Short Technical Report

Use of Short-Lived Green Fluorescent Protein for the Detection of Proteasome Inhibition

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ABSTRACT

Human embryonic kidney (HEK293) cells were stably transduced with a retroviral vector containing an expression cassette for a short-lived green fluorescent protein (d2EGFP) and the neomycin resistance gene (Neo^r). When Neo^r HEK293 clones were treated with proteasome inhibitors, lactacystin or MG132, an increase in the constitutive levels of d2EGFP expression was observed. Based on flow cytometry, proteasome inhibitors induced a 5- to 10-fold increase in the fluorescent intensity of d2EGFP in HEK293 cell clones. However, in the presence of proteasome inhibitors, HEK293 clones showed a 4- to 6.5-fold increase in d2EGFP concentration as determined by western blot analysis. Our data suggest that d2EGFP is a useful indicator of proteasome inhibition. Therefore, stable expression of d2EGFP in mammalian cells is potentially useful for high-throughput screening of cDNAs or pharmaceutical drugs that repress proteasome functions in vivo.

INTRODUCTION

Proteasome is an intracellular proteolytic system that degrades polyubiquitinated proteins and recycles the ubiquitin moiety. It is a large complex consisting of the core catalytic domain (20S particle) and the regulatory docking domain (19S particle) that functions as a platform for the recognition and entry of ubiquitinated proteins. The core catalytic domain is capped on each end by a 19S structure, consisting of several ATPases and receptors for proteasome substrates (10). In contrast, the ubiquitin system consists of an array of three enzymes, E1, E2, and E3, which collectively function to attach ubiquitin moieties to proteins destined for degradation by the proteasome (2).

Proteasome inhibitors are valuable experimental tools for probing the mechanisms of intracellular protein degradation. As a microbial product, lactacystin is a specific and irreversible inhibitor of proteasome activity via covalent linkage to the active site of β -subunits, a component of the 20S catalytic core (4,6). In contrast, MG132 is a peptide aldehyde that reversibly inhibits proteasome activity by functioning as a transition state analogue (4,6) for β -subunits. While lactacystin inhibits both the chymotrypsin and trypsin activities of β -subunits with different kinetics, the MG132 primarily

inhibits the chymotrypsin activity of proteasome (4,6).

We report here the development of a quantitative assay for the rapid detection of ubiquitin-proteasome inhibition in vivo. Dantuma et al. (3) have recently demonstrated the utility of an EGFP fusion protein containing an N end rule degradation signal to monitor proteasome inhibition by way of transfection studies. We, on the other hand, show the utility of EGFP fusion protein containing a proteolytic signaling domain "PEST sequence" to measure proteasome inhibition quantitatively. We made use of stable expression of a short-lived green fluorescent protein (d2EGFP) in human embryonic kidney (HEK293) cells to test its fluorescent intensity when proteasome is inhibited. d2EGFP is the same as EGFP, except it has a 39 amino acid insertion at its C-terminus that is originated from the mouse ornithine decarboxylase (ODC) gene (7). The aforementioned insert contains the proteolytic signaling domain or PEST sequence from the C-terminus coding region of ODC gene.

Our study shows that stable expression of d2EGFP in mammalian cell lines can be used as a sensitive indicator of ubiquitin-proteasome inhibition. Furthermore, it is feasible to use this system for high-throughput screening of cDNAs or pharmaceutical agents that inhibit proteasome functions in vivo.

MATERIALS AND METHODS

Cells

HEK293 and a 293-derived amphotropic retroviral packaging (Phoenix) cells were grown in DMEM (Life Technologies, Rockville, MD, USA) containing 10% fetal bovine serum, streptomycin (100 µg/mL), and penicillin (100 U/mL). Both cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Construction of Retroviral Vectors

The retroviral backbone plasmid, pLNCX, was obtained from Clontech Laboratories (Palo Alto, CA, USA). Plasmid pLNCX contains a neomycin resistance (Neo^r) gene driven by the long terminal repeat (LTR) promoter from the murine leukemia virus (MuLV), followed by the cytomegalovirus (CMV) immediate early promoter. The construction of the PN-1 vector was described elsewhere (8). *Bam*HI (blunt-ended with Klenow)/*Not*I or *Pin*AI/*Xho*I coding fragment of d2EGFP (pd2EGFP; Clontech Laboratories) or EGFP (pEGFP-C1; Clontech Laboratories) gene, respectively, was cloned into the *Stu*I-*Not*I or *Pin*AI-*Xho*I multiple cloning sites (MCS) of the plasmid PN-1. The resulting constructs, in which the CMV promoter drives the expression of EGFP and d2EGFP, were designated as pPN-5 and pPN-25, respectively.

