were fixed in Bouins fluid and the percentage

of differentiation determined by dividing the percentage of differentiation in the treated population by the percentage of differentiation in the positive control.

Methods for specific experiments

For experiments to determine the effect of *p*-F-L-Phe on macrostomal differentiation, stomatin-stimulated cells were exposed to different concentrations of this inhibitor for 480 min. A non-drug treated population of stomatin-stimulated microstomal cells was used as the positive control while a population of microstomal cells suspended in inorganic medium (IM) was the negative control. At the end of 480 min, the cells were fixed in Bouin's fluid and the percentage of macrostomal differentiation determined. For this and for the following experiments, the data are expressed as the percentage of differentiation in the drug treated populations, as compared to differentiation in the positive control.

In order to determine whether or not L-Phe could reverse the effect of *p*-F-L-Phe, the following experiment was performed. One ml of stomatin stimulated microstomal cells was suspended in each of 12 (120 x 20 mm) Kimax culture tubes. The first group of differentiating microstomal cells were exposed to 2 or 4 mM *p*-F-L-Phe (final concentration) for 480 minutes. The second group of microstomal cells were exposed to 2 or 4 mM *p*-F-L-Phe (final concentration) for 60 minutes post stomatin addition, followed by the addition of 21 mM L-Phe (final concentration). To a third group of non-drug treated differentiating stomatin stimulated microstomal cells, 21 mM L-Phe was added. The fourth group of stomatin induced cells served as the positive control. A fifth population of non-stomatin treated cells suspended in IM acted as a negative control (data not shown).

In order to determine if the D-isomer of *p*-F-Phe, or D-Phe were effective in preventing or sparing differentiation of macrostomal cells, respectively, the following experiment was performed. One ml of stomatin treated microstomal cells were suspended in each of the 14 (120 x 20 mm) Kimax culture tubes. The first group of differentiating microstomal cells were exposed for 480 minutes following stomatin addition to 2 mM (final concentration) of D or L-isomers of *p*-F-Phe. The second group of stomatin-stimulated microstomal cells were exposed to 2 mM (final concentration) of D or L-isomer of *p*-F-Phe for 60 minutes, followed by addition of 21 mM (final concentration) of L-Phe. The third group of microstomal cells were exposed to 2 mM (final concentration) of the D or L-isomers of *p*-F-Phe for 60 min followed by addition of 21 mM (final concentration) of D-Phe. A fourth group of stomatin stimulated cells was the positive control.

In order to determine the effect of 30 minute pulses of *p*-F-L-Phe throughout the differentiation cycle, the following experiment was performed. One-ml of stomatin-stimulated differentiating cells was suspended in each of 16 (120 x 20 mm) culture tubes. A total of 7 pulses of the inhibitor were given, beginning at 60 min intervals, post stomatin treatment and compared to a positive control.

RESULTS AND DISCUSSION

We determined that *p*-F-L Phe effectively prevented macrostomal differentiation in a concentration-dependent manner (Table I). Two or 4 mM *p*-F-L-Phe were selected for

experimentation because they prevented 96 and 97 % inhibition of macrostomal differentiation, respectively. It has been reported that this inhibitor interferes with gene expression by preventing formation of phenylalanine acyl-t-RNA as proposed by Maclean and Huber (1971). Next we determined if the effect of *p*-F-L-Phe could be reversed by addition of L-phenylalanine (L-Phe). Table II shows that while 2 or 4 mM *p*-F-L Phe prevented 99 and 98 % differentiation, respectively, addition of 21 mM L-phenylalanine reversed inhibition in both cases. Understandably, the level of reversal was correlated with the ratio of inhibitor to metabolite. L-Phe reversal of microstomal cells treated with 4 mM *p*-F-L-Phe (ratio 5.25 to 1) was 27 % lower than that treated with 2 mM *p*-F-L-Phe where the ratio is 10.5:1. Interestingly, transforming cells treated with 21 mM L-phenylalanine differentiated at 74 % of control values. Stomatin has been shown to contain many products of nutrient quality including amino acids, lipids in addition to nucleic acid derivatives (Buhse, 1965). Thus, one possible explanation is that the nutrient value of stomatin was increased by the addition of 21 mM L-Phe, so that the ratio of the active principle (hypoxantine-uracil-iron) to nutrients was reduced decreasing the level of differentiation.

