Establishing ocular cell lines to monitor the ubiquitin-proteasome system (UPS) activity in cells with fluorescent GFP reporters

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Abstract:

Background: The Ubiquitin-proteasome system (UPS) plays a critical role in nearly every biological process that includes the removal of undesired damaged proteins. Reduction of UPS capacity is associated with aging and several age-related diseases. In order to understand the relationship between the accumulation of damaged proteins and aging or age-related diseases a reliable experimental system is necessary for measuring capacity of UPS. We establish an experimental procedure to measure capacity of UPS based on a GFP-fusion UPS reporter, Ub-G76V-GFP, constitutively expressed in Hela cells, a cell line prepared by other groups (Dantuma et al., 2000). We develop a method for cyclohexamide chase assay of the UPS reporter by utilizing fluorescence spectroscopy.

Methods and Findings: Cyclohexamide (CHX) chase assay of Ub-G76V-GFP was performed to measure the degradation kinetics of the UPS reporter. CHX chase assay was chosen as a method in order to eliminate the concern about the potential changes in the transcriptional rate of the UPS reporter. We used fluorescence spectrometry (fluorescence plate reader) and Western blotting to measure the level of Ub-G76V-GFP. The Hela cells constitutively expressing Ub-G76V-GFP were first incubated for 3 hours with a proteasome inhibitor, MG132, in order to accumulate the reporter to an observable level. After MG132 was removed, cells were incubated with or without cyclohexamide or cyclohexamide and MG132 combined. These cells were harvested at 0, 2, 4 and 6 hours during incubation and lysed in a buffer containing detergent (1% Triton X-100 and 0.03% SDS), and the level of Ub-G76V-GFP in the whole cell lysate and detergent soluble fraction was measured. With cyclohexamide alone, most Ub-G76V-GFP was degraded within 4 hours. Without cyclohexamide, most of Ub-G76V-GFP was still degraded in 6 hours. As expected, MG132 significantly blocked the degradation of Ub-G76V-GFP, although some of the Ub-G76V-GFP (~40%) was still degraded over 6 hours. Essentially, Ub-G76V-GFP in the whole cell lysate and detergent soluble fraction showed the same degradation rate, and almost no Ub-G76V-GFP was detected in the detergent insoluble fraction.

Conclusions: In this project, a method of monitoring the UPS reporter was established. This procedure can be used in the future to examine the effect of aging and protein –damaging stress on the capacity of the UPS. When using this method, we recommend performing the experiment within 4 hours because of the rapid degradation kinetics of Ub-G76V-GFP.

Introduction:

Cells have two major proteolytic machines for removing damaged proteins. The two main systems consist of the lysosomal proteolytic system (LPS) and the ubiquitin proteolytic system (UPS). The lysosomal proteolytic pathway contains lysosomes, which are organelles needed for the degradation of old and damaged proteins. The ubiquitin-proteasome system (UPS) plays an important role in biological processes pertaining to the degradation of undesired short-lived proteins by the proteolytic activity of the proteasome (Keller, 2004).

The deterioration of the UPS capacity is related to aging and several age-related diseases such as AMD and neurodegeneration (Ehrlich, 2008). In these conditions, the capacity of intracellular proteolysis is diminished by protein damaging stresses such as oxidations, protein-modification, and protein precipitation. Measuring the capacity of UPS is desirable to understand the relation between accumulation of damaged proteins and aging or age-related diseases. However, the detection of the impaired UPS is a huge challenge due to the complexity of this process. It consists of multiple steps catalyzed by different

enzymes (Beckman, 1998). Examination of the UPS capacity is possible when measuring the degradation rate of proteins that are exclusively degraded by the UPS.

We establish an experimental procedure to measure capacity of UPS based on a GFP-fusion UPS reporter, Ub-G76V-GFP, constitutively expressed in Hela cells, which was established Dantuma and other co-workers (Dantuma et al., 2000). A UPS reporter is a protein that is exclusively degraded by the UPS. This reporter is easy to examine when accumulating inside the cells. Groups such as Salomon (Salomon et al., 2009) and Nonaka (Nonaka et al., 2009) monitored the steady level (level at one time point) of the UPS reporter. However, a recent report demonstrated that proteasome inhibition increases transcription of the UPS reporter construct, in which the expression is driven by the cytomegalovirus promoter (Alvarez-Castelao, 2009).

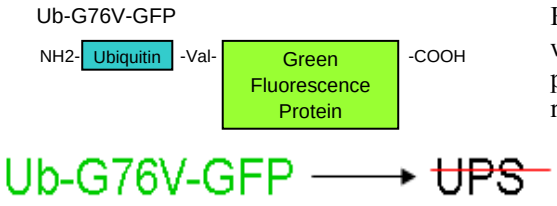

Figure 1: Ub-G76-GFP reporter. UPS reporter used to perform CHX chase assay to measure degradation kinetics of reporter. If the UPS is blocked then Ub-G76V-GFP will accumulate within the cells

It is known that without adding proteasome inhibitor, the UPS reporter is constitutively expressed. Since the degradation rate is rapid (faster than the rate of synthesis), the level of UPS reporter is small, lower than out lowest detection level. Therefore to make the UPS reporter accumulate, the proteasome must be inhibited. When the proteasome (end-point of the UPS) is inhibited, the degradation of the UPS is inhibited. Synthesis still continues so the UPS reporter accumulates. It is expected that the rate of degradation is proportional to the concentration of the UPS reporter. To model this relation, a first order kinetics model is used in which $-dA/dt=k[A]$, where [A] is the concentration of the UPS reporter in the cell and k is the rate constant.

