ABSTRACT

To determine whether "fresh" protein synthesis is required for flagella regeneration, cultures of *Chlamydomonas reinhardii* were deflagellated and treated with 10µg/mL of cycloheximide, a protein synthesis inhibitor, or 3 mg/mL of colchicine, a substance that prevents all growth of flagella. Non-deflagellated cultures were inoculated with the same reagents. Over a two hour span, flagella lengths in untreated deflagellated cells reached 9±2 µm in length whereas the lengths in cycloheximide treated deflagellated cells only reached 3±2 µm. The non-deflagellated cultures, cycloheximide and untreated, did not change significantly for the duration of the experiment ($p > .05$). The non-deflagellated, colchicine treated culture underwent significant ($p < .05$) decline in flagella length from 10±1 µm at time zero to 8±2 µm at 120 m. This difference may be attributable to sampling errors to be eliminated by further sampling. Results suggest that new protein synthesis is not required for flagella regeneration and that numerous control mechanisms affect the steady state of tubulin in cells.

INTRODUCTION

Tubulin is a well-characterized protein in all eukaryotic cells responsible for numerous cell functions as a microtubule component including (but not limited to), cell division, intracellular transport, and motility. This study addressed the regulation of microtubule assembly from alpha- and beta-tubulin in unicellular, photosynthetic, flagellated protist, *Chlamydomonas reinhardii*. Understanding this process is critical to understanding basic cell function.

Chlamydomonas reinhardii was chosen because its two flagella are formed by the tubulin/microtubule assembly process of interest. It is easily grown in large, uniform amounts, and is readily available in numerous related laboratory species which can be used to study gene expression. The genetic and physical simplicity of *Chlamydomonas reinhardii* should allow its study to shed light on similar microtubule assembly processes in more complex organisms.

The necessity of tubulin in cell function leads to questions concerning the mechanisms that control the microtubule assembly. The likely existence of multiple control and regulatory mechanisms affecting flagella regeneration requires understanding the influence of a fundamental necessity for this process: protein synthesis. This study specifically questioned whether or not “fresh” protein synthesis must occur prior to re-growth of flagella.

Cycloheximide was added to a deflagellated culture of *Chlamydomonas reinhardii*. This experiment group was supported by two deflagellated control groups: an untreated positive control, and a colchicine-treated negative control group. The positive control group tested if the deflagellated *Chlamydomonas Reinhardii* would regrow flagella at all if left untreated. Colchicine, obtained from *Cochicum autumnale*, is known to stop all microtubule assembly. The negative control group thus created a standard for 100% cessation of flagella regrowth. Three non-deflagellated cultures were also prepared. One was left untreated to control for changes in

lengths of present flagella. The other two were treated with cycloheximide and colchicine, respectively, to determine if these substances affected pre-existing flagella. Original sample of the organism was deflagellated using polytron tissue homogenizer. Deflagellated cultures were sampled every 10 minutes and flagella lengths notated. Non-deflagellated cultures were sampled at the beginning and end of the experiment. Samples were fixed for observation using an aldehyde fixative.

MATERIALS AND METHODS

Culture Conditions

Chlamydomonas reinhardii and spring water used for propagation were provided by Carolina Biological Supply Co., NC. Motile *Chlamydomonas reinhardii* were increased by adding spring water to an active culture and exposing the culture to bright sunlight.

Deflagellation

Tissue homogenization was conducted with a Janek and Kunkel Ultra-Turrax Polytron tissue disruptor retrofitted with a 20-mm shaft from Tekmar, based in Cincinnati, OH. One hundred mL of *Chlamydomonas reinhardii* culture in a 250 mL beaker was homogenized by the polytron for 15 s on setting "70." This process was repeated 8 times at 1 m intervals. Suspension was then moved to six 15 mL centrifuge tubes and spun down for 3 minutes at high speed in an International Model HN tabletop centrifuge. The pellets were recombined in the spring water suspension at 2.5 times the original concentration of *Chlamydomonas*.

