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Ubiquitin-mediated Proteolysis During Gametogenesis in *Chlamydomonas reinhardtii*

by

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**Introduction:**

Scientists in the field of cell biology have focused considerable attention on a unicellular species of green algae known as *Chlamydomonas reinhardtii*. This organism is relatively common in bodies of fresh water and some types of soil, but most of the attention is due to its value as a model for biochemical studies. This laboratory has undertaken a broad study of the species including exploration into a special class of proteins, called heat shock proteins, and certain biochemical processes of the organism. The work herein described is a part of this latter study, and also a preliminary step in a project that will continue at least through the summer of 1997.

The life cycle of *Chlamydomonas reinhardtii* includes three stages: a vegetative stage, a gamete stage, and a zygote stage. Zygotes are formed when gametes of the two mating types (mt+ and mt-) fuse. Vegetative cells can be transformed into gametes by starving the cells of a nitrogen source. This transformation is called gametogenesis. Researchers in Japan have demonstrated that a decrease in the concentration of intracellular ammonium ions is responsible for triggering gametogenesis.

Gametogenesis involves, among other things, significant changes in the protein profile of the cells. The changes observed occur by recycling of proteins; no net synthesis is observed. This process required that old proteins be degraded and new proteins made.
from the free amino acids generated. This degradative pathway could be either lysosomal, proteasomal, or some combination of the two.

The proteasomal degradative pathway has generated a great deal of excitement in recent years after discoveries were made linking it to an extremely diverse set of biological processes. The pathway is mediated by a protein called ubiquitin which, as its name implies, is found ubiquitously in eukaryotic cells. A protein enters this proteolytic pathway when it is multi-ubiquitinated, which is to say several ubiquitin molecules become covalently bound to the protein in question. Multi-ubiquitination targets the protein to a 26S proteasome (roughly one third the size of a ribosome) which contains many proteolytic enzymes. If a protein is only mono-ubiquitinated, however, it often becomes a target for de-ubiquitinating enzymes and is thus spared degradation. A number of ATP-dependent proteases are arranged into a 20S cylinder with the active sites facing the core. Ubiquitinating enzymes tag proteins with several ubiquitin molecules, then those proteins are denatured by other enzymes and fed into the core of the 20S cylinder like a piece of thread from a spool. The 20S proteolytic core degrades the proteins into short oligopeptides. One notable example of this ATP-dependent, ubiquitin-mediated proteolytic pathway is in cells that have been heat shocked. Due to its role in recycling the amino acids of proteins that have been damaged by high temperature, ubiquitin is produced in greater concentration in heat shocked cells, but is also produced under normal cellular conditions.

The experiments described below are designed to determine what role, if any, ubiquitin plays in the process of gametogenesis in Chlamydomonas reinhardtii. These experiments are specifically designed to detect any major changes in ubiquitin concentration, or changes in ubiquitinated proteins.
Based on the observed protein turnover during gametogenesis, it is expected that ubiquitin plays a significant role. The strategy for determining whether or not this expectation is true involves several stages. First, a baseline for comparison must be amassed in the form of full protein profiles at various stages of gametogenesis. Once this has been completed, and the protein turnover findings have been corroborated, the investigation will turn to the specific role of ubiquitin. First, does the concentration of ubiquitin change during the course of gametogenesis? Does the profile of ubiquitinated proteins change? Can one protein’s degradation be followed throughout gametogenesis as a marker for cell differentiation?

The scope of this project includes the elucidation of the protein profiles of the cells, analysis of major changes, and analysis of the profile of ubiquitinated proteins in a sample. These objectives were achieved using the methods outlined below.

**Methods:**

I. Induction of gametogenesis:

Synchronous vegetative cultures of *Chlamydomonas reinhardtii* were kept in an incubator at 23°C, suspended in a Medium I (Sager and Granick), and subjected to a cycle 12 hours of light followed by 12 hours of dark for at least 48 hours before use in an experiment. Also, air is bubbled in a stream into the vials of every culture to provide a ready supply of carbon dioxide. To induce gametogenesis, cultures at $1-5 \times 10^6$ cells/mL, were centrifuged (Centra, 2400 rpm, 20 min., 20°C) out of this Medium I and resuspended in a nitrogen-free variant of Medium I (NFM) and kept in constant light. NFM is identical in every way to normal Medium I except that the nitrogen source, ammonium nitrate, is withhold.
from the solution. When the cells were resuspended in NFM, they were brought to a concentration of $3 \times 10^6 \text{cells/mL}$. Gametogenesis takes about 15 hours from the introduction of the cells into NFM until they are fully competent as gametes. In this experiment, the cells were allowed to incubate in NFM with bubbling under constant light for about 20 hours in order to ensure that gametes were formed.