Alvarez-Castelao B. et al. measured the rate of degradation by cyclohexamide chase assay. They found that monitoring the steady level of the UPS reporter is misleading. It was determined that the increase in the level of the UPS reporter is due to the increase of transcription activity and not the inhibition of the UPS reporter's degradation when the proteasome was inhibited in the cell expressing the UPS reporter. They recommended that UPS degradation should be examined when transcription level does not change (Alvarez-Castelao, 2009). Therefore cyclohexamide chase assay is a good option to monitor the UPS reporter because it eliminates the contribution of transcription level changes. This allowed for the assumption that the change in levels of the UPS reporter can be due to degradation since protein synthesis is blocked by cyclohexamide.

Not many groups have reported a study based on cyclohexamide chase assay of the UPS reporter; therefore this method was first tested to make sure that reporter degradation could be properly measured. In addition, FACS analysis has been done in most studies to monitor the GFP-fusion UPS reporter, but because of the availability of equipment, the GFP-fusion UPS reporter was measured by using fluorescence of plate reading and Western blotting.

Methods:

Overview

Cyclohexamide (CHX) chase assay of Ub-G76V-GFP was performed to measure degradation kinetics of the reporter. CHX chase assay was chosen as a method in order to eliminate concern about potential changes in transcriptional rate of UPS reporter. In order to examine the GFP-fusion UPS reporter through cyclohexamide chase assay. We used Hela cells stably transfected with a construct that constitutively expresses Ub-G76V-GFP. This cell line was prepared and provided by Dr. Nico P. Dantuma's group at Karlinska Institute in Sweden.

Preparation of cells in cyclohexamide chase analysis

Twenty-four hours before the experiment, 2 to 3 x 10^6 cells were plated onto 60 mm dishes. These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The purpose of counting the cell number for the 0 hour samples was to determine the amount of lysis buffer. Based on a preliminary experiment, it was found that dissolving about 10^6 cells in 100 µL of lysis buffer is optimum.

In the cyclohexamide chase assay (see scheme below), the Hela cells constitutively expressing Ub-V76G-GFP were first incubated for 3 hours with MG132, in order to accumulate the reporter to an observable level. The cells that were used as a control with no-MG132 were not incubated with the proteasome inhibitor. The purpose of this negative control, a set of cells incubated with fresh media without MG132, allowed for the determination of background fluorescence produced by cellular materials other than GFP. The cells were washed with about 2 mL of serum free DMEM for 4 times. The removal of MG132 allowed the degradation of the UPS reporter and inhibition to no longer take place, activating the UPS. The cells were then incubated for an additional 1 hour to allow the diffusion of MG132. Afterwards media was removed. All the T0 samples were placed on ice. After MG132 was removed, cells were incubated with or without cyclohexamide or cyclohexamide and MG132 combined. To the remaining plates, 3 mL of complete DMEM containing no inhibitor, 100 µg/mL cyclohexamide, or 100 µg/mL cyclohexamide and 10 µM of MG132 combined were added and incubated at 37°C.

In the first experiment, the cells were collected only at 0 (T0) and 4 (T4) hours. The 4 replicates were prepared for each condition as shown in Table 1. In the second experiment, the cells were collected at 0, 2, 4, and 6 hours. For this run, 2 to 3 replicates were prepared for each condition as shown in Table 2.

The samples were observed under the microscope before harvesting. Phase contrast images and fluorescence images were taken using the fluorescence microscope setting FITC. In order to harvest the cells the plates were washed with PBS and scrapped with rubber policeman. One dish of the cells (typically the 0 hour cells pre-incubated with MG132 like the other samples), were harvested by trypsinization. The numbers of cells were counted in order to determine the volume of lysis buffer to be added (procedure is described below). The samples were centrifuged at maximum speed for 5 minutes and washed three times by PBS. The samples were stored at -80 °C.

Preparation of the cell lysate

The cell samples were thawed and lysed in a lysis buffer (20mM Tris-HCl, 2mM EGTA, 2mM EDTA, 30mM NaF, 30mM $Na4O_7P_2$, 2mM Na_2VO_4, 1% Triton X-100, 0.03mM SDS, 10mM N-ethylmaleimine, 1mM AEBSF, pH 7.4). Approximately, 100µl of the lysis buffer was added to every 10^6 cells. The cells were lysed by pippeting up and down. The soluble supernatant was prepared by centrifuging the cell lysate at 16,000 x g at 4C for 10min and taking supernatant. The insoluble pellet was used to measure amount of DNA in the lysate as described below.

Measuring level of Ub-G76V-GFP

We used fluorescence spectrometry (fluorescence plate reader) and Western blotting to measure level of Ub-V76G-GFP. Using the plate reader the fluorescence was measured at excitation of 485/40nm and emission of 530/40nm. The level of Ub-G76V-GFP in the whole cell lysate and detergent soluble fraction was measured. For Western blotting, 10µg of total proteins were loaded onto each lane of 12% acrylamide SDS-PAGE gel. After the proteins were transferred to a nitrocellulose membrane, GFP was probed by an anti-GFP antibody (AbCam Boston MA).