Retroviral Production and Transduction

Retroviral production was carried out according to the manufacturer's specifications (Clontech Laboratories). Briefly, each recombinant plasmid was co-transfected with an expression plasmid for vesicular stomatitis virus G glycoprotein (VSV-G) into Phoenix cells using a calcium phosphate transfection kit (Promega, Madison, WI, USA). Culture supernatant containing recombinant virus was collected and titered on human fibrosarcoma cell line (HT1080) based on neomycin selection (1). Monolayer cultures of HEK293 cells were transduced at a multiplicity of infection of 1 in the presence of 8

µg/mL Polybrene (Sigma, St. Louis, MO, USA). Neo^r HEK293 cell clones were selected in the presence of 900 µg/mL of the neomycin analogue, G418 (Life Technologies).

Fluorescent Microscopy

HEK293-PN25-C3 cells (5×10^4) were seeded in chamber slides (Nalge Nunc International, Naperville, IL, USA) one day before treatment with 2.5 µM lactacystin or MG132. One day after treatment, cells were fixed in 4% paraformaldehyde in PBS for 10 min at -20°C. Cells were rinsed in PBS twice, and a coverslip was then placed on each slide using mounting medium. Slides were examined immediately by a fluorescent microscope (Zeiss, Oberkochen, Germany). All pictures were taken at 400× magnification by a Model F Reflex Nikon® camera.

Western Analysis

Cell pellets were resuspended in lysis buffer [150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.05 mM PMSF, 0.5 mM dithiothreitol (DTT), 0.5% Nonidet® P-40, 0.5% sodium deoxycholate, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA)] and subjected to four rounds of freeze/thawing in a dry ice/ethanol and a 37°C water bath, respectively. Samples were then spun down in a microcentrifuge (14000× g) for 15 min at 4°C to pellet cell debris, and supernatants were collected for protein analysis. Protein concentrations were calculated using a Bradford assay (Sigma). Indicated amounts of each sample were resolved by gel-electrophoresis (12% polyacrylamide, 29:1 acryl:bisacryl), and blotted to polyvinylidene difluoride (PVDF) membrane using a transfer apparatus (both from Bio-Rad Laboratories, Hercules, CA, USA). Protein blots were blocked in solution A (5% milk in PBS and 0.2% Tween® 20) at room temperature for 1 h on a plate rocker. Next, blots were incubated with a 1:100 dilution of rabbit anti-GFP antibody (Clontech Laboratories) in solution B (1% milk in PBS and 0.2% Tween 20) at room temperature for 2 h on a plate rocker. After washing three times in so-

lution C (0.2% Tween 20 in PBS) at room temperature, protein blots were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:10 000 dilution) at room temperature in solution D (2.5% milk in PBS and 0.2% Tween 20) for 1 h and subsequently washed four times in solution C at room temperature. Western blot detection of CyP-A protein was performed exactly as described previously (8). Protein bands were detected using an ECL® kit (NEN® Life Science Products, Boston, MA, USA). Blots were scanned and quantitated using a Fluor-S™ MultiImage System and Quantity One® software (both from Bio-Rad Laboratories), respectively.

Flow Cytometric Analysis

HEK293 clones were plated in tissue culture dishes (5×10^5 /6-cm dish) and one day later treated with the indicated amounts of either lactacystin or MG132 (Biomol, Plymouth Meeting, PA, USA). The untreated and treated cells were trypsinized 20 h later, rinsed in growth medium (DMEM and 10% FBS), and resuspended in fresh medium for flow cytometry. All samples were analyzed in the presence of propidium iodide (2 µg/mL) using a Coulter EPICS™ XL-MCL model flow cytometer (Beckman Coulter, Miami, FL, USA) at the University Of Colorado Health Sciences Center core facility. There was no significant cell death 20 h after treatment with lactacystin or MG132.