II. Verification of mating competency:

To verify that gametes had been produced, vegetative cells of both mating types were first incubated separately in NFM for ~20 hours. After that time, the cells were centrifuged again and resuspended in a smaller volume to bring the cells to a concentration of $1 \times 10^7 \text{cells/mL}$. The two mating types were then combined in a 1:1 ratio. Aliquots of living cells were fixed with Lugol’s iodine were taken at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20 minutes, and analyzed by phase contrast microscopy (100x) to monitor cell agglutination, and zygote formation. A living sample was also observed in a similar manner, and yielded the same results.

III. Harvest of cells:

The harvesting of cells was a multi-step process beginning with the isolation of the cells from their medium using centrifugation (Sorvall, 10 min., 10000 rpm, 4°C). Then the cells were either stored in the freezer, or, if time permitted, immediately subjected to a solution of 1% SDS + 0.6 M DTT (sodium dodecyl sulfate, and dithiothreitol) which dissolves the cell pellet and breaks up much of the quaternary and even tertiary structure of the proteins in the solution. The ratio of SDS-DTT to cells was $1.9 \mu\text{L per million}$
cells. The tubes containing the cells and SDS-DTT were mixed on a rocker in Oak Ridge tubes for 1 hour at room temperature. Then the solution was transferred to a microfuge tube and put on the 100°C heat block for two minutes. Following a two minute centrifugation in the microfuge, the supernatant was transferred to another microfuge tube. This concentrate contains most of the proteins, the pellet was usually quite small and contained mostly unbroken cells. A Bradford protein assay was then performed to determine the protein concentration of each sample (a standard curve was created using gamma globulin; slope = 0.006 Abs/µg).

IV. SDS-PAGE:

Polyacrylamide gels (12.5% running and 3.5% stacking, Laemmli) were usually cast on the same day that the gels were run, but sometimes the day before. The Bio-Rad Mini Protean II system was used. Based on the concentrations calculated by the Bradford assay, volumes of protein concentrate were used in sample preparation so that 35 µg of protein was loaded into each well in the gels bound for Coomassie staining, whereas 50 µg of protein was loaded into each well of the gels bound for western blotting. Samples were prepared to reach a final volume of 25 µL (sample, deionized water, and 4x protein sample buffer). The samples were then heated on the 100°C block for 2 min., allowed to cool to room temperature, and loaded onto the gel. The gels were run at 200 volts usually for about 40-45 minutes.
V. Coomassie staining:

After separation of the proteins, some gels were immersed in Coomassie stain for periods of time ranging from 1 hour to overnight depending on the freshness of the stain (the older the stain, the less potent). After staining, the entire gel is blue, so the gels are transferred to a destaining solution which destains the portions of the gel not containing protein. After this procedure, bright blue bands are visible for analysis. The gels were dried down for storage using the Bio-Rad gel dryer (SDS PAGE, 80°C, 90 minutes).

VI. Western blotting:

The proteins from the gels were electrophoretically transferred to a nitrocellulose membrane (100 volts for 90 minutes). After transfer, the membrane was put in blocking solution (3% Carnation nonfat dry milk dissolved in phosphate buffered saline) which attached proteins in a non-specific way all over the membrane. The membrane was then washed in PBS and incubated with 1:250 solution of primary antibody in PBST (Sigma anti-ubiquitin from rabbit, # U-5379) for at least 1 hour at room temperature with rotation in the Bellco micro-hybridization oven. After this incubation, the membrane was washed in PBST (PBS, 0.2% nonfat dry milk, and 0.1% Tween 20) three times for 10 minutes each and incubated with 1:5000 solution of secondary antibody in PBST (Sigma anti-rabbit IgG alkaline phosphatase conjugate, # A-0418) for 90 minutes with rotation in the hybridization oven. Another four 5 minute washes with PBST followed this incubation and then the membrane was incubated with a color reaction solution for periods of time ranging from 5 minutes to 15 minutes depending on the speed of the reaction for a particular blot. For a long time there was a great deal of difficulty in getting reproducible
results that showed any sort of banding pattern when the membranes were probed with anti-ubiquitin antibodies, but finally it seems that the problem was with the blocking step. When the membranes are in the blocking solution for about 2.5 to 3 hours at room temperature, clear banding patterns emerge at the end of the procedure. When the membranes were in blocking solution overnight at 4°C, however, no usable results were gathered. This probably means that the lower temperature prevents the binding of proteins to the surface of the membrane, so when the membrane is incubated in the antibodies, they act like a blocking solution and bind non-specifically as well as specifically. The result is greatly reduced resolution.