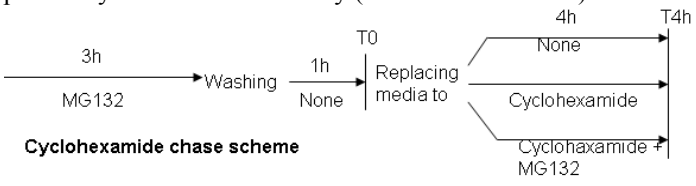

Figure 2: General Procedure for CHX chase assay

Time (hours)	Condition:	Number of dishes:
0 Control	No MG132	8 dishes (harvested at T0)
0	MG132 incubated	4 dishes (one dish, number of cells will be counted after trypsinization)*
4	No inhibitors	4 dishes
4	Cyclohexamide	4 dishes
4	Cyclohexamide + MG132	4 dishes

Table 1: Cyclohexamide chase analysis of Ub-G76V-GFP at 0 and 4 hours

* Counting the number of cells gave a rough estimate of volume of lysis buffer needed to be added to cells (done in every CHX chase assay)

Time (hours):	Condition:	Number of dishes:
0 Control	No MG132	2 dishes (harvested at T0: A1, A2,)
0	MG132	2 dishes (one plate will be counted after trypsinization, serves as a standard (A1, A2))
2	No inhibitor	2 dishes (A1, A2)
2	Cyclohexamide	2 dishes (A1, A2)
2	Cyclohexamide + MG132	2 dishes (A1, A2)
4	No inhibitor	1 dish (A)
4	Cyclohexamide	1 dishes (A)
4	Cyclohexamide + MG132	1 dishes (A)
6	No inhibitor	1 dish (A)
6	Cyclohexamide	1dishes (A)
6	Cyclohexamide + MG132	1 dishes (A)

Table 2: Cyclohexamide chase analysis of Ub-G76V-GFP at 0, 2, 4, and 6 hours

Determination of DNA contents in the cell lysate

The amount of DNA in the cell lysate was determined by a dye called Hoechest 33258. This dye exhibits significant increases of fluorescence when binding to double strand DNA (Tonary, 2006). Preliminary analysis showed that Hochest 33258 increased its fluorescence when mixed with sonicated insoluble pellet. Almost no increase of fluorescence was observed when mixed with the soluble fraction. In the CHX chase assay, the insoluble pellet was washed once with 50mM sodium phosphate pH7.5 2M NaCl and sonicated in 200μl of the same buffer for 10 second. 10μl of sonicated pellet suspension was mixed with 100μl of 10μg/ml Hoechest 33258 prepared in the same buffer. As a standard, 2.5 μg, to 40 μg of sonicated salmon sperm were also prepared and mixed with Hoechest 33258. The mixture was places on a 96 well plate and at 360/40 nm and emission 460/40 nm fluorescence was measured.

Determination of Ub-G76V-GFP remaining at each time point

The following procedure was first performed in order to determine the GFP fluorescence at T0 where the maximum level of Ub-V76G-GFP was expected. In addition, the background fluorescence from cellular materials without GFP was examined. Lastly, this procedure was done to analyze and ensure that the amount of Ub-V76G-GFP in the cell lysate and fluorescence were linearly related. The "T0 samples" were a set of cells were collected after 3 hours incubation with MG132 followed by additional 1 hour incubation without MG132. These cells were treated in the same manner as cells collected at later time points. The "T0 negative control samples" were another set of cells that were incubated for 3 hours without MG132 and collected after additional 1 hour incubation.

From these two sets of cells, whole cell lysate or soluble fraction sample were prepared as mentioned above. Cell lysates from the "T0 samples" were serially diluted in the lysate of the "T0 negative control samples" in total 50μl as shown in Figure 3A. The fluorescence corresponding to GFP in each dilution showed a linear relation with volume of lysate from the T0 samples or amount of DNA in the "T0 samples" (Figure 3B left).

This graph (shown in Figure 3B left) was converted into a standard curve (Figure 3B right) in order to determine percentages of Ub-G76V-GFP in a sample with respect to the "T0 sample". "100% T0" was defined as the average fluorescence from 50μl of the cell lysate from T0 samples. "0% T0" was defined as the average fluorescence from 50μl of the cell lysate from "T0 negative control samples" (This represents background fluorescence without GFP). When Ub-G76V-GFP is degraded during cyclohexamide chase assay, fluorescence at a time point should fall between "100% T0" and "0% T0". Similarly, "50% T0" was defined as average fluorescence from the sample in which 25μl of lysate from the "T0 sample" was mixed with 25ul of lysate from the "T0 negative control samples". This way, X-axis unit was converted from "μg of DNA" to "% T0" as shown in Figure 3B.