The results presented in Table III shows that while *p*-F-L-Phe prevented 98 % of stomatin- treated cells from differentiating into the macrostomal cell-type, the D-form of this drug had no effect. In fact, cells treated with *p*-F-D-Phe differentiated at a higher level than the control. Furthermore, the D-Phe isomer failed to reverse inhibition of differentiation caused by *p*-F-L-Phe while the L-form caused reversal. These results are consistent with the fact that the D-isomers of amino acids are not metabolized by cells (Stryer, 2000). Taken together, the effects of *p*-F-L-Phe appear to be specific in blocking protein synthesis. Consistent with this conclusion is our observation that the inhibitory effect is reversed following addition of L-Phe.

We have identified a period of time between 150-180 min post-stomatin treatment where the levels of macrostomal differentiation are reduced as compared to the non-treated controls (Table IV). There appears to be little effect of the analogue on differentiation before or after this window. Most interestingly, as shown in Fig. 2, this window corresponds exactly to the same period of sensitivity induced by application of heat shocks to differentiating cells (Holsen and Buhse 1969). In fact, the shape of the curves generated by these two different perturbations are remarkable similar in most all details (Fig. 2). This observation is consistent with the hypothesis that proteins required for differentiation of the macrostomal phenotype are synthesized during this window of time. Additionally, studies by Buhse (1967b), who blocked macrostomal differentiation using inhibitors of transcription and translation and Cameron and Buhse, (1968) who showed that rounds of RNA and protein synthesis occurred early in differentiating cells, supports the idea that macrostomal differentiation is dependent upon protein synthesis. Additionally, it has been shown that differentiating microstomal cells challenged by sub-lethal heat shocks between 150-180 min following stomatin addition caused them to abandoned the macrostomal pathway and switch to the division pathway (Holsen and Buhse, 1969). Whether or not differentiating cells treated in this time period with *p*-F-L-Phe also switch to the division pathway has yet to be determined.

Future investigations plan to focus on the differentiation-specific proteins that are synthesized between 150-180 min. Several studies identify possible differentiation-specific

protein candidates. Although Buhse and Williams (1982) using 1-D SDS PAGE reported no significant differences in the pattern of cytoskeletal or membrane proteins between the microstomal and macrostomal forms of *T. vorax*, Gulliksen et al. (1984) using 2-D SDS PAGE reported a total of 52 proteins isolated from the oral apparatus of the macrostomal form that were not found in the microstomal form. Yang and Ryals (1994) reported a critical role for ongoing glycosyl phosphatidyl inositol units (GPI-anchored protein synthesis) and assembly during differentiation. Zhang and Smith-Somerville (1996) report the appearance of several polypeptides in stomatin-stimulated microstomal cells that are absent from uninduced control cells. Most recently, Green et al., (2000) reported a putative macrostomal specific protein sequence derived from a gene that was transcribed during macrostomal differentiation. Using radiolabeled amino acids, it should be possible to identify proteins synthesized between 150 and 180 min for both differentiating cells suspended in stomatin and for dividing cells suspended in inorganic medium.

Although the two curves presented in Fig. 2 are strikingly similar and suggest that synthesis of macrostomal-specific proteins are inhibited by heat shocks or by *p*-F-L-Phe, the mechanism of action of these treatments might be different. *p*-F-L-Phe may inhibit the level of protein synthesis directly by blocking or lowering the levels of translation of all proteins including those required for macrostomal formation. It is equally possible that both heat and *p*-F-L-Phe induce formation of stress proteins. The heat shock treatment causes formation of stress proteins called heat shock proteins (Linquist,1986). Additionally, Verbeke et al. (2001), reported that stress proteins can form in response to treatment with amino acid analogues. Under this scenario stress proteins would be produced by both treatments. Stress proteins induced by heat shocks have been described from both *Tetrahymena thermophila* (Williams and Nelsen, 1997) and *T. vorax* (Buhse and Williams 1984). It should be possible to determine their mechanism of action using radiolabeled amino acids and comparing the pattern of protein synthesis induced by heat or *p*-F-L-Phe. If the protein synthesis patterns induced by these treatments show synthesis of a few similar proteins at the expense of other synthesis, then it is highly likely that both treatments function by inducing stress proteins.