Culture Preparation, Sampling Intervals

Deflagellated cells were split into three separate cultures. One culture was treated with 10 µg/mL cycloheximide, another with 3 mg/mL colchicine. The remaining culture was left untreated. The same setup applied to three non-deflagellated cultures. The cycloheximide and colchicine were purchased from Sigma Chemical Co., St. Louis, MO. The six cultures were kept under heavy light during experimentation. They were removed from direct light for sampling. All deflagellated cultures were sampled every 10 m for 2 h. Non-deflagellated cultures were sampled at the beginning and at the end of the experiment.

Sampling Procedure

Four drops of each culture were Pasteur pipetted into individual Eppendorf tubes for sampling. An equal volume of aldehyde fixative was added to each sample under the fume hood. The aldehyde fixative was composed of 2.4 mL 37.6% formaldehyde, 1.2 mL 25% glutaraldehyde, and 170 µL 1M NaCl brought to 10 mL total solution with distilled water. Each fixed sample was agitated gently and then allowed to sit undisturbed for two minutes for fixation to complete. Samples were then distributed for scoring.

Scoring Procedure

Teams prepared wet mounts with one drop of fixed sample and viewed them under 400x phase contrast. One drop of Lugol's Iodine (6% KI, 4% iodine crystals, in distilled water, stirred and shielded from light overnight) was used to stain samples if scoring was not possible. Samples were scored immediately if Lugol's Iodine was used. Cells were chosen at random for scoring. Straightest flagellum on each cell was measured even if cell had two flagella. One hundred division ocular micrometers were calibrated to 2.5 µm per division at 400x.

RESULTS

Deflagellated Cultures

Observations of the cycloheximide treated deflagellated culture demonstrate that flagella regrew despite the presence of the protein synthesis inhibitor, cycloheximide (Fig.1). Flagella regrowth in the untreated deflagellated culture surpassed both the colchine- and cycloheximide-treated deflagellated cultures. The lengths of flagella in the cycloheximide culture were consistently less than one-half the length of flagella in the untreated deflagellated culture. Rapid growth of flagella in these two cultures was observed through 40 m of sampling before a visible plateau in flagella length was reached. No significant growth was noted in the deflagellated, colchicine treated culture so no graph of its data is presented.

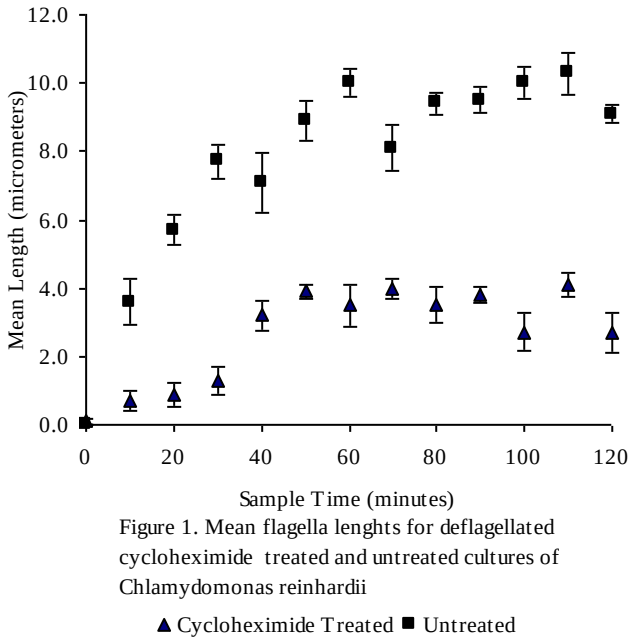

Figure 1. Mean flagella lenghts for deflagellated cycloheximide treated and untreated cultures of Chlamydomonas reinhardii

Non-Deflagellated Cultures

Results indicate (Table 1) that the flagella lengths of untreated and cycloheximide treated, non-deflagellated cultures were not significantly affected by the duration of the experiment ($p > 0.05$). In both cases, the null hypothesis was accepted. Colchicine, however, significantly ($p < .05$) decreased flagella length over the duration of the experiment (Table 1).