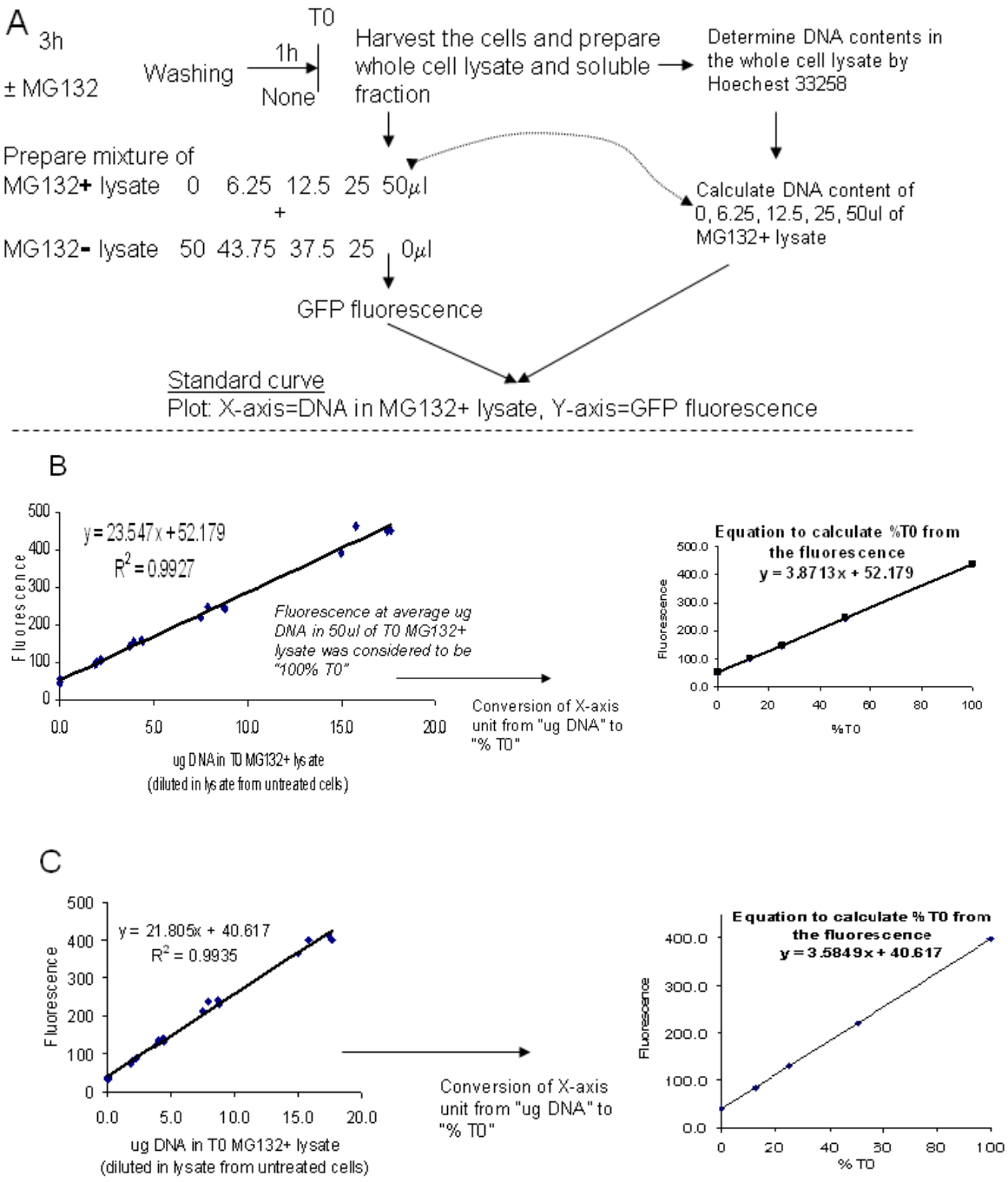

Figure 3. Standard curve to determine percentage of Ub-G76V-GFP remaining at each time point A. A scheme for generating a standard curve. Whole lysate or soluble fraction from the cells incubated with MG132 was serially diluted with lysate from the control cells, which was not incubated with MG132. (These cells were collected at time zero during cyclohexamide chase assay). Fluorescence of GFP was plotted against amount of DNA in the corresponding volume of the MG132-treated cells. **B.** A representative standard curve for the whole cell lysate from 4 dishes of cells. **C.** A representative standard curve for the soluble fraction from 4 dishes of cells.

To obtain a GFP signal from the same number of cells, the fluorescence in each sample were normalized by amount of DNA in the cell lysate[1]. By plugging in normalized GFP into the standard curve, percentages of Ub-G76V-GFP were extrapolated.

Results:

CHX chase analysis for 0 and 4 hours

First, to obtain a roughly asses the degradation rate of Ub-G76V-GFP, we conducted cyclohexamide chase assay by measuring GFP immediately after MG132 was removed. 4 hours after the cells were incubated with cyclohexamide. To examine if cyclohexamide actually blocked the synthesis of Ub-G76V-GFP, one set of cells were incubated without any inhibitor. To ensure Ub-G76V-GFP was degraded by the proteasome, another set of the cells were incubated with cyclohexamide and MG132 combined. 4 dishes (i.e. 4 replicates) of the cells were prepared for each condition to examine how much error was associated with this procedure. The results are shown in Figures 4 and 5. In the presence of cyclohexamide most of the Ub-G76V-GFP was degraded within 4 hours and about 50% of the Ub-G76V-GFP was degraded without any inhibitors added (no cyclohexamide or MG132). When the cells were incubated with cyclohexamide and MG132 combined, no degradation of Ub-G76V-GFP was observed proving that the Ub-G76V-GFP degradation is dependent on the proteasome.

In Figure 4, it can be noted that the minor bands of protein react with anti-GFP antibody (the blue arrows indicate this). These proteins accumulate when the cells are incubated with MG132 implying these are fragments of GFP. In the insoluble fraction image, no bands are seen of the 0 and 4 hour except the samples with MG132. The 4 hour samples treated with MG132 and cyclohexamide show a faint band. Twice the amount for the insoluble fraction was loaded compared to the soluble fraction. If the same amount of sample was loaded then no bands are seen in the insoluble fraction (already tested in a previous experiment, data is not shown). This corresponds to the minor band at ~26 kD shown in the soluble fraction at 4 hours. Since these bands are consistent with one another, this suggests that this minor band goes into the insoluble fraction but is still degraded slowly by the proteasome. The minor bands are not simply leftover fragments of the soluble fraction that were washed off. If that were the case, then a major band of Ub-G76V-GFP (the big band slightly below 47 kD in the whole cell) should also be present in the insoluble fraction. The major band of the soluble fraction shows essentially the same results as the whole cell lysate. The loading control in this figure was used to show that the proteins from the same number of cells are loaded on the blot. It can be seen that the levels of GAPDH does not change significantly in the cell samples so it is implied that GAPDH is degraded slowly in the cells. This amount would not change much during the CHX chase assay.

In Figure 5, the amount of Ub-G76V-GFP remaining at 4 hours was examined. In this particular experiment, a standard curve was created by serial dilution of the lysate from the 4 dishes independently. The data from the 4 dishes were combined as explained in the legend of Figure 5.