CONCLUSIONS

We have identified a critical time period between 150-180 min following stomatin addition, when most *p*-F- L-Phe-treated cells are no longer competent to form the macostomal cell type. Failure of differentiating cells to express these proteins during this time results in a pathway switch leading to cell division. Based on these results we conclude that this window of competence is correlated with expression of differentiation-specific proteins. These findings will allow us to identify the elements responsible for differentiation of the macrostomal phenotype by focusing on the proteins that are synthesized during this critical window.

ACKNOWLEDGEMENTS

The authors thank Dr. Norman E. Willians, Department of Biological Sciences, University of Iowa and Dr. Harriett E. Smith-Somerville, Department of Biology, University of Alabama for their comments and suggestions in the preparation of this manuscript.

FOOTNOTES

[1] The active principle of stomatin is as an iron chelate of hypoxanthine and uracil (Smith-Somerville et al., 2000).

[2] Experiments "pulsing" the protein synthesis inhibitor cycloheximide failed because removal of the drug required several sequential washing steps that were accomplished by centrifugation. This treatment prevented macrostomal differentiation in all cells including the positive control.

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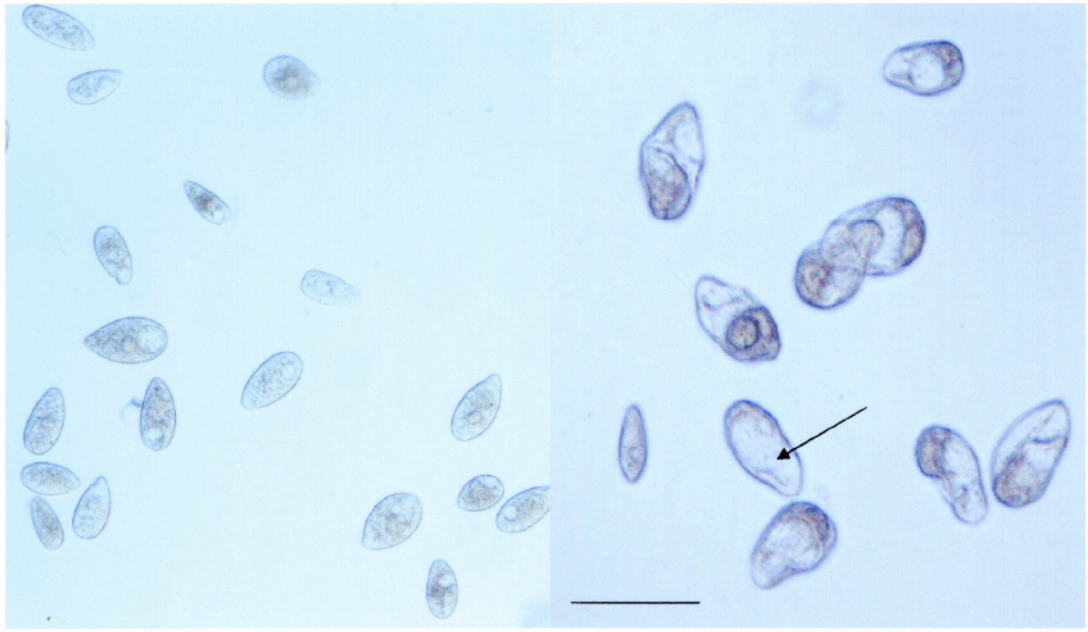
Figure 1. A comparison of microstomal and macrostomal cells. A). A population of microstomal cells suspended in inorganic medium. The microstomal cell is small and is a filter feeder capable of ingesting small particles including bacteria. (B). A population of macrostomal cells induced by the transforming principle stomatin. The macrostomal cell type a voracious carnivore is larger than the microstomal form with most of its volume occupied by a prey receptacle called the cytopharyngeal pouch (arrow). The macrostomal form feeds by capturing and ingesting prey and digesting them in the cytopharyngeal pouch. Note that several macrostomes have ingested microstomal cells. Bar = 100uM.