Table 1. Student's T-test Results for Non-deflagellated Cultures of Chlamydomonas reinhardii

Culture	Time (mins)	Mean ± Standard Deviation	p-Value
Untreated	0	9±1 µm	0.83
	120	9±1 µm	
Cycloheximide treated (10µg/mL)	0	8±1 µm	0.5
	120	8±0 µm	
Colchicine treated (3mg/mL)	0	9±1 µm	0.03
	120	7±1 µm	

p-Value calculated using unpaired, 2-tailed, student's t-test

DISCUSSION

Deflagellated Cultures

The results clearly suggest that "fresh" protein synthesis is not required for flagella regeneration in *Chlamydomonas reinhardii*. If new synthesis was required, the deflagellated, cycloheximide treated culture and the deflagellated, colchicine treated culture should return identical results. Figure 1 demonstrates that the former culture still grew flagella, but to shorter lengths than the deflagellated, untreated culture. For this to occur, the existence of some unassembled form of microtubule components (tubulin) is likely.

However, Figure 1 also indicated that both cultures initially grew quickly but reached a plateau after 40 m. This limited growth of flagella implies that the assembled and unassembled forms of tubulin are in a steady state with one another. Following amputation, the steady state of assembled and unassembled tubulin is disrupted and tubulin assembly commences. This shift toward assembled tubulin formation triggers the synthesis of tubulin itself. Because synthesis is a result of a shift toward tubulin assembly, protein synthesis itself is the unlikely choice for a signal to commence flagella regeneration.

However, flagella lengths in Figure 1 did not ascend immediately to a maximum length. Thus, the rate of regeneration of flagella is likely limited by the concentration of enzymes or other substance(s) responsible for tubulin assembly as well as the maximum rate of formation of assembled tubulin. The rapid shift in the tubulin steady state due to flagella amputation causes these substances to produce assembled tubulin at the maximum rate physically possible.

The observed plateau in both cultures from Figure 1 suggests that a mechanism controls the maximum flagella length for *C. reinhardii*. A tubulin steady state is a viable explanation. In the protein synthesis-inhibited cycloheximide culture, the concentration unassembled tubulin

must decrease due to its conversion to an assembled form. As the concentration of the latter form increases, the two reach equilibrium – a steady state. However, for the steady state to exist in the untreated culture, a regulation mechanism must control the concentration of unassembled tubulin. Negative feedback inhibition is the mechanism that maintains this concentration through the inhibition of translation by its product – unassembled tubulin. Thus, as assembled tubulin concentration increases, it, too reaches a steady state with its unassembled form. At this steady state, assembled tubulin concentration does not increase and flagella growth stops. This coincides with the observed relationship between the lengths of flagella in both cultures (Fig. 1).

Non-Deflagellated Cultures

The non-deflagellated cultures coincided with expectations with the exception of the colchicine treated culture. The t-test results for the untreated and cycloheximide treated cultures (Table 1) indicate that neither culture experienced significant flagella length changes from the beginning of the experiment to the end. The colchicine culture returned a significant change in mean flagella length from the beginning of the experiment to the end (Table 1; $p < .05$). This, too, can be explained by the steady state theory. Preassembled tubulin can change back to an unassembled form, thereby decreasing the length of flagella. However, in the presence of colchicine, these now soluble heterodimers bind to the colchicine cap the microtubules as they are assembled, stopping growth, and leaving the microtubules shorter than they originally were. Colchicine may also directly affect flagella lengths beyond the ability to cap addition of heterodimers. If colchicine causes flagella damage, the validity of the experimental group is put at question because the deflagellated, colchicine treated culture is no longer a definitive zero-growth standard.

This study raises the issue of what effects the direct addition of unassembled tubulin would have on flagella length because the next step in understanding microtubule assembly is characterizing the control mechanisms that maintain the steady state within the cell. Halting the negative feedback inhibition mechanism should demonstrate the existence of other regulatory mechanisms if the flagella do not grow unrestricted.