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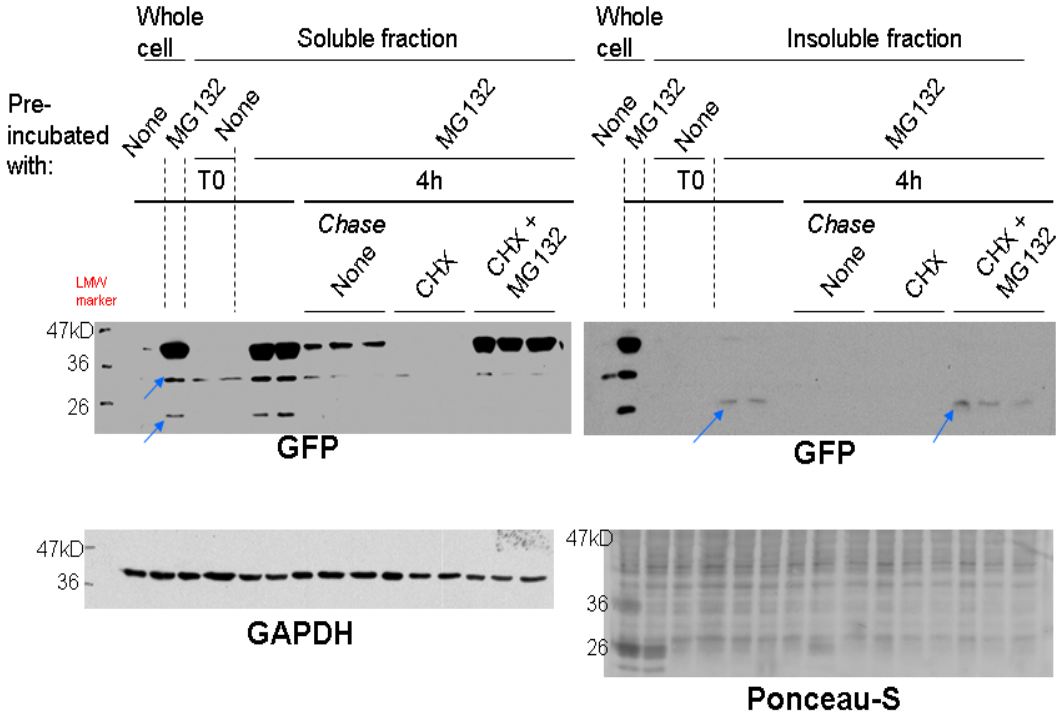
[1] A sample calculation for normalizing fluorescence: First the average amount of DNA in 50µl of whole cell lysate from the "T0 samples" was determined. In the experiment shown in Figure 3, it was 16.4µg, and this was used as a reference point. Next, fluorescence and the amount of DNA in 50µl of whole cell lysate from one of the samples collected at 4 hours was found to be 221 (arbitrary unit) and 14.6mg respectively. The fluorescence was scaled in this particular sample so this sample would contain 16.4µg of DNA (221 (a.u) x (16.4µg / 14.6µg) = 238). This value (238) was plugged in the standard curve shown in Figure 3B to determine the percentage of Ub-G76V-GFP remaining in this sample.

Figure 4. Western blot analysis of Ub-G76V-GFP. 10μg of total protein from the whole cell lysate (T0 only) and the soluble fraction was loaded on each lane. For the insoluble percentage, the volume was double of the whole cell lysate volume loaded. (Loading volume 4 times more for the insoluble fraction showed the same result (data not shown)) GFP was probed by anti-GFP antibody. GAPDH and Ponceau S staining of the same blot are shown as loading controls. No GAPDH was detected from the insoluble fraction (not shown). Arrows indicate the minor species with GFP immuno-reactivity that accumulated in the presence of MG132. Duplicates of each condition were loaded.



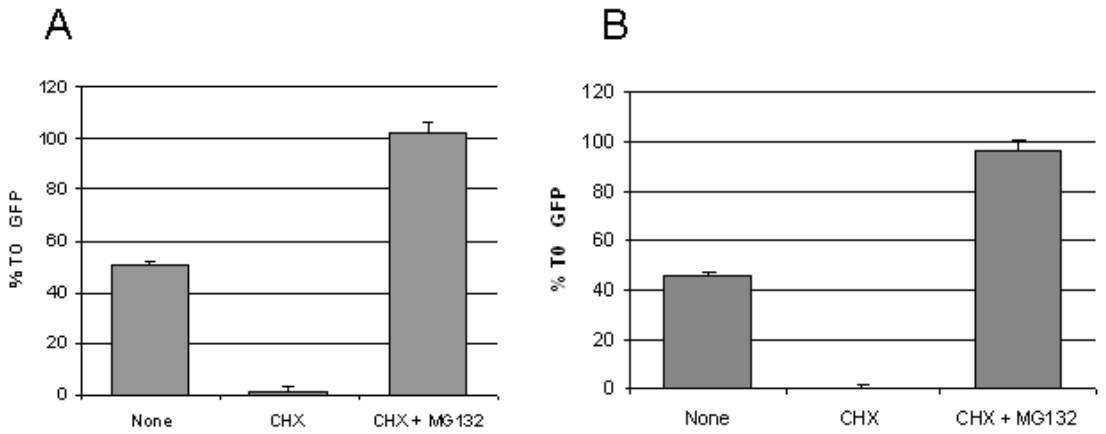


Figure 5. Ub-G76V-GFP remaining at 4 hours A. in the whole cell lysate B. and the soluble fraction at 4 hours during cyclohexamide chase analysis. Amount of Ub-G76V-GFP was determined by fluorescence spectroscopy (plate reader) and normalized by DNA content in the lysate. Percentage of Ub-G76V-GFP at 4 hours with respect to the amount of Ub-G76V-GFP at time zero was calculated. None: Cells incubated without any inhibitor after removal of MG132. CHX: Cells incubated with cyclohexamide. CHX + MG132: Cells incubated with cyclohexamide and MG132 combined. Data represent average 4 replicates of cells treated with out without inhibitor independently. Error bars represents standard deviation.