A. Microstomal cells **B. Macrostomal cells**

Figure 2. A comparison of the effect of 30 min sub-lethal heat shocks (Holsen and Buhse, 1969) to the effect of 30 min pulses of para-fluoro-L-phenylalanine (present study). The drug was applied for 30 min at the times indicated during macrostomal differentiation (60-270 min). These results are plotted against the % of control differentiation. Note the similarity of the curves and the fact that both treatments have their major inhibitory effect between 150-180 min following addition of stomatin.

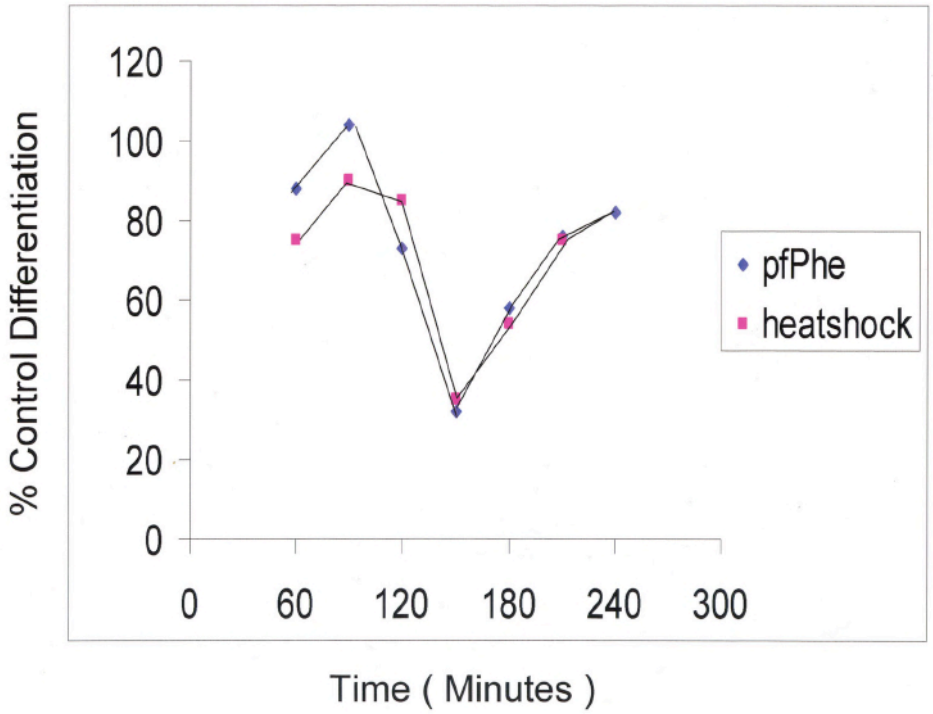


Table I. The effects of different concentrations of *p*-fluoro-L-phenylalanine (*p*-F-L-Phe) on stomatin-stimulated macrostomal differentiation.

Drug concentration (mM)	% Positive control	Standard Error of the Mean (SEM)
0.5	14	1.9
1.0	9	1.3
2.0	4	0.8
4.0	3	0.4

Table II. The effect of L-phenylalanine (L-Phe) on stomatin treated differentiating microstomal cells suspended in *p*-F-L-Phe.

Group	Treatment	% control
1	2 mM *p*-F-L-phe	0.4
	4 mM *p*-F-L-phe	0.2
2	2 mM *p*-F-L-phe + 21 mM L-Phe	103
	4 mM p- F-L-phe + 21 mM L-Phe	76
3	21 mM L-phe	74

Table III. The effect of the D or L isomer of *p*-F-Phe and Phe on stomatin-stimulation macrostomal differentiation.

Groups	Treatment	% control
1	2mM *p*-F-L-Phe	2
	2mM *p*-F-D-Phe	117
2	2mM *p*-F-D-Phe + 21mM L-Phe	70
	2mM *p*-F-L-Phe + 21mM L-Phe	86
3	2mM *p*-F-D-Phe + 21mM D-Phe	102
	2mM-*p*-F-L-Phe + 21mM D-Phe	3

Table IV. The effect of 30 min pulses of *p*-F-L-Phe given to cells at different times during the stomatin-induced differentiation.

Time of administration (min)	% control
60-90	88
90-120	104
120-150	73
150-180	32
180-210	58
210-240	76
240-270	82