VII. Heat shock conditions:

In heat shock experiments cells were incubated in a circulating water bath at 37°C for 1 hr., with bubbling as usual. This temperature was chosen based on the report by Shimogawara and Muto\(^8\) that when the temperature is raised above 37°C the concentration of ubiquitinated proteins declines rapidly. After heat shock, the cells were usually harvested immediately, but sometimes they were incubated at 26°C again for some period of time and then harvested. For control purposes, a concentration of 3x10^6 cells/mL was used as the target concentration.

VIII. Radioisotopic pulse labeling:

Radioactive sulfur, \(^{35}S\), was used in these experiments which were designed to determine which proteins are synthesized during a certain period of time. In order to maximize the amount of radioisotope taken up, the cells were incubated overnight in a variant of Medium I that had no MgSO\(_4\) and therefore no source of sulfur(MgCl\(_2\) was used as a
substitue source of Mg$^{2+}$). This medium was called sulfur free medium (SFM). On the day of the labeling, the cells were counted and $1.5 \times 10^7$ cells per experimental tube were removed from the stock culture, centrifuged (Centra, 2400 rpm, 20 min., 20°C), and resuspended in 5 mL of fresh SFM to bring the concentration to $3.0 \times 10^6$ cells/mL. There were always at least two cultures of cells, one experimental (heat shock, 37°C) and a control (26°C). To these tubes 75 µCi of $^{35}$SO$_4$ were added to reach a final concentration of 15 µCi/mL, and the cells were incubated with bubbling at their respective temperatures for 1 hour. At the end of the hour, the cells were harvested according to the usual protocol (the waste went into special radioactive waste containers). After the harvesting was completed, the amount of radioactivity in the samples was assayed using a scintillation counter. This step is analogous to the Bradford protein assay for non-radioactive samples. This process was done by first spotting 1 µL of protein samples onto duplicates of small filter paper discs (Whatman 3MM). The discs were then soaked in solutions of trichloroacetic acid (TCA) to precipitate the protein from solution. The first solution was 10% TCA, the second and third were both 5% and all washes were for 10 minutes each. All of the TCA precipitation was done on ice in order to minimize the loss of protein into solution. The final wash of the discs was with 95% ethanol for 10 minutes at room temperature, after which the discs were placed in a drying oven at 150°C until the ethanol had all evaporated. They were then put in a scintillation vial with 8 mL of Scintiverse scintillation cocktail and run on program A7 (for $^{35}$S) on the biochemistry lab's scintillation counter. The concentration of radioactivity in the protein samples was then calculated based on the scintillation data, and the samples were then loaded onto a 12% polyacrylamide gel for electrophoresis (see above). Radioactive $^{14}$C markers were
used instead of the usual rainbow markers. To check for spills of radioactivity, swipes were made with small paper disks of the workspace and put in scintillation cocktail (8 mL) and read in the scintillation counter on program A7 just like the other samples. If the readings were above background for any of the specified areas, then they were cleaned according to the standard operating procedures of the lab.

IX. Immunoprecipitation

This technique involves the use of anti-ubiquitin antibodies, the same as in the western blotting, to pull ubiquitin and ubiquitin conjugates out of a crude protein solution. In western blotting, one separates all of the proteins from a crude extract using SDS-PAGE and then visualizes those areas where ubiquitin can be found. The advantage of immunoprecipitation is that the separation is performed on a purified sample, so the results are cleaner.