CHX chase analysis for 0, 2, 4, 6 hour samples

In this experiment, the main goal was to examine the amount of Ub=G76V-GFP in the cells at each time point. The amount of DNA was measured in order to normalize the GFP fluorescence so that the GFP fluorescence from the same number of cells could be compared between the different time points or different conditions. The amount of protein was measured in order to load the same amount of protein from each time point or each condition. This normalization assumed that the total amount of protein per cell did not change significantly during the experiment. Figure 8 displays the image of anti-GAPDH blot of the whole cell lysate and detergent soluble fraction samples for both 0 and 4 hours. This Western blot semi-quantitatively analyzes the amount of GFP remaining at each time point. GAPDH or Ponceau S is used as a loading control, which shows that more ore less equal amounts of proteins are loaded. Western blot gives information about anti-GFP.

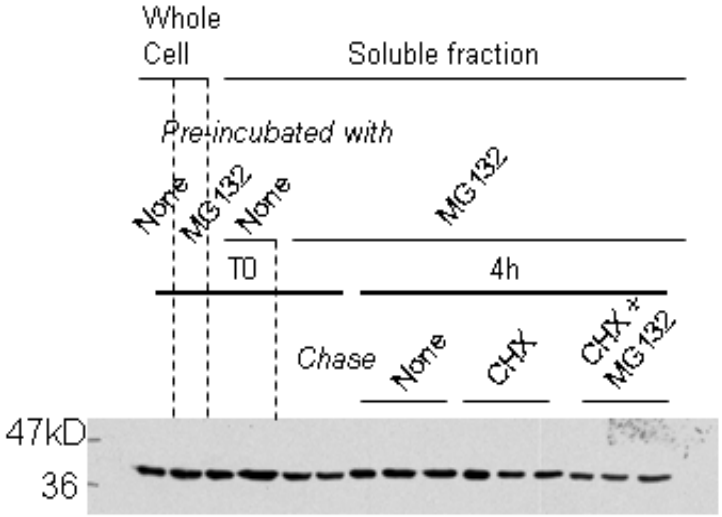


Figure 6. Anti-GAPDH Western blot image of the soluble fraction for the CHX chase analysis. Samples for the 0 and 4 hours time points were loaded.

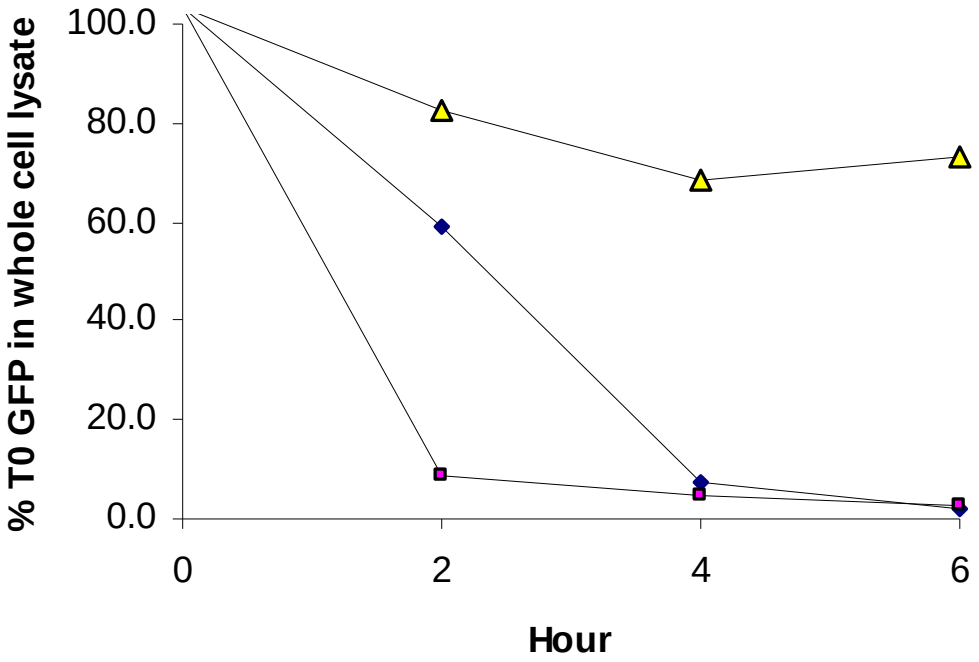


Figure 7. Degradation of Ub-G76V-GFP over 6 hours. With cyclohexamide, most of the reporter was degraded within 2 hours. Without inhibitor most of the reporter degraded within 4 hours. As seen there is not much difference between cyclohexamide alone and no inhibitor at 4 hours. Degradation was blocked by MG132 showing proteasomal degradation of the reporter. "% T0" was the determined amount of Ub-G76V-GFP remaining with respect to the beginning of cyclohexamide chase assay from the same number of cells. Value was expressed in terms of a percentage.

In order to obtain a visual analysis for monitoring the activity of Ub-GFP reporter, phase contrast and fluorescence images were taking using a fluorescence microscope as shown in Figure 8.