RESULTS AND DISCUSSION

We were interested in developing a quantitative assay to screen for cDNAs whose expression in sense or antisense orientation would result in inhibition of proteasome functions in vivo. We reasoned that such an assay would also be highly useful for the rapid detection of pharmaceutical compounds that interfere with proteasome functions. GFP has been used extensively in the development of a variety of assays (5). Its N- and C-termini are amenable to modification without significant change in fluorescent intensity. EGFP is highly stable with a half-life of over 24 h in eukaryotic cells (5). However, insertion

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of the PEST sequence from the mouse ODC gene at the C-terminus of EGFP is reported to reduce its half-life to less than 2 h (7).

We hypothesized that cells expressing d2EGFP, when treated with proteasome inhibitors, should fluoresce at a higher intensity as compared to the untreated control cells. To test this hypothesis, we constructed a retroviral vector (PN-25) that contains an expression cassette for d2EGFP driven by the

CMV promoter as described in Materials and Methods. This vector also contains a Neo^r gene driven by the LTR promoter of MuLV. PN-25 vector was used to transduce HEK293 cells, and several Neo^r clones were isolated in the presence of the neomycin analogue, G418. We also established HEK293 Neo^r clones transduced with PN-5 vector containing the CMV-driven expression cassette for the stable EGFP.

Based on fluorescent microscopy

(data not shown), the fluorescent intensity of HEK293 cells transduced with PN-25 vector is significantly lower than the fluorescent intensity of HEK293 cells transduced with PN-5 vector. However, the fluorescent intensity of HEK293-PN25 cells treated with proteasome inhibitors (lactacystin or MG132) appeared to be approximately equivalent to HEK293-PN5 cells. To determine the magnitude of increase in d2EGFP fluorescence mediated by lactacystin or MG132, two independent clones of HEK293-PN25 cell lines (C1 and C3) were treated with each inhibitor separately and analyzed by flow cytometry. In Figure 1, the histogram profiles of the steady state levels of d2EGFP expression for each clone are overlapped to depict the change in cell fluorescence in the presence or absence of inhibitors. Whereas untreated C1 and C3 clones showed different baseline mean fluorescent intensities, treatment with proteasome

Figure 1. Flow cytometric profile of d2EGFP expression in HEK293 cell lines treated with the proteasome inhibitors. Two independent clones of HEK293 cells [C1 and C3 (5×10^5 cells/6-cm dish)] were cultured and the next day left untreated or treated with 2.5 and 5 μ M of either lactacystin or MG132. Cells were analyzed by flow cytometry for the fluorescent level of d2EGFP 20 h after treatment. Histogram profiles of the steady state levels of d2EGFP expression are shown in each panel. Untreated C1 and C3 cell lines are 95% and 97% positive for d2EGFP expression, respectively. Both lactacystin and MG132 significantly increased the fluorescent intensity of C1 and C3 clones. Filled gray histograms for each panel represent HEK293 untransduced cells. Black, blue, and green lines represent the d2EGFP expression profile of HEK293 clones treated with zero, 2.5, and 5 μ M of the indicated proteasome inhibitor, respectively. Mean, median, and peak channel fluorescence data for C1 and C3 cell lines were determined using CellQuestTM software (BD Biosciences, San Jose, CA, USA): untreated (left panels, C1 = 142/92/106; C3 = 180/132/83), 2.5 μ M LS (C1 = 222/134/82; C3 = 386/289/194), 5 μ M LS (C1 = 366/250/313; C3 = 1172/835/1094), untreated (right panels, C1 = 104/81/83; C3 = 143/97/79), 2.5 μ M MG132 (C1 = 363/266/271; C3 = 300/243/252), and 5 μ M MG132 (C1 = 487/368/537; C3 = 313/266/261). PI and LS denote proteasome inhibitor and lactacystin, respectively.