In the procedure used for this research, chemical methods were not enough to lyse the strong *Chlamydomonas* cell walls, so a mechanical method was used. This method is called nebulization and requires a device called a nebulizer (made by Bio-Neb). The process involves misting a liquid culture of *Chlamydomonas*. During this misting, the pulling apart of water droplets creates shearing forces that tear the cells open and release the contents into solution. This was performed with a flow rate of 6 L/min of nitrogen while the cells were suspended in RIPA cell lysis buffer (1 mL/15x10^6 cells). After the lysate was collected, it was centrifuged (Sorvall, 14,000 rpm, 15 min, 4°C) to remove the solid particles. The supernatant contained most of the cells' proteins. All of the rest of the procedure was performed at low temperature (i.e. on ice). Anti-ubiquitin antibody (60 µL) was added to the supernatant and incubated on a rocker for 2 hrs. on ice. After this
period of time, 50 µL of a slurry of Sepharose beads with Protein A adhered to their surface was added to the solution. This was allowed to incubate under the same cold conditions as before for 1 hr. Then the tube was centrifuged (Sorvall, 14,000 rpm, 12 min., 4°C) and the supernatant removed and discarded. The pellet contained the beads with all of the ubiquitinated proteins attached because the Protein A bound to the anti-ubiquitin antibodies which were bound to the ubiquitinated proteins. The pellet was washed with RIPA buffer (500 µL) and recentrifuged (~1-2 minutes, conditions as before). This washing process was done a total of six times. At this point 100 µL of Laemmli protein sample buffer (without β-mercaptoethanol) was added to the pellet. The tube was placed in a boiling water bath (on a heat block) for 5 minutes, then vortexed at 1/2 speed and centrifuged again (14,000 rpm, 1 min., 4°C). The supernatant, immunoprecipitate, was then transferred to a clean microfuge tube. At this point it was ready to be run on a gel.

Results:

Coomassie-stained gels indicate significant changes in the protein profile of *Chlamydomonas reinhardtii* cells as they differentiate from vegetative to gametic cells. These changes are seen as differences in the intensity of certain bands of protein (Figure 1). There is a general trend of bands of lower molecular weights darkening and higher molecular weights disappearing. For instance, the vegetative lane and the gametic lane differ drastically in the regions around 75 kD and 15 kD *.

* All molecular weights are based on the weights provided by Bio-Rad for their color marker proteins. (From the top of the gel they are 208, 144, 87, 44.1, 32.7, 17.7, 7.1 kD)
While the changes in protein profile were significant, most of them occurred between the 12 hour time point and the 20 hour (full gamete) time point. Another time course experiment was conducted to investigate this apparently critical period of time. This experiment was only performed once and, thus, yielded inconclusive results, but it appears that the changes may be quite sudden. Jones observed that fully differentiated gametes appear very suddenly at some time between 12 and 15 hours after the onset of nitrogen starvation. Based on this new evidence, it could be that the entire differentiation process takes place suddenly sometime between 12 and 15 hours, instead of slowly over the entire time period and culminating at that time. More experimentation would be needed to confirm that hypothesis however.
After confirming that there were major differences between the protein profiles of the gametes and vegetative cells, the next step was to try to correlate bands from the Coomassie staining with bands from the western blot.

The initial plan for this technique was to trace the degradation of ubiquitinated proteins by following individual bands that decreased in intensity of color from the first time point in gametogenesis through the full gamete time point. Figure 2 is a drawing showing this predicted banding pattern.

![Figure 2: Predicted Western Blot Pattern Showing the Ubiquitin-Mediated Degradation of a Hypothetical Protein During Gametogenesis](image)

The actual western blot indicated a significant presence of ubiquitin in all samples, but no evidence of the predicted banding pattern (Figure 3). As with Coomassie staining, a western blot was performed examining the time interval of 12 hours to 15 hours. That blot looked very similar to the one presented in Figure 3, so, even though most of the changes probably occur between 12 and 15 hours, those changes do not follow the banding pattern predicted in Figure 2.
One explanation for the accumulation of protein at the top of the gels would be that the multi-ubiquitinated proteins appear to have very high molecular weights, even though the actual proteins themselves may not. There is very little separation of these high molecular weight proteins, so they appear as a “blob” and their degradation becomes impossible to follow. There are some lower molecular weight bands (40-80 kD) that do appear in the blot (they are faint and not visible in the reproduction given here), but they do not dissipate during gametogenesis and therefore must not be degraded. These bands could be examples of mono-ubiquitinated proteins that are de-ubiquitinated.

We know that the western blot method is working because there is a control lane of just ubiquitin. In that lane one can clearly see a band of low molecular weight free ubiquitin and then
several bands of higher molecular weight ubiquitin conjugates where ubiquitin has bound to itself. This lane encompasses the full range of molecular weights of the proteins under investigation (~5 kD - ~200 kD).

Immunoprecipitation was used to get a cleaner picture of the same information sought with western blotting, those proteins that have ubiquitin conjugated to them. The procedure will truly be put to the test during the summer of 1997, but a trial run using vegetative cells yielded remarkably clear results. Despite significant, correctable losses of sample volume before analysis, the gel showed the typical broad band at the top of the gel and two lower molecular weight bands that were not discerned from the western blots. The gel was stained using a silver staining procedure and then dried. Unfortunately, during the drying process, the gel became too dark to be reproducible for this paper, so a diagram of the results is shown instead (Figure 4). A preliminary evaluation of the old western blots indicates that the two lower molecular weight bands may actually be present, but were obscured by background color.