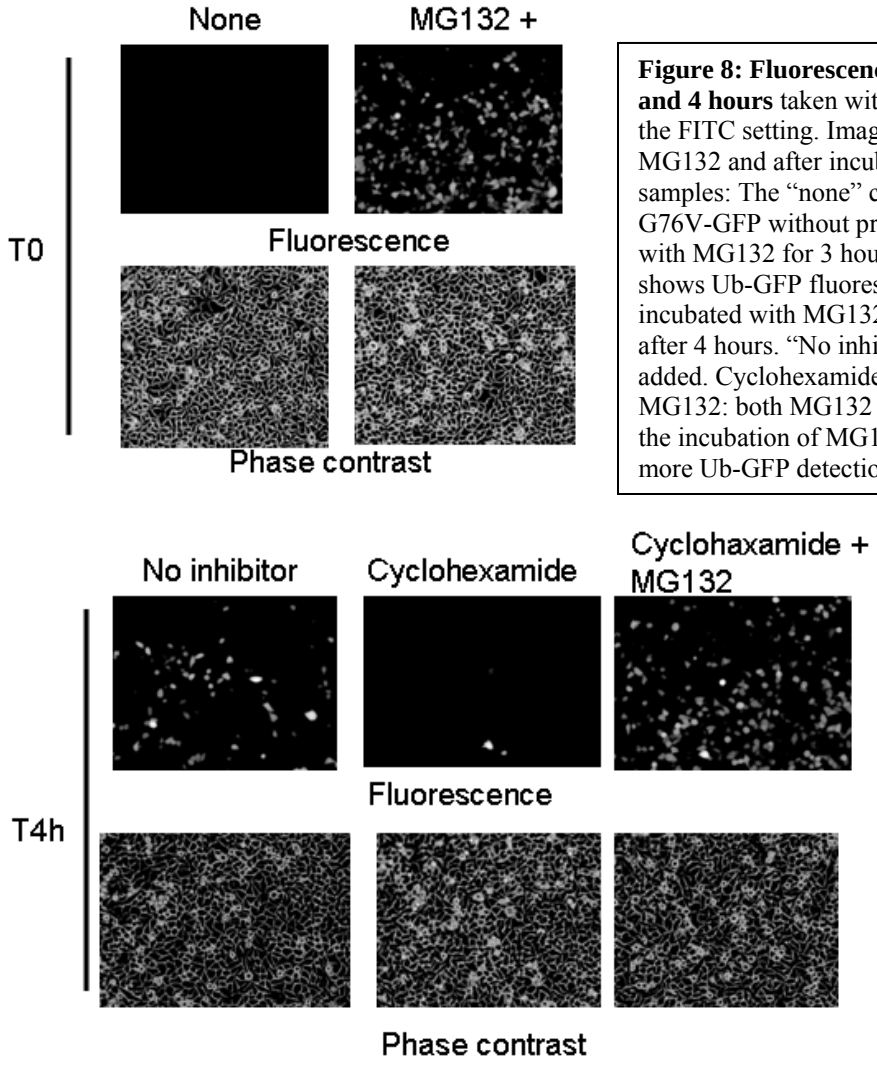


Figure 8: Fluorescence and phase contrast images at 0 and 4 hours taken with the fluorescence microscope using the FITC setting. Images were taken before incubation with MG132 and after incubation with or without CHX. T0 samples: The "none" cells are Hela cells expressing Ub-G76V-GFP without pre-incubation with MG132. Incubation with MG132 for 3 hours and 1 hour with serum free media shows Ub-GFP fluorescence. T4 samples: All samples pre-incubated with MG132. Figures all showed fluorescence after 4 hours. "No inhibitor": no MG132 or cyclohexamide added. Cyclohexamide: just CHX. Cyclohexamide + MG132: both MG132 and cyclohexamide. The image with the incubation of MG132 and cyclohexamide experienced more Ub-GFP detection than the other conditions.

Discussion:

When examining the fluorescence and phase contrast images obtained after incubation with or without MG132 for 3 hours and media for 1 hour, GFP signals can be analyzed. Without the addition of MG132, no GFP signal was detected as shown in the "none" image in Figure 8. These cells were stably transfected contained a piece of DNA that expressed the Ub-G76V-GFP gene, which was inserted into the chromosome of the construct. Ub-G76V-GFP accumulates after 3 hour incubation with MG132 as shown in the T0 fluorescence image. After the 1 hour incubation with media there was still a significant level of GFP signal present as shown in Figure 8. The additional 1 hour incubation without MG132 allowed for the remaining MG132 to diffuse out of the cells. Washing the MG132 out of the cells allowed for protein degradation to occur and this additional 1 hour incubation allowed the cells to begin the degradation process again. A set of cells were harvested immediately after the 1 hour incubation, these samples were considered to be time zero (T0). The rest of the cells were incubated for 4 hours with media containing either no cyclohexamide, 100μg/mL cyclohexamide, or 100 μg/mL cyclohexamide and 10 μM MG132. In 4 hours most of the observable GFP signal was gone in the cells incubated with CHX. However, no major changes of GFP were noted when MG132 was added to CHX showing that Ub-G76V-GFP was degraded

by the proteasome. The four replicates of cells incubated without any inhibitor after removal of MG132 at the 3 hour time point as well as the cells incubated with cyclohexamide and MG132 combined, both experienced GFP signal. Cells incubated with cyclohexamide did not, which proves that cyclohexamide stopped protein synthesis. The GFP in the cells without any inhibitors (no CHX and no MG132) shows a decrease in GFP signal in 4 hours. This signifies that from 0 to 4 hours, the rate of Ub-G76V-GFP degradation is faster than its synthesis. When looking at the level of Ub-G76V-GFP at 0 and 4 hours, the average rate of degradation within 4 hours was only examined. Therefore another experiment was repeated looking at samples from 0 to 6 hours.

Figure 7 shows that without the presence of cyclohexamide most of the Ub-G76V-GFP degraded within 6 hours. As expected MG132 blocked protein degradation significantly even through some of the Ub-G76V-GFP was still degraded at 6 hours. In cells incubated with cyclohexamide alone about 90% of the Ub-G76V-GFP was degraded within 2 hours, after two hours the degradation rate slowed. The cells labeled as "no incubator" had degradation occur within 4 hours. The decreasing degradation rate was due to the fact that not much Ub-G76V-GFP was left in the cells to be degraded. The samples in the 0 to 6 hour experiment gave an average level of Ub-G76V-GFP from 0 to 6 hours; however, it was difficult to analyze so many samples at once. Conducting the experiment at 0 and 4 hours only allowed for more dishes to be handled per condition for more accurate results.