Figure 2. Fluorescent microscopy of HEK293-PN25-C3 cell line treated with the proteasome inhibitors. HEK293-PN25-C3 clonal cells were untreated (a) or treated with 2.5 μ M lactacystin (b) or 2.5 μ M MG132 (c) as described in Materials and Methods. Pictures were taken 24 h after treatment at 400 $\times$ magnification.

Figure 3. Western blot detection of EGFP and d2EGFP in the stably transduced HEK293 cell lines. HEK293-PN25-C3 clonal cells were cultured and the next day left untreated or treated with either lactacystin or MG132, exactly as described in Figure 1. Cell pellets were processed for the detection of EGFP, d2EGFP, and CyP-A by western blotting as described in Materials and Methods. Total protein (20 μg) for each sample was analyzed for the detection of EGFP or d2EGFP. On a separate blot, 5 μg total protein for each sample was analyzed for the detection of CyP-A protein. HEK293-PN5 cells stably express EGFP, which migrates at a lower molecular weight as compared to d2EGFP containing the mouse ODC's PEST sequence. UT denotes the untransduced HEK293 cells.

inhibitors significantly increased the fluorescent intensity of both clones. In the case of C3 clone, approximately a 10-fold increase in the fluorescent intensity of d2EGFP was detected in the presence of 5 μM lactacystin. The C3 clone showed a dose-dependent increment in fluorescent intensity between 0 and 5 μM lactacystin (data for 2.5 and 5 μM are shown). Figure 2 compares the fluorescent intensity of d2EGFP in C3 clone by fluorescent microscopy when cells were untreated or treated with 2.5 μM of either lactacystin or MG132. Augmented fluorescence of d2EGFP is probably due to the accumulation of d2EGFP molecules that are not being degraded in the presence of proteasome inhibitors. To verify this, total protein from untreated and lactacystin- or MG132-treated cells were analyzed by western blot analysis for the detection of d2EGFP (Figure 3). The levels of d2EGFP in HEK293-PN25-C3 cells in-

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creased 4- or 6.5-fold when cells were treated with 2.5 or 5 μ M lactacystin, respectively. This suggests that d2EGFP is not only a suitable indicator of proteasome inhibition but also is a good reporter for the magnitude of lactacystin-mediated proteasome inhibition. Similarly, MG132 treatment elevated the levels of d2EGFP in HEK293-C3 clone by approximately 2-fold either at 2.5 or 5 μ M concentration (Figure 3). However, MG132 treatment did not impart a dose-dependent inhibition of proteasome activity, at least within the concentration range (0.0 to 5 μ M) examined. One possible explanation for this is that MG132 is known to be less specific inhibitor of proteasome as compared to lactacystin (6). However, more clonal HEK293-PN25 cell lines need to be tested to confirm this observation. To verify that d2EGFP degradation is mediated by proteasome, we examined the levels of an endogenous protein, cyclophilin A (CyP-A), which is devoid of proteasome recognition se-

quence (i.e., PEST). No significant change in the levels of CyP-A protein was detected under all the above experimental conditions (Figure 3).

Several signaling domains other than PEST sequences are known to target intracellular proteins to the ubiquitin-proteasome-mediated pathway (2,10). It is therefore feasible to fuse these signaling domains with EGFP to assess other aspects of the ubiquitin-proteasome pathway. Indeed, Dantuma et al. (3) have recently reported a study in which an N-end rule domain for protein degradation was tagged to EGFP to monitor the magnitude of proteasome inhibition in the presence of MG132 or Z-L3-VS. GFP has also been used to probe the prokaryotic proteasome-like system. Weber-Ban et al. (9) demonstrated that GFP was recruited to the bacterial ClpAP machinery for degradation when an 11-residue ssrA peptide was attached to its terminus. In summary, we developed a sensitive assay using constitutively expressed d2EGFP in mammalian cells to quantitate proteasome inhibition *in vivo*. Furthermore, stable expression of d2EGFP in different mammalian cell lines may become useful for high-throughput screening of cDNAs and pharmaceutical agents that inhibit the ubiquitin-proteasome system.

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