![Diagram of the Results from Immunoprecipitation](image-url)

Figure 4: Diagram of the Results from Immunoprecipitation

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In a last effort to glean some more usable information out of the first experimental approach, a very involved study was conducted using vegetative cells (heat shocked and control), cells 12 hours into gametogenesis (control cells saved from this summer; cells that were heat shocked and immediately harvested; and cells that were heat shocked, put back under the hood to continue gametogenesis and then harvested), and full gametes. Unfortunately, the gels (Figure 5) do not show much difference between lanes. The western blot (Figure 6) especially seems to be the same banding pattern over and over again. In the coomassie gel, there are some differences, but they are mostly in band intensity.

Figure 5: Coomassie Blue Stained Gel of Samples From Second Time Course
A sample of protein (from cells 12 hours into gametogenesis; 12 old) from the summer of 1996 was used as a control to compare the results from the summer to those from this experiment. For some reason, it appears that much of the protein degraded. In that lane there is a pale smear down the lane with a couple of darker bands, but nothing like what that same sample looked like over the summer.

Developing a somewhat different approach, radioisotopic pulse labelling experiments were conducted to establish the differences in protein synthesis between heat shocked and control cultures. The differences between control and heat shocked cells were dramatic, although the film did not reproduce well and is not included here. The two lanes of heat shocked cells showed massive accumulation of high molecular weight proteins (~ 200 kD) compared to the control, but virtually no other protein at all.
**Discussion:**

The experiments presented here do not fully answer the question of what role ubiquitin plays in gametogenesis, but they have led to promising ideas for further experimental strategies.

One of the biggest problems with the first experimental strategy outlined above is that the ubiquitination equalizes the molecular weights of the proteins. Because all bands are stained the same color in both Coomassie staining and western blotting, there is no way to distinguish the proteins from each other after this ubiquitination. To help remedy this problem, radioactive pulse labeling and autoradiography will be used to distinguish between old and new proteins. This technique will also be used in conjunction with heat shock experiments.

The initial goal of this project was to identify proteins which enter the proteasomal degradative pathway by correlating Coomassie-stained gels with anti-ubiquitin-probed Western blots. Of course, this investigative method has not worked because of the unexpected equalization of the protein molecular weights. This method would have been nice because it offered a broad spectrum approach to answering the questions posed in this investigation, namely, what is the role of ubiquitin in gametogenesis? The immunoprecipitation technique holds still some promise for this project.

One possible strategy would be to purify the protein extract by immunoprecipitation and then run the sample out on a gel for a very long time. This strategy would forfeit many of the low molecular weight proteins, but it might also separate the very high molecular weight proteins. This would be an acceptable tradeoff because it appears that most of the ubiquitin conjugates are of high molecular weight.
Some preliminary work has been done on specific proteins, namely α-tubulin and cytochrome b6/f, both of which decrease in concentration during gametogenesis. Also it is hoped that the relative activity of ubiquitin can be measured using one of these proteins as a reference.

The more promising lead is with cytochrome b₆/f which has been shown to be selectively degraded during gametogenesis. If it can be demonstrated that this degradation is by the proteasomal pathway, then, by following the degradation, it should be possible to elucidate the period of ubiquitin’s activity in gametogenesis.

The protein complex cytochrome b₆/f is present in the cell in very small quantities. The complex is involved in the photosynthetic electron transport chain and is located in the thylakoid membranes of chloroplasts. To monitor its presence will probably require differential centrifugation techniques followed by gel electrophoresis and staining with a cytochrome specific stain. All of these are possible, and the staining procedure, which would be the limiting factor, has already been tested in this laboratory with good results.

Some recent research in other laboratories investigating ubiquitin has involved the use of proteasome inhibitors. The benefit of these inhibitors in this project would be that they eliminate one of the complicating factors. A major problem in determining which proteins are tagged by ubiquitin is that as soon as a protein is tagged it is immediately degraded. If this degradation could be halted, then there would be a build-up of ubiquitinated proteins, and the analysis would be greatly simplified.

Also the question could be approached from a completely different angle using a proteasome inhibitor. Some indicator of differentiation, such as mating competency, could
be used to determine whether or not gametogenesis can occur if the proteasome is inhibited. If differentiation did not occur, then it would be obvious that the proteasome, and consequently ubiquitin, are essential to gametogenesis.