The observation that Ub-G76V-GFP degrades even with MG132 suggests that there might be a proteolytic system other than the proteasome that degrades Ub-G76V-GFP. This point was not proven, but is important to consider for future repeats. In addition, as mentioned that the rate of degradation for cyclohexamide alone is rapid from 0 to 2 hours and slows down afterwards indicating that the rate of degradation is proportionally correlated with the amount of Ub-G76V-GFP present in the cells. This implies that when Ub-G76V-GFP is abundant, the rate is fast, but when its level is low, the rate of degradation is slow.

After the cells were lysed with buffer, the GFP fluorescence was measured from the whole cell lysate and the detergent soluble fraction by a fluorescence plate reader. Figure 5 shows the amount of Ub-G76V-GFP remaining at 4 hours in the whole cell lysate and the soluble fraction. The amount of Ub-G76V-GFP measured by fluorescence spectroscopy (plate reader) was normalized by DNA content in the lysate. The percentage of Ub-G76V-GFP at 4 hours regarding the amount of Ub-G76V-GFP at time zero was calculated.

In addition, the same average μg of DNA and protein in the soluble fraction of the T0 samples incubated with MG132 lysate were used to determine the μg of DNA and protein from the fluorescence measured as shown in Figure 3. In all the graphs, the amount of lysate and GFP fluorescence showed a linear relation in the range of sample volume used in the experiment. From the insoluble fraction, DNA content was measured. During optimization of measuring DNA, it was observed that most of the DNA remains in the insoluble fraction. GFP fluorescence was normalized based on DNA content knowing that the number of cells and DNA content are proportionally related.

GFP in the soluble and insoluble fractions were probed by Western blotting using an anti-GFP antibody. Essentially, it was proven that the whole cell lysate and the detergent soluble fraction showed similar results as seen from fluorescence spectrometry. Roughly the same amount of Ub-G76V-GFP was detected in both parts. In the second experiment analyzing samples from 0 to 6 hours, Western blotting detected small GFP-positive bands in the insoluble fraction and these bands were stabilized by MG132. This suggested that a small percentage of Ub-G76V-GFP fragments seen in the whole cell lysate go into the insoluble fraction and are still degraded by the proteasome. On the other hand, no intact Ub-G76V-GFP was observed in the insoluble fraction even if these intact species were more abundant. Figure 4 shows that in the insoluble fraction there were almost no proteins that reacted with anti-GFP. It was also found that no GAPDH was detected from the insoluble fraction. This signified that there was hardly any Ub-G76V-GFP in the insoluble fraction. As seen from both Figure 4 and 6 the signal detected for both the whole cell lysate and soluble fraction were roughly the same, and GAPDH was detected in both. As seen in Figure 4, the bands appearing at 26kD and 36 kD gave a rough estimation of the molecular weight of the proteins. It is known that the theoretical mass of GFP is about 28 kD and that of ubiquitin is about 8.5 kD. When summed together the total molecular weight is about 39 kD. Western blot is an advantageous method because it allows us to see these fragments where as most methods will not.

Conclusion:

In this project, I established a method to measure the capacity of UPS based on a GFP-fusion UPS reporter, Ub-G76V-GFP, constitutively expressed in Hela cells. Cyclohexamide chase assay was chosen as the method of preference because it eliminated the concern relating to the transcriptional rate of the UPS reporter. Essentially using this inhibitor allowed for us to block protein synthesis allowing for the degradation kinetics of the reporter to be examined.

The method established utilizes fluorescence spectrometry (fluorescence plate reader) and Western blotting to measure level of Ub-V76G-GFP. Through the accumulation of the reporter with the use of MG132, Hela cells constitutively expressing Ub-G76V-GFP were examined. By washing the MG132 after 3 hour incubation cells could degrade the UPS reporter. Levels of Ub-G76V-GFP in the whole cell lysate and detergent soluble fraction were measured through analyzing fluorescence with a plate reader. With cyclohexamide alone it was found that most Ub-G76V-GFP was degraded within 4 hours. Without cyclohexamide, most of Ub-G76V-GFP was still degraded within 6 hours. As expected, MG132 significantly blocked degradation of Ub-G76V-GFP, although some Ub-76G-GFP was still degraded over 6 hours. About 60% of Ub-G76V-GFP was degraded in 4 hours without any inhibitor. About 5% of Ub-G76V-GFP was degraded with cyclohexamide and MG132 combined. If this experiment were conducted again the 2 hour samples would be advantageous to examine mainly because rapid degradation of all sample conditions occur at this point. It can be concluded that Ub-G76V-GFP is rapidly degraded, and its degradation is mostly dependent on the proteasome. The Ub-G76V-GFP in the whole cell lysate and detergent soluble fraction samples showed similar results, however hardly any Ub-G76V-GFP was detected in the detergent insoluble fraction.

By establishing such a protocol to monitor UPS reporter degradation, one can understand the effect of cellular stress such as protein-damaging agents and aging on UPS capacity. It is recommended to perform the experiment within 4 hours because of very rapid degradation kinetics of Ub-G76V-GFP. This method established allows for error of 4 replicates was between 1 to 9%. In addition, this procedure allows for the reliable measurement of the degradation rate of Ub-G76V-GFP, as shown in Figure 7.

Even though FACS analysis is often used for assaying UPS reporters, the method established can monitor the behavior of the reporter, which FACS analysis may not be able to do. Further experiments can examine the UPS reporter degradation in older cells in the presence of stress as well as the mRNA levels using real-time PCR. Additionally, measuring Ub-G76V-GFP in soluble and insoluble fractions using buffer without detergent can be performed. This will allow for the examination of reporter degradation in the membrane fraction and the soluble (cytosolic) fraction. Lastly, similar experiments can be carried out using other UPS reporters